Plant Mol Biol (2017) 95:111–121
DOI 10.1007/s11103-017-0640-6

Evaluation of the mature grain phytase candidate *HvPAPhy_a* gene in barley (*Hordeum vulgare* L.) using CRISPR/Cas9 and TALENs

Inger B. Holme[1] · Toni Wendt[1] · Javier Gil-Humanes[2,3] · Lise C. Deleuran[4] · Colby G. Starker[2] · Daniel F. Voytas[2] · Henrik Brinch-Pedersen[1]

Received: 1 April 2017 / Accepted: 20 July 2017 / Published online: 28 July 2017

Abstract In the present study, we utilized TALEN- and CRISPR/Cas9-induced mutations to analyze the promoter of the barley phytase gene *HvPAPhy_a*. The purpose of the study was dual, validation of the PAPhy_a enzyme as the main contributor of the mature grain phytase activity (MGPA), as well as validating the importance of a specific promoter region of the *PAPhy_a* gene which contains three overlapping cis-acting regulatory elements (GCN4, Skn1 and the RY-element) known to be involved in gene expression during grain filling. The results confirm that the barley PAPhy_a enzyme is the main contributor to the MGPA as grains of knock-out lines show very low MGPA. Additionally, the analysis of the *HvPAPhy_a* promoter region containing the GCN4/Skn1/RY motif highlights its importance for *HvPAPhy_a* expression as the MGPA in grains of plant lines with mutations within this motif is significantly reduced. Interestingly, lines with deletions located downstream of the motif show even lower MGPA levels, indicating that the GCN4/SKn1/RY motif is not the only element responsible for the level of *PAPhy_a* expression during grain maturation. Mutant grains with very low MPGA showed delayed germination as compared to grains of wild type barley. As grains with high levels of preformed phytases would provide more readily available phosphorous needed for a fast germination, this indicates that faster germination may be implicated in the positive selection of the ancient *PAPhy* gene duplication that lead to the creation of the *PAPhy_a* gene.

Keywords Barley · CRISPR/Cas9 · GCN4/Skn1/RY motif · *HvPAPhy_a* · Mature grain phytase activity · TALENs

Introduction

Implementation of sequence specific nucleases (SSNs) for targeted mutagenesis has now been successfully achieved in many important crops (Voytas 2016; Weeks et al. 2016; Zhang et al. 2016a). Targeted mutagenesis relies on the ability of SSNs to recognize and cleave a specific site within a genome and thereby create a double strand break (DSB) at the targeted site. Mutations are then induced during the repair of the DSB through the non-homologous end-joining pathway of the cell. This repair pathway can be imprecise and may result in the generation of mutations at the DSB in the form of deletions, insertions and/or substitutions. Presently, nucleases that can be designed to cleave at specific sites in a genome include meganucleases, zinc finger nucleases, transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) (Bibikova et al. 2003; Bogdanove and Voytas 2011; Epinat et al. 2003; Jinek et al. 2012).

Electronic supplementary material The online version of this article (doi:10.1007/s11103-017-0640-6) contains supplementary material, which is available to authorized users.

✉ Inger B. Holme
inger.holme@mbg.au.dk

[1] Department of Molecular Biology and Genetics, Research Centre Flakkebjerg, Aarhus University, Slagelse, Denmark

[2] Department of Genetics, Cell Biology and Development and Center for Genome Engineering, University of Minnesota, Minneapolis, MN, USA

[3] Present Address: Calyxt Inc., New Brighton, MN, USA

[4] Department of Agroecology, Research Centre Flakkebjerg, Aarhus University, Slagelse, Denmark

Currently, the most widely used SSNs for targeted mutagenesis in plants are TALENs and CRISPR/Cas9 as molecular constructs for both tools are easy to assemble and can be designed to target almost any location in a genome. Even though only recently developed, both tools have been used successfully to improve different crops, such as the generation of plants resistant to diseases through knock-out of the susceptibility genes in rice (Li et al. 2012), and wheat (Wang et al. 2014), generation of oilseeds with decreased levels of polyunsaturated fats through knock-out of the fatty acid desaturase gene in soybean (Haun et al. 2014) and Camelina (Jiang et al. 2016) and generation of potatoes with reduced levels of acrylamide after frying by knocking-out the vacuolar invertase gene (Clasen et al. 2016).

Targeted mutagenesis is primarily used to improve crops by knocking-out undesirable genes, but a few reports have highlighted its potential for promoter targeting (Duan et al. 2016; Jia et al. 2016; Li et al. 2012). Mutations targeted directly to specific promoter sequences of an endogenous gene can be used as alternatives to the traditional promoter analysis that utilizes the integration of a reporter gene that is expressed by an altered promoter of interest. However, using targeted mutagenesis to induce mutations directly at the promoter site has the advantage that potentially altered expression will result in a gene specific phenotype and thereby provide information about the impact of the regulatory element. Furthermore, lines homozygous for the mutations can be multiplied and maintained and used for further experiments investigating the impact of the mutations under different conditions.

In the present study we used TALENs and CRISPR/Cas9 to analyze the promoter of the barley phytase gene *HvPAPhy_a*—thought to be a main contributor to the preformed phytase present in the mature barley grain. Phytases are of outmost importance for providing bioavailable phosphate for grain germination as they initiate the sequential liberation of orthophosphate groups from phytate accounting for 70–80% of the total P in the cereal grains (Brinch-Pedersen et al. 2002). In the Triticeae tribe including wheat, barley and rye, grains contain pre-formed phytases accumulated in the aleurone layer and scutellum during grain development (Dionisio et al. 2007, 2011). Substantial phytase activity is therefore present in mature grains constituting the mature grain phytase activity (MGPA). By contrast, non-Triticeae cereals such as maize, rice and oat have very little MGPA and thus for the vital phosphor supply during germination, they are fully depending on *de novo* phytase synthesis (Brinch-Pedersen et al. 2014).

Phytase activities in cereals are derived from the multiple inositol polyphosphate phosphatase (MINPP) HAP phytase and the purple acid phosphatase phytases (PAPhy) (Dionisio et al. 2007, 2011). In Triticeae, both consist of a set of paralogs: MINPP_a, MINPP_b and PAPhy_a and PAPhy_b. Expression analysis have shown that the a-types are preferentially expressed during grain filling whereas the b-types are preferentially expressed during germination (Dionisio et al. 2007, 2011). Purification of phytases from wheat bran and germinating barley grains has revealed the PAPhy as the major contributor to the MGPA in wheat and barley while MINPP only makes a minor contribution (Greiner et al. 2000; Madsen et al. 2013; Nakano et al. 1999). However, the individual contributions from PAPhy_a versus PAPhy_b to the MGPA have so far not been assessed.

The coding sequences of the two genes are similar but the promoter sequences are very different (Online Resource 1). Promoter analysis using the online tool PlantCare shows that both promoters contain a conserved core with two TATA-boxes but that the cis-acting regulatory elements of the promoters upstream to this are strikingly different (Madsen et al. 2013). The *PAPhy_b* promoters contain elements responsive to gibberellin and its antagonist abscisic acid. These types of arrangements are typical for hydrolases expressed during germination. The *PAPhy_a* promoters contain a conserved arrangement of overlapping cis-acting regulatory elements (GCN4, Skn1 and the RY-element) spanning a total of 13 bp. The three elements are known to be implicated in gene expression during grain filling (Baumlein et al. 1992; Fauteux and Stromvik 2009; Muller and Knudsen 1993). Thus, the webtool promoter analysis of *PAPhy_a* and *PAPhy_b* strongly indicate that the three overlapping cis-acting regulatory elements (GCN4, Skn1 and the RY-element) of *PAPhy_a* are responsible for expression during grain filling and that PAPhy_a therefore is the main contributor to MGPA in Triticeae.

