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Cisgenic barley with improved phytase activity

Inger B. Holme*, Giuseppe Dionisio, Henrik Brinch-Pedersen, Toni Wendt, Claus K. Madsen, Eva Vincze and Preben B. Holm

Faculty of Science and Technology, Department of Molecular Biology and Genetics, Research Centre Flakkebjerg, Aarhus University, Slagelse, Denmark

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*Correspondence (Tel +45 89993742;
fax +45 89993501;
email inger.holme@agrsci.dk)
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Summary

The *cisgenesis* concept implies that plants are transformed only with their own genetic materials or genetic materials from closely related species capable of sexual hybridization. Furthermore, foreign sequences such as selection genes and vector-backbone sequences should be absent. We used a barley phytase gene (*HvPAPhy_a*) expressed during grain filling to evaluate the *cisgenesis* concept in barley. The marker gene elimination method was used to obtain marker-free plant lines. Here, the gene of interest and the selection gene are flanked by their own