The purpose of this study was to use TALENs and CRISPR/Cas9 to validate the PAPhy_a enzyme as the main contributor of the MGPA and also validate the importance of the promoter region of the *PAPhy_a* gene including the three overlapping cis-acting regulatory elements (GCN4, Skn1 and the RY-element). We chose *HvPAPhy_a* as target because barley is a well-characterized diploid member of the Triticeae tribe with an efficient *Agrobacterium*-mediated transformation system.

Materials and methods

TALEN and CRISPR/Cas9 assembly and vector constructions for plant transformation

The target regions for both TALEN and CRISPR/Cas9 were in the same area of the HvPAPhy_a promotor (Fig. 1). Sequence specific TALEN and CRISPR/Cas9 constructs were designed based on the promoter sequence of barley

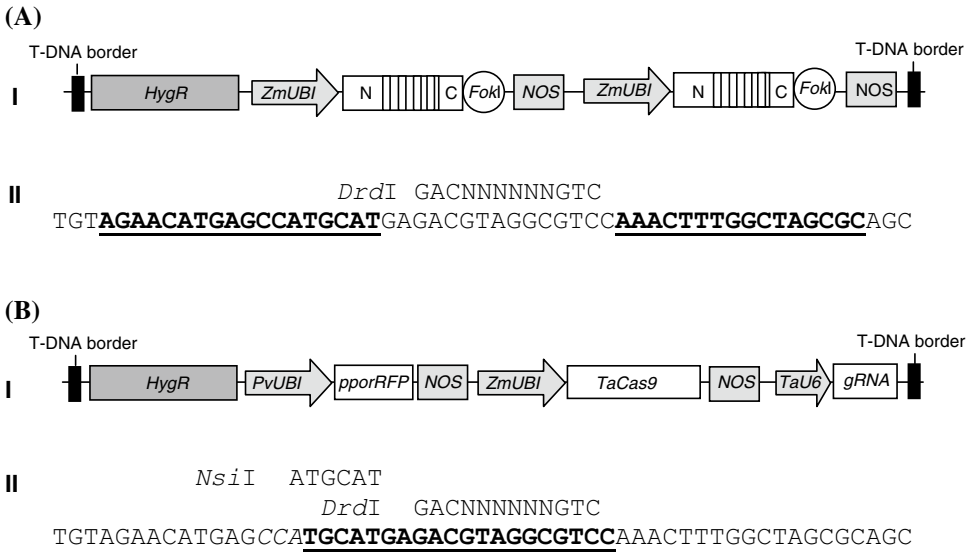

Fig. 1 Schematic presentation of the T-DNA in the vector constructs used for TALEN- and CRISPR/Cas9-induced mutations. Both constructs contain the selection gene for hygromycin resistance (HygR). **a** (*I*) T-DNA of the TALEN-construct. Both TALEN arrays are flanked by a *Zea mays* ubiquitin (ZmUBI) promoter and a NOS terminator. (*II*) The *Drd*I restriction site used to detect mutations at the target site. Sequences in *bold* and *underlined* represent the binding sites of the two TALENs. **b** (*I*) T-DNA of the CRISPR/Cas9 construct containing the reporter gene for red fluorescence protein *ppor*RFP controlled by the *Panicum virgatum* ubiquitin (PvUBI) promoter and a NOS terminator, the TaCas9 nuclease (codon optimized for *Triticum aestivum*) controlled by the *Zea mays* ubiquitin promoter and terminated by the NOS terminator and the *Triticum aestivum* U6 promoter controlling the gRNA. (*II*) The *Nsi*I restriction site used to detect mutations at the target site in T_0 and the *Drd*I restriction site used in combination with the *Nsi*I restriction site to detect mutations at the target site in T_1- and T_2-progeny. The PAM is indicated in *italics* and the *bold* and *underlined* sequence represents the sgRNA binding site

cultivar Golden Promise. The TALEN design and assembly using Golden Gate cloning was previously described in detail by Wendt et al. (2013). We used the TALENs with the highest cleavage activity according to a yeast-based assay (Wendt et al. 2013). The TALEN arrays were assembled in the truncated pTAL backbone (NΔ152/CΔ63) as described by Christian et al. (2012). The sequences of TALEN target sites are shown in Fig. 1a. The left TALEN array was assembled into pZHY500 (Zhang et al. 2013) and the right TALEN array was assembled into pZHY501 (Zhang et al. 2013). The left TALEN was ligated into pTW115 (a gateway compatible entry vector based on pZHY013 containing only FokI-left) using the *Xba*I and *Bam*HI restriction sites in both vectors. The right TALEN was ligated into pTW116 (a gateway compatible entry vector based on pZHY013 containing only FokI-right) using the *Xba*I/*Bam*HI and *Nhe*I/*Bgl*II restriction sites in both vectors. The left TALEN array including FokI-left was subsequently cloned into the destination vector pTW155 using LR clonase. The vector pTW155 is a plant transformation vector, modified from pH2GW7 (Karimi et al. 2002), containing a ubiquitin promoter and NOS terminator for the expression of the TALEN array and a hygromycin resistance gene controlled by a 35S promoter for the selection of transgenic plants. The right TALEN was cloned into pTW156, a minimal destination plasmid for the cloning of the right TALEN array into an ubiquitin promoter and NOS terminator cassette. The right TALEN array (including FokI-right and flanked by an ubiquitin promoter and NOS terminator) was ligated into pTW155 using the *Sac*I and *Apa*I restriction sites on both plasmids. The resulting plasmid contains both TALEN arrays individually expressed by a ubiquitin promoter and NOS terminator within the T-DNA of the plant transformation vector (Fig. 1a).

For the CRISPR/Cas9 a synthetic guide RNA (sgRNA) (5′-GGACGCCTACGTCTCATGCA 3′) was designed to target the GCN4/Skn1/RY motif (Fig. 1b), and cloned into the entry vector pJG85 under the expression of the wheat U6 polymerase III promoter (TaU6). In a second plasmid (pJG80), a wheat codon optimized Cas9 (TaCas9) was cloned under the expression of the ZmUbi1 promoter. Using Gateway cloning, pJG85 and pJG80 were cloned into the destination vector pANIC6A (Mann et al. 2012) (Fig. 1b). The T-DNA of pANIC6A also contains a hygromycin resistance gene controlled by the rice actin 1 promoter and a Porites porites red fluorescent protein gene controlled by the *Panicum virgatum* PvUbi1 promoter (Fig. 1b).

The plant transformation vectors were subsequently transformed into the *Agrobacterium* strain AGL0 using the freeze/thaw method. Positive colonies were selected on medium with rifampicin (25 µg/ml) and the selection antibiotic of the vectors i.e. spectinomycin (50 µg/ml) for the TALEN conctruct and kanamycin (50 µg/ml) for the CRISPR/Cas9 conctruct.

Plant transformation

Barley cultivar Golden Promise was grown in growth cabinets at 15 °C during the day and 10 °C at night with a 16 h light period and a light intensity of 350 µ E m^{-2} s^{-1}. Immature embryos isolated 12–14 days after pollination were used for *Agrobacterium*-mediated transformation following the procedure of Holme et al. (2012). Callus was induced on infected immature embryos on hygromycin-containing medium, and plantlets resistant to hygromycin were regenerated.

Molecular analysis

Plant material for molecular analysis was collected from leaves of T_0, T_1-and T_2-progenies. Genomic DNA was isolated using the FastDNA™ Kit (MP Biomedicals). A PCR/Restriction screen assay was used to identify the mutations. A PCR-product of 1051 bp containing the TALEN target site was amplified using the forward primer: 5′-taatctggccgaaccttgtta-3′; and the reverse primer: 5′-agaatcaatgccttgccatc-3′. Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used for these PCR-reactions according to the manufacturer's instructions. The PCR-products were digested for 1 h at 37 °C with the restriction enzymes for the restriction site sequences predicted to be disrupted by the TALENs (DrdI) or Cas9 (for T_0: NsiI and for segregation analysis of T_1-plants: NsiI and DrdI) (Fig. 1). Both restriction enzymes cut the PCR-product into two fragments of almost the same size (DrdI: 529 and 522 bp; NsiI: 524 and 527 bp). Digestion profiles were analyzed on 1% agarose gels. Undigested bands were gel purified (GenElute™ Gel Extraction Kit, Sigma-Aldrich) and cloned using the TOPO Zeroblunt® cloning kit (Invitrogen). For each PCR product amplified from the T_0-plants, 4–10 clones were picked and sequenced. Cloned products were sequenced and analyzed by alignment to the wild type. For T_1- and T_2-progenies, the amplified PCR product was isolated, cloned and sequenced without prior digestion by restriction enzymes. The presence of the T-DNA in T_1- and T_2-progenies of mutated T_0-plants was assessed by PCR amplification of the hygromycin resistance gene using the forward primer 5′-actcaccgcgacgtctgtcg-3′ and the reverse primer 5′gcgcgtctgctgctccata'3 to amplify a 727-bp fragment of the hpt gene (Vain et al. 2003).

Phytase activity in grains of mutated T_1-lines

Phytase activity was analyzed in crude protein extracts from milled mature T_2-grains of T_1-lines according to Brinch-Pedersen et al. (2000) and Engelen et al. (1994). Random samples of 20–25 grains from each T_1-line were milled and the flour used for phytase activity assays. Average phytase activity in grains of all plants was determined from four repetitions.

Promoter analysis

Cis-acting regulatory elements within the first 325 bp of the *HvPAPhy_a* promoter were identified using the online tool PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Evaluation of barley mutant oxygen consumption during germination

Respiration of barley grains from the control (non-mutated wild type) and the two mutation T_1-lines L1 and L3 was measured via a Q2 instrument (http://www.astec-global.com). The principles of the instrument in relation to grain respiration are described in Bradford et al. (2013). For each barley line, respiration of 15 individual grains was measured. With the purpose of using grains with an approximate uniform grain weight and embryo development, the grains were individually weighed before the experiment (Fig. 4). Each grain was placed in a 1.5 ml well containing 0.7 ml of a water solidified with 1% agar, prepared as described by Erlang-Nielsen (2014). The covering of the well has a membrane containing a metal organic dye that changes its fluorescence properties in proportion to the oxygen concentration. As the grain respires, it depletes the oxygen in the sealed well, and thereby increases the fluorescence of the dye. Measurements of oxygen relative content in all wells were automatically determined every half hour. The measurements were performed at 20 °C for 108 h.

Statistics

The Welch's t-test for unequal variances was employed in the mature grain phytase activity experiments to test significant differences in mature grain phytase activities between the non-transformed control plants and T_2-grains from the mutated T_1-lines.

Results

Selection of T_1- and T_2-lines homozygous for mutations induced by TALENs and CRISPR/Cas9

Sequence specific TALENs and CRISPR/Cas9 were designed to target and induce mutations within a region of the *HvPAPhy_a* promoter thought to be important for expression of the gene. For the knockout of the *HvPAPhy_a* gene we relied on the generation of large deletions extending from the targeted area within the promoter into the coding region of the *HvPAPhy_a* gene instead of targeting the coding sequence directly. In this way we would be sure that no truncated gene was expressed leaving traces of activity.

Primary transformants (T_0) were generated by *Agrobacterium*-mediated transformation with the TALEN and CRISPR/Cas9 constructs illustrated in Fig. 1a, b, respectively. A total of 47 and 64 hygromycin resistant T_0-plants were generated from 750 to 320 embryos transformed with the TALEN- and CRISPR/Cas9 constructs, respectively. Mutations were identified by PCR/Restriction digest analysis (PCR/RE). Based on this analysis, 43 and 44% of the T_0-plants obtained with the TALEN- or CRISPR/Cas9-constructs were considered mutated (Online Resource 2). Undigested PCR-products were isolated and TOPO cloned from 15 T_0-plants with mutations induced by each of the TALEN or CRISPR/Cas9 construct. From each T_0-plant, 4–10 TOPO clones were picked and sequenced. Based on these sequences, five T_0-plants with TALEN-induced mutations and four T_0-plants with CRISPR/Cas9-induced mutations showing only one or two mutated alleles were self-pollinated (Table 1). Progenies were subsequently analyzed for segregation of the alleles using PCR/RE in order to identify T_1-lines homozygous for the mutations present in the T_0-plants. The PCR-products from the T_1-lines identified as homozygous were sequenced to confirm that they were homozygous for the mutation (for examples see Online Resource 3). Interestingly, two of the T_0-plants selected for further propagation and already identified as homozygous mutants in the T_0-generation (T_0 2–01 and T_0 4–01, Table 1) was confirmed to be homozygous mutants in the T_1-progeny analysis (Table 1). The generation of inheritable homozygous mutations as early as in the first generation has also been observed in several other targeting

Table 1 Sequences of T_0-plants selected for further propagation and the sequences of the T_1-and T_2-progenies homozygous for specific mutations selected for promoter analysis

T_0-ID	Allele Types	Sequences of T_0-plants	Allele types	Sequences of T_1- and T2-plants homozygous for a T_0-mutation	Line no[a]
T_0 TALENs			**T_1 and T_2 TALENs**		
1-01	Phy1 -14	CATGAGCCATGCATGAG----------ACTTT	Phy1 -14	CATGAGCCATGCATGAG----------ACTTT	L6
			Phy2 -14	CATGAGCCATGCATGAG----------ACTTT	
	Phy2 WT	CATGAGCCATGCATGAGACGTAGGCGTCCAAACTTT			
2-01	Phy1 -21	CATGAGCCATGCA----------TT	Phy1 -21	CATGAGCCATGCA----------TT	L5
			Phy2 -21	CATGAGCCATGCA----------TT	
	Phy2 -21	CATGAGCCATGCA----------TT			
3-01	Phy1 -110,+4	----------GTCCAAACTTT	Phy1 -110,+4	----------GTCCAAACTTT	L3
			Phy2 -110,+4	----------GTCCAAACTTT	
	Phy2 WT	CATGAGCCATGCATGAGACGTAGGCGTCCAAACTTT			
4-01	Phy1 -4	CATGAGCCATGCATGAGAC----GCGTCCAAACTTT	Phy1 -4	CATGAGCCATGCATGAGAC----GCGTCCAAACTTT	L7
			Phy2 -4	CATGAGCCATGCATGAGAC----GCGTCCAAACTTT	
	Phy2 -4	CATGAGCCATGCATGAGAC----GCGTCCAAACTTT			
5-01	Phy1 -362	----------	Phy1 -362	----------	L1
			Phy2 -362	----------	
	Phy2 -21	CATGAGCCATGCA----------TT			
T_0 CRISPR/Cas9			**T_1 and T_2 CRISPR/Cas9**		
6-01	Phy1 +1	CATGAGCCATGCaATGAGACGTAGGCGTCCAAACTTT	Phy1 +1	CATGAGCCATGCaATGAGACGTAGGCGTCCAAACTTT	L11
			Phy2 +1	CATGAGCCATGCaATGAGACGTAGGCGTCCAAACTTT	
	Phy2 -9	CATGAGCCATGC---------AGGCGTCCAAACTTT			
7-01	Phy1 -1	CATGAGCCATGCA-GAGACGTAGGCGTCCAAACTTT	Phy1 -1	CATGAGCCATGCA-GAGACGTAGGCGTCCAAACTTT	L12
			Phy2 -1	CATGAGCCATGCA-GAGACGTAGGCGTCCAAACTTT	
	Phy2 -11	CATGAGCC----------GTAGGCGTCCAAACTTT	Phy2 -11	CATGAGCC----------GTAGGCGTCCAAACTTT	L9
			Phy1 -11	CATGAGCC----------GTAGGCGTCCAAACTTT	
8-01	Phy1 -11	CA----------TGAGACGTAGGCGTCCAAACTTT	Phy1 -11	CA----------TGAGACGTAGGCGTCCAAACTTT	L10
			Phy2 -11	CA----------TGAGACGTAGGCGTCCAAACTTT	
	Phy2 WT	CATGAGCCATGCATGAGACGTAGGCGTCCAAACTTT	Phy1 -387[b]	----------	L2
			Phy1 -387[b]	----------	
9-01	Phy1 -31	----------GTCCAAACTTT	Phy1 -31	----------GTCCAAACTTT	L4
			Phy2 -31	----------GTCCAAACTTT	
	Phy2-12,+11	CATGAGCtttg-gtgccttGTAGGCGTCCAAACTTT	Phy1 -12,+11	CATGAGCtttg-gtgccttGTAGGCGTCCAAACTTT	L8
			Phy2 -12,+11	CATGAGCtttg-gtgccttGTAGGCGTCCAAACTTT	

[a]Correspond to line numbers in Figs. 2, 3 and 4

[b]A mutation not present in T_0 was identified in T_1 and inherited to T_2

mutation studies (Zhang et al. 2014; Ma et al. 2015). In parallel with the segregation analysis, the corresponding T_1-progenies were also tested for the presence of T-DNA using PCR with primers for the hygromycin resistance gene. When possible only T-DNA-free T_1-lines were selected (for examples see Online Resource 3). The latter was not always possible due to poor grain setting, so for some mutant lines selection of lines without T-DNA was postponed to the T_2-generation (See Online Resource 3).

The twelve mutated T_1-lines that were selected (Table 1) showed mutations also identified in their parent lines. The only exception was line L2 where a new mutation was identified in the T_1-progeny. This indicates that the CRISPR/Cas9 construct remained active during the development of T_0-plant 8–01 and induced a new mutation in the wild type chromosome in tissue also participating in the production of the gametes. All twelve selected T_1-lines were self-pollinated and the sequencing of T_2-progeny confirmed the inheritance of the mutations to the T_2-generation.

Phytase activities in grains of T_1-lines homozygous for mutations

The region from position −188 to −244 of the different homozygous mutations found in the twelve T_1-lines are shown in Fig. 2. This region includes three overlapping regulatory elements consisting of the GCN4-like element with the sequence TGAGCCA (also identified as TGAGTCA), the Skn1-like element with the sequence GCCAT (also identified as GTCAT) and the RY-element with the sequence CATGCATG. These three elements (Fig. 2a) are known to be important for seed specific expression (Baumlein et al. 1992; Fauteux and Stromvik 2009; Madsen et al. 2013; Muller and Knudsen 1993). As only the smaller mutations of −1 to −31 bp can be fully displayed in Fig. 2b, a schematic presentation of the mutations of the different lines and their position in relation to the *HvPAPhy_a* genomic clone are illustrated in Fig. 3. The area of the *HvPAPhy_a* clone affected by the mutations is shown in Fig. 3b covering the first 325 bp of the promoter and the first 160 bp of the first exon. The positions of the two TATA-boxes and a G-box within this part of the promoter are also shown.

One of the most important findings of this study comes from the large deletions found in line L1 and L2. Line L1 contains a 362 bp deletion from −270 bp of the promoter into the first 93 bp of exon1 and line L2 contains a deletion of 387 bp from −229 bp of the promoter into the first 158 bp of exon1 of the *HvPAPhy_a* gene (Fig. 3c). These lines show very low MGPAs of 30 and 80 FTU/kg flour, respectively, as compared to the wild type control with a MGPA of 1910 FTU/kg flour (Fig. 3d). Thus the deletions within these lines clearly confirm that the *PAPhy_a* phytase of barley is the main contributor to the phytase activity of the mature grains. The residual MGPA still present in the grains of these lines could be caused by a weak expression of the *HvPAPhy_b* gene during grain development (Dionisio et al. 2011) and the *HvMINPP_a* phytase gene which is also expressed in the mature barley grains at a low level (Dionisio et al. 2007).

The remaining ten lines only contain mutations within the promoter. Mutation lines L3 to L10 all have significantly lower MGPAs as compared to the non-mutated control (Fig. 3d). Line L3 contains a large deletion of 110 bp ranging from position −213 to −321 plus a 4 bp insertion at position −321 (Fig. 3c) and has a low MGPA of 213 FTU/kg flour (Fig. 3d). This indicates that this area of the promoter which includes the three overlapping elements (GCN4/Skn1/RY) is very important for the expression level of the *HvPAPhy_a* phytase gene. Likewise, mutation line L4 with a smaller deletion of 31 bp ranging from position −213 to −243 also deleting the (GCN4/Skn1/RY) motif shows a low MGPA of 490 FTU/kg flour. Therefore, the low MGPA of lines L3 and L4 could indicate (as we were expecting) that the deletion of the GCN4/Skn1/RY motif causes large reductions in MGPA. However, for both lines nucleotides upstream and downstream of this motif are also deleted which could also be important for the expression of the gene (Figs. 2, 3). In support of this, line L5 with a 21 bp deletion of the promoter (position −203 to −222) that includes only the last G in the RY element but does not affect any nucleotide of the GCN4 and Skn1 elements, still shows a very low MGPA of 172 FTU/kg flour (Fig. 3d). In addition, line L6 and L7 containing deletions of 14 and 4 bp both starting five nucleotides downstream of the GCN4/Skn1/RY motif show low MGPAs of 488 and 213 FTU/kg flour, respectively (Fig. 3d). Thus, positions downstream of the GCN4/Skn-1/RY motif are very important for the expression level of the *HvPAPhy_a* gene in the mature grain.

The GCN4/Skn1/RY motif is, however, also important for the MGPA level as lines L8, L9 and L10 with mutations affecting almost exclusively the GCN4/Skn-1/RY motif, all show significantly lower MGPAs than the wild-type control (Fig. 3d). Lines L8 and L9 contain deletions and insertions ranging from positions −219 to −230 and −220 to −230, respectively (Fig. 2). These lines show MGPAs of 855 and 1247 FTU/kg flour, respectively. Furthermore, line L10 having a −11 bp deletion spanning the whole GCN4/Skn-1/RY motif except for the first and last bp (−222 to −232, Fig. 2b) show a MGPA of 1163 FTU/kg flour.

Lines L11 and L12 only have a single bp insertion and deletion at position −225 and −224, respectively, which

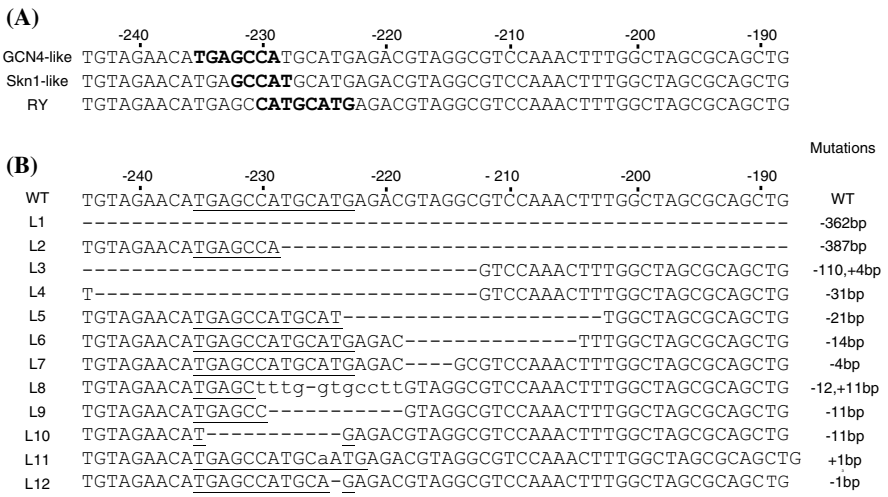

Fig. 2 Sequence alignment of the 57 bp target region from position −188 to −244 of the wild type and T_1-lines homozygous for mutations induced by either TALENs or CRISPR/Cas9. **a** In *bold*, position of the three overlapping promoter regulatory elements GCN4, Skn1 and RY present in this area of the promoter. **b** Sequences of the wild type (WT) and T_1-lines with mutations. *Underlined sequences* represent the remaining nucleotides of the three overlapping promoter regulatory elements GCN4, Skn1 and RY. Nomenclature for sequences: non-mutated bp: *upper case lettering*; deleted bp: "−"; insertions bp: *lowercase lettering*. Nomenclature for mutations: (−) indicates deletions followed by the number of bp deleted, (+) indicates insertions followed by the number of bp inserted

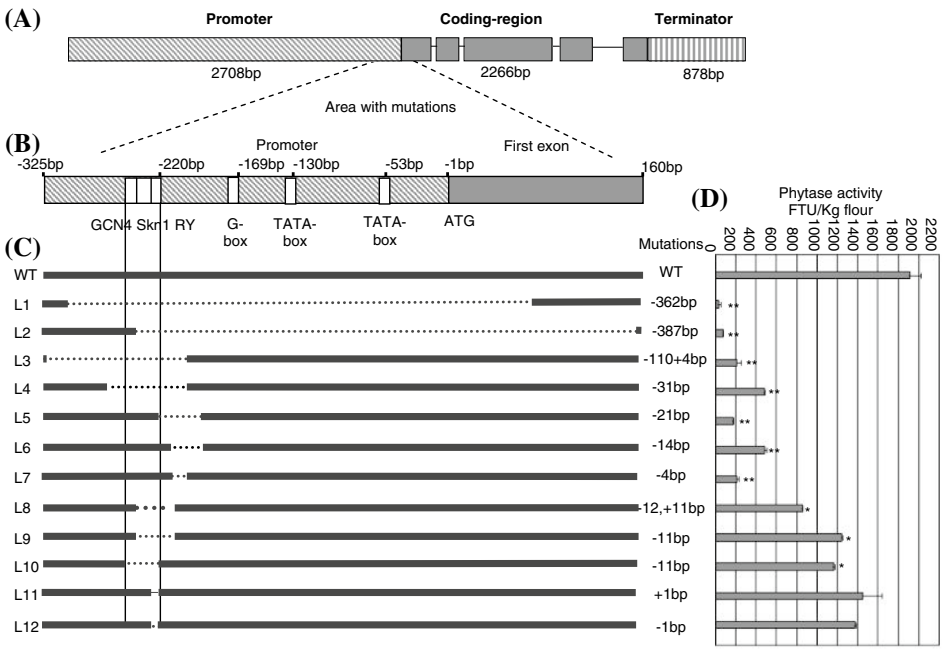

Fig. 3 Schematic presentation of induced mutations in different barley lines within the *HvPAPhy_a* genomic clone and the corresponding mature grain phytase activity of these lines. **a** The *HvPAPhy_a* genomic clone. **b** Area with mutations. The positions of the TATA-boxes, the GCN4/Skn1/RY motif and the G-box are indicated. **c** Schematic presentation of the wild type and the mutations in twelve T_1-lines. (−) indicates deletions followed by the number of bp deleted, (+) indicates insertions followed by the number of bp inserted. **d** The corresponding mature grain phytase activities of the wild type and twelve T_1-lines. *Bars* represent standard errors (SE). *Asterisks* indicate the significance levels: $*p < 0.05$ and $**p < 0.01$

result in changes at the 3′end of the RY element (CATGCA ATG and CATGCAG instead of CATGCATG) (Fig. 2). These two mutated lines do not show significant differences in MGPA levels as compared to the wild type control. This could indicate that the last two nucleotides of RY-element are not critical for the function of element as previously found by Reidt et al. (2000).

Overall, the mutations within the promoter area show that all lines with a very reduced MGPA as compared to the wild type (L3 to L7), contain deletions which include the four base pair deletion of line L7 starting five nucleotides downstream of the GCN4/Skn1/RY motif (−218 to −215, Fig. 2b). This could indicate the presence of an additional element which in combination with the GCN4/Skn1/RY motif is important for the full expression of the

HvPAPhy_a gene in the developing grain. However, no homology to currently known regulatory elements could be identified in this area.

Evaluation of barley mutant oxygen consumption during germination

The influence of MGPA levels on the time period required for germination was investigated using homozygous T_2-grains from mutation lines L1 and L3 showing MGPA of 30 and 213 FTU/kg flour, respectively, and compared to the germination of grains from the wild type control with a MGPA of 1910 FTU/kg flour. The fitted curves in Fig. 4 show the respiration pattern of grains from the three lines as an average of the 15 grains for each line. The grains from the non-mutated wild type reach R50 (the time required for the seed to reduce the initial oxygen level by 50%) after approximately 44 h, whereas grains from mutant line L1 reach R50 after approximately 46 h. Grains from mutant line L3 reach R50 after 52 h. The delay in reduction in the initial oxygen level gets more pronounced between the grains from the three lines with time. The grains from the non-mutated line have reduced the initial oxygen level by 100% after 72 h. In mutant line L1 and L3, these were 84 and 88 h, respectively.

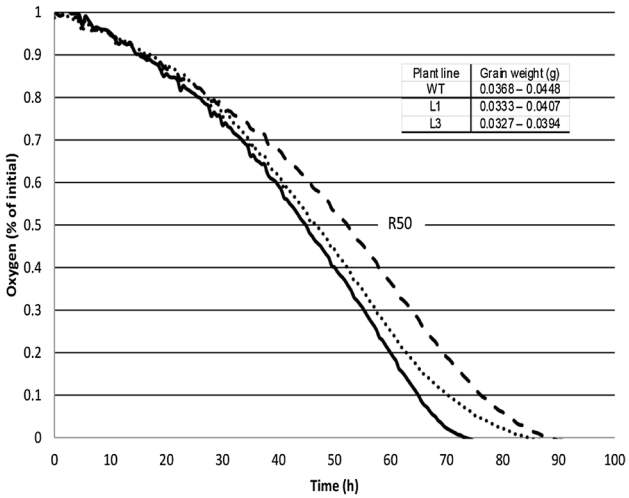

Fig. 4 Oxygen consumption curves for three barley lines imbibed at 20 °C. The initial oxygen percentage is normalized to 100% (equivalent to 21% air composition). R50 is the time required for the seed to reduce the initial oxygen level by 50%. *Solid line* wild type, *dotted line* mutation line L1, *dashed line* mutation line L3. The table shows the range of grain weight for the three plant lines used for the germination assay

Discussion

In the present study we used TALEN- and CRISPR/Cas9-induced mutations to investigate the significance of the *HvPAPhy_a* gene and specific sequences within the promoter associated with the regulation of phytase activity in the mature grain.

A prerequisite of this study was the transmission of the TALEN- and CRISPR/Cas9-induced mutations to the next generations. In a previous study we used a TALEN construct with both TALEN monomers controlled by a single 35S promoter, resulting in all T_0-plants showing mosaic mutation distribution (Wendt et al. 2013), and no transmission of the mutations to the next generation. However, in the present study, using the ZmUbi promoter to drive the expression of each TALEN monomer, TALEN-induced mutations found in the T_0 generation were stably transmitted to subsequent generations. Similar to the present study, constructs with a promoter (35S and/or ubiquitin) in front of each TALEN monomer were found to result in TALEN-induced mutations with inheritance in barley (Gurushidze et al. 2014), rice (Li et al. 2012; Zhang et al. 2016b) and maize (Char et al. 2015). This implies that a stronger promoter activity than a single 35S-promoter might be needed in cereals to ensure high expression in the reproductive tissue and inheritance of TALEN-induced mutations. Yet, the inclusion of a ubiquitin promoter in front of each TALEN monomer might not be crucial as successful *OsBADH2* knockouts with inheritance to the next generation were obtained with one ubiquitin promoter driving the expression of the two TALEN monomers (Shan et al. 2015). A similar construct was also successfully used to induce knock-outs of all three homoeoalleles *Tamlo* genes in wheat which were all inherited to the next generation (Wang et al. 2014).

The CRISPR/Cas9 induced mutations of the present study were also stably inherited to the next generation in the plants selected for further propagation. The use of both a TALEN- and a CRISPR/Cas9 construct targeting the same promoter area generated T_1-lines with a higher diversity of mutations than when only one of the SSNs was used. Interestingly, the TALEN- and the CRISPR/Cas9 constructs used in the present study resulted in almost the same mutation efficiencies (43 and 44%, respectively). These high frequencies and the fact that restriction sites were identified right at the predicted cleavage sites of both SSNs facilitated the selection of T_1-lines homozygous for mutations. As mentioned above inheritance of TALEN-induced mutations in barley was previously demonstrated using microspore derived haploid callus to knockout a GFP-gene (Gurushidze et al. 2014). Also, stable inheritance of CRISPR/Cas9-induced mutations in barley was previously reported using immature embryos as starting material (Lawrenson et al. 2015; Kapusi et al. 2017). In the study by Lawrenson et al. (2015) the target was the *HvPM19* gene encoding a putative plasma membrane protein expressed during embryo

development and dormancy (Ranford et al. 2002). However, as not all copies of the gene were targeted in the same mutant plant, no phenotype was reported. In the study by Kapusi et al. (2017) the target was the putative barley endo-N-acetyl-β-D-glucosaminidase gene. No phenotype has so far been reported for the plants generated in that study.

The most noticeable phenotypes in the present study were the dramatically reduced MGPAs in grains of two T_1-lines, L1 and L2, both carrying knocked-outs of the *HvPAPhy_a* gene caused by deletions spanning parts of the promoter and the first exon. The MGPA was reduced from 1910 FTU/kg flour in wild type to 30 and 80 FTU/kg in line L1 and L2, respectively. This clearly confirms HvPAPhy_a's role as the main contributor to the MGPA in barley, while the other phytases present in barley — HvPAPhy_b, MINPP_a and MINPP_b—make very little contribution to the MGPA. Orthologous of all four genes have been identified in several Triticeae tribe cereals including *Triticum aestivum* and *Secale cerale* (Dionisio et al. 2011, 2007; Madsen et al. 2013) and therefore, *PAPhy_a* might be the main mature grain phytase in these species too.

The targeted area included the three GCN4/Skn-1/RY overlapping elements which are the only known elements important for seed specific expression within the promoter of the *PAPhy_a* genes of Triticeae (Madsen et al. 2013). The GCN4 and the Skn-1 elements are often present either separately or together in promoters of genes encoding seed storage proteins (Fauteux and Stromvik 2009; Juhasz et al. 2011; Muller and Knudsen 1993; Onate et al. 1999; Wu et al. 1998, 2000). The RY-element is present in many promoters of seed-specific genes in both dicots and monocots and is thought to be a positive regulator of seed-specific expression and a negative element repressing expression in non-seed tissues (Baumlein et al. 1992; Forster et al. 1994; Fujiwara and Beachy 1994). While lines with mutations within this motif showed significant reduction in MGPA, our results indicate that the GCN4/SKn1/RY elements are not the only elements responsible for the level of *HvPAPhy_a* expression during seed maturation. Lines with deletions starting five nucleotides downstream of the GCN4/Skn1/RY motif showed even lower mature grain phytase activity levels. Specifically deletions including the four nucleotides at position −218 to −215 (GTAG) seems to be crucial for the level of phytase activity in the mature grain. As no known regulating elements could be identified in this area of the promoter, further studies will have to verify if these nucleotides are part of an element regulating the level of *HvPAPhy_a* expression. Since PAPhy_a phytase accumulates in the aleurone layer and scutellum during grain development (Dionisio et al. 2011), other cis-acting elements regulating aleurone and scutellum specific expression may be present.

The presence of off-target mutations cannot be ruled out without complete sequencing of the genome of each of these independent mutant lines. Thus, we cannot definitively show that the phenotypes we observe are due to the observed mutations in *HvPAPhy_a*. However, since almost all the mutated lines generated in this study have a significant effect on the MGPA we argue that off-targets within the genome by either TALENs or CRISPR/Cas9, if they exist, do not affect the phenotypes.

The plant lines with mutations causing very low phytase activities of this study could help unravel the reason why members of the Triticeae tribe possess an extra phytase gene (*PAPhy_a*) not present in other cereals. The additional phytase type *PAPhy_a* present in Triticeae is thought to have evolved by a single-gene segmental duplication of the ancestral *PAPhy* gene about 32–54 million years ago (Madsen et al. 2013). The reason for an evolutionary positive selection of this extra phytase gene in the Triticeae is unknown. Several studies have shown that high phosphorus content of seeds allow a faster seed germination and a faster seedling establishment which ultimately will result in higher yield (Grant et al. 2001; Robinson et al. 2012; White and Veneklaas 2012). As the preformed grain phytases synthesized by the *PAPhy_a* gene result in an early phosphorous supply, we compared the germination time of grains from non-mutated lines and grains from mutant lines exhibiting very low MGPA. We observed delayed germination in mutated lines with low MGPA. Future studies using the mutation lines of the present study could help reveal if seedling establishment and grain yield is also affected by the preformed phytase from the *PAPhy_a* gene or if other factors are responsible for the positive selection of the extra *PAPhy* gene.

In conclusion, this study confirms that the PAPhy_a enzyme of barley is the main contributor to the MGPA. The analysis of the *HvPAPhy_a* promoter area containing the GCN4/Skn1/RY regulatory elements for seed specific expression shows that these elements are important but that other elements are required to obtain MGPA levels as high as the wild type. The MGPA of the Triticeae species have a very important influence on the nutritional value of the grains. Humans and monogastric animals basically have no phytase activity in their digestive tract and the MGPA is therefore entirely responsible for providing bio-available phosphate from the phytic acid in the grains (Brinch-Pedersen et al. 2014). However, higher MGPA levels than present in most Triticeae cereals are required to avoid the supply of extra rock phosphate to the feed. Knowledge about the exact gene responsible and its regulation is therefore valuable when attempting to increase the MPGA levels either through traditional breeding or biotechnology approaches. Further studies identifying all promoter

elements important for the MGPA levels would promote these approaches.

Acknowledgements The authors thank Rikke B Jacobsen, Lis B Holte and Ole B Hansen for skilful technical assistance. The research was funded by a grant to IBH from the Danish Ministry of Food, Agriculture and Fisheries (3304-FVFP-09-B-006), a Grant to IBH from Brd. Hartmann's foundation (A27246) and Grants to DFV from the National Science Foundation (IOS-1444511 and IOS-1339209). Javier Gil-Humanes acknowledges the Fundación Alfonso Martin Escudero for his post-doctoral fellowship.

Author contributions IBH, TW, JG-H and LCD all contributed to the experimental part of this research. CGS, DFV and HB-P advised on the experimental part. IBH and HB-P wrote the initial draft for the paper and the initial draft was carefully revised by JG-H, TW, CGS, LCD and DFV.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Baumlein H, Nagy I, Villarroel R, Inze D, Wobus U (1992) *Cis*-analysis of a seed protein gene promoter: the conservative RY repeat CATGCATG within the legumin box is essential for tissue-specific expression of a legumin gene. Plant J 2:233–239. doi:10.1046/j.1365-313X.1992.t01-45-00999.x

Bibikova M, Beumer K, Trautman JK, Carroll D (2003) Enhancing gene targeting with designer zinc finger nucleases. Science 300:764. doi:10.1126/science.1079512

Bogdanove AJ, Voytas DF (2011) TAL effectors: customizable proteins for DNA targeting. Science 333:1843–1846. doi:10.1126/science.1204094

Bradford KJ, Bello P, Fu JC, Barros M (2013) Single-seed respiration: a new method to assess seed quality. Seed Sci Technol 41:420–438. doi:10.15258/sst.2013.41.3.09

Brinch-Pedersen H, Olesen A, Rasmussen SK, Holm PB (2000) Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an Aspergillus phytase. Mol Breed 6:195–206. doi:10.1023/A:1009690730620

Brinch-Pedersen H, Sørensen LD, Holm PB (2002) Engineering crop plants: getting a handle on phosphate. Trends Plant Sci 7:118–125. doi:10.1016/S1360-1385(01)02222-1

Brinch-Pedersen H, Madsen CK, Holme IB, Dionisio G (2014) Increased understanding of the cereal phytase complement for better mineral bio-availability and resource management. J Cereal Sci 59:373–381. doi:10.1016/j.jcs.2013.10.003

Char SN, Unger-Wallace E, Frame B, Briggs SA, Main M, Spalding MH, Vollbrecht E, Wang K, Yang B (2015) Heritable site-specific mutagenesis using TALENs in maize. Plant Biotechnol J 13:1002–1010. doi:10.1111/pbi.12344

Christian ML, Demorest ZL, Starker CG, Osborn MJ, Nyquist MD, Zhang Y, Carlson DF, Bradley P, Bogdanove AJ, Voytas DF (2012) Targeting G with TAL effectors: a comparison of activities of TALENs constructed with NN and NK repeat variable di-residues. PLoS ONE 7(9):e45383. doi:10.1371/journal.pone.0045383

Clasen BM, Stoddard TJ, Luo S, Demorest ZL, Li J, Cedrone F, Tibebu R, Davison S, Ray EE, Daulhac A, Coffman A, Yabandith A, Retterath A, Haun W, Baltes NJ, Mathis L, Voytas DF, Zhang F (2016) Improving cold storage and processing traits in potato through targeted gene knockout. Plant Biotechnol J 14:169–176. doi:10.1111/pbi.12370

Dionisio G, Holm PB, Brinch-Pedersen H (2007) Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) multiple inositol polyphosphate phosphatases (MINPPs) are phytases expressed during grain filling and germination. Plant Biotechnol J 5:325–338. doi:10.1111/j.1467-7652.2007.00244.x

Dionisio G, Madsen CK, Holm PB, Welinder KG, Jørgensen M, Stroger E, Arcalis E, Brinch Pedersen H (2011) Cloning and characterization of purple acid phoshatase phytases from wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), maize (*Zea maize* L.) and rice (*Oryza sativa* L.). Plant Physiol 156:1087–1100. doi:10.1104/pp.110.164756

Duan Y, Li J, Qin R, Xu R, Li H, Yang Y, Ma H, Li L, Wei P, Yang J (2016) Identification of a regulatory element responsible for salt induction of rice *OsRAV2* through ex situ and in situ promoter analysis. Plant Mol Biol 90:49–62. doi:10.1007/s11103-015-0393-z

Engelen AJ, van der Heeft FC, Randsdorp PH, Smit ELC (1994) Simple and rapid-determination of phytase activity. J AOAC Int 77:760–764

Epinat JC, Arnould S, Chames P, Rochaix P, Desfontaines D, Puzin C, Patin A, Zanghellini A, Pâques F, Lacroix E (2003) A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells. Nucl Acids Res 31:2952–2962. doi:10.1093/nar/gkg375

Erlang-Nielsen M (2014) Plant nutrition and health. Dissertation, Aarhus University

Fauteux F, Stromvik M (2009) Seed storage protein gene promoters contain conserved DNA motifs in *Brassicaceae, Fabaceae* and *Poaceae*. BMC Plant Biol 9:126. doi:10.1186/1471-2229-9-126

Forster C, Arthur E, Crespi S, Hobbs SLA, Mullineaux P, Casey R (1994) Isolation of a pea (*Pisum sativum*) seed lipoxygenase promoter by inverse polymerase chain reaction and characterization of its expression in transgenic tobacco. Plant Mol Biol 26:235–248. doi:10.1007/BF00039535

Fujiwara T, Beachy RN (1994) Tissue-specific and temporal regulation of a β-conglycinin gene: roles of the RY repeat and other *cis*-acting elements. Plant Mol Biol 24:261–272. doi:10.1007/BF00020166

Grant CA, Flaten DN, Tomasiewicz DJ, Sheppard SC (2001) The importance of early season phosphorus nutrition. Can J Plant Sci 81:211–224. doi:10.4141/P00-093

Greiner R, Jany KD, Larsson MA (2000) Identification and properties of myo-inositol hexakisphosphate phosphohydrolases (phytases) from barley (*Hordeum vulgare*). J Cereal Sci 31:127–139. doi:10.1006/jcrs.1999.0254

Gurushidze M, Hensel G, Hiekel S, Schedel S, Valkov V, Kumlehn J (2014) True-breeding targeted gene knock-out in barley using designer TALE-nuclease in haploid cells. PLoS ONE 9(3):e92046. doi:10.1371/journal.pone.0092046

Haun W, Coffman A, Clasen BM, Demorest ZL, Lowy A, Ray E, Retterath A, Stoddard T, Juillerat A, Cedrone F, Mathis L, Voytas DF, Zhang F (2014) Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol J 12:934–940. doi:10.1111/pbi.12201

Holme IB, Dionisio G, Brinch-Pedersen H, Wendt T, Madsen CK, Vincze E, Holm PB (2012) Cisgenic barley with improved phytase activity. Plant Biotechnol J 10:237–247. doi:10.1111/j.1467-7652.2011.00660.x

Jia H, Orbovic V, Jones JB, Wang N (2016) Modification of the PthA4 effector binding elements in Type I CsLOB1 promoter using Cas9/sgRNA to produce transgenic Duncan grapefruit

alleviating XccΔpthA4:dCsLOB1.3 infection. Plant Biotechnol J 14:1291–1301. doi:10.1111/pbi.12495

Jiang WZ, Henry IM, Lynagh PG, Cormai L, Cahoon EB, Weeks DP (2016) 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

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821. doi:10.1126/science.1225829

Juhasz A, Makai S, Sebestyen E, Tamas L, Balazs E (2011) Role of conserved non-coding regulatory elements in LMW glutenin gene expression. PLoS ONE 6(12):e29501. doi:10.1371/journal.pone.0029501

Kapusi E, Corcuera-Gomez M, Melnik S, Stoger E (2017) Heritable genomic fragment deletions and small indels in the putative ENGase gene induced by CRISPR/Cas9 in barley. Front Plant Sci 8:540. doi:10.3389/fpls.2017.00540

Karimi M, Inzé D, Depicker A (2002) Gateway vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci 7:193–195. doi:10.1016/S1360-1385(02)02251-3

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

Li T, Liu B, Spalding MH, Weeks DP, Yang B (2012) High-efficiency TALEN-based gene editing produces disease-resistant rice. Nat Biotech 30:390–392. doi:10.1038/nbt.2199

Ma X., Zhang Q., Zhu Q., Liu W., Chen Y., Qiu R., Wang B., Yang Z., Li H., Lin Y., Xie Y., Shen R., Chen S., Wang Z., Chen Y., Guo J., Chen L., Zhao X., Dong Z., and Liu Y.-G. (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

Madsen CK, Dionisio G, Holme IB, Holm PB, Brinch-Pedersen H (2013) High mature grain phytase activity in the Triticeae has evolved by duplication followed by neofunctionalization of the purple acid phosphatase phytase (*PAPhy*) gene. J Exp Bot 64:3111–3123. doi:10.1093/jxb/ert116

Mann DGJ, LaFayette PR, Abercrombie LL, King ZR, Mazarei M, Halter MC, Poovaiah CR, Baxter H, Shen H, Dixon RA, Parrott WA, Stewart Jr CN (2012) Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.). Plant Biotechnol J 10: 226–236. doi:10.1111/j.1467-7652.2011.00658.x

Muller M, Knudsen S (1993) The nitrogen response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. Plant J 4:343–355. doi:10.1046/j.1365-313X.1993.04020343.x

Nakano T, John T, Tokumoto E, Hayakawa T (1999). Purification and characterization of phytase from wheat bran of *Triticum aestivum* L. cv. Nourin #61. Food Sci Technol Res 5:18–23.

Onate L, Vicente-Carbajosa J, Lara P, Diaz I, Carbonero P (1999) Barley BLZ2, a seed-specific bZIP protein that interacts with BLZ1 in vivo and activates transcription from the GCN4-like motif of B-hordein promoters in barley endosperm. J Biol Chem 274:9175–9182. doi:10.1074/jbc.274.14.9175

Ranford JC, Bryce JH, Morris PC (2002) PM19, a barley (*Hordeum vulgare* L.) gene encoding a putative plasma membrane protein, is expressed during embryo development and dormancy. J Exp Bot 53:147–148. doi:10.1093/jexbot/53.366.147

Reidt W, Wohlfarth T, Ellerstrom M, Czihal A, Tewes A, Ezcurra I, Rask L, Baumlein H (2000) Gene regulation during late embryogenesis: the RY motif of maturation-specific gene promoters is a direct target of the FUS3 gene product. Plant J 21:401–408. doi:10.1046/j.1365-313x.2000.00686.x

Robinson WD, Carson I, Ying S, Ellis K, Plaxton WC (2012) Eliminating the purple acid phosphatase ATPAP26 in *Arabidopsis thaliana* delays leaf senescence and impairs phosphorus remobilization. New Phytol 196:1024–1029. doi:10.1111/npi.12006

Shan Q, Zhang Y, Chen K, Zhang K, Gao C (2015) Creation of fragrant rice by targeted knockout of the OsBADH2 gene using TALEN technology. Plant Biotechnol J 13:791–800. doi:10.1111/pbi.12312

Vain P, Afolabi AS, Worland B, Snape JW (2003) Transgene behavior in populations of rice plants transformed using a new dual binary vector system: pGreen/pSoup. Theor Appl Genet 107:210–217. doi:10.1007/s00122-003-1255-7

Voytas DF (2016) Editorial prerogative and the plant genome. J Genet Genom 43:229–232. doi:10.1016/j.jgg.2016.03.004

Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, Qiu J (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

Weeks DP, Spalding MH, Yang B (2016) Use of designer nucleases for targeted gene and genome editing in plants. Plant Biotechnol J 14:483–495. doi:10.1111/pbi.12448

Wendt T, Holm PB, Starker CG, Christian M, Voytas DF, Brinch-Pedersen H, Holme IB (2013) TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. Plant Mol Biol 83:279–285. doi:10.1007/s11103-013-0078-4

White PJ, Veneklaas EJ (2012) Nature and nurture: the importance of seed phosphorus content. Plant Soil 357:1–8. doi:10.1007/s11104-012-1128-4

Wu C, Suzuki A, Washida H, Takaiwa F (1998) The GCN4 motif in a rice glutelin gene is essential for endosperm-specific gene expression and is activated by Opaque-2 in transgenic rice plants. Plant J 14:673–683. doi:10.1046/j.1365-313x.1998.00167.x

Wu C, Washida H, Onodera Y, Harada K, Takaiwa F (2000) Quantitative nature of the Prolamin-box, ACGT and AACA motifs in a rice glutenin gene promoter: minimal *cis*-element requirements for endosperm-specific gene expression. Plant J 23:415–421. doi:10.1046/j.1365-313x.2000.00797.x

Zhang Y, Zhang F, Li X, Baller JA, Qi Y, Starker CG, Bogdanove AJ, Voytas DF (2013) Transcription activator-like effector nucleases enable efficient plant genome engineering. Plant Physiol 161:20–27. doi:10.1104/pp.112.20517

Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, Mao Y, Yang L, Zhang H, Xu N, Zhu K-J (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 12:797–807. doi:10.1111/pbi.12200

Zhang D, Zhenxiang L, Li J (2016a) Targeted gene manipulation in plants using CRISPR/Cas technology. J Genet Genom 43:251–262. doi:10.1016/j.jgg.2016.03.001

Zhang H, Gou F, Zhang J, Liu W, Li Q, Mao Y, Botella JR, Zhu J (2016b) TALEN-mediated targeted mutagenesis produces a large variety of heritable mutations in rice. Plant Biotechnol J 14:186–194. doi:10.1111/pbi.12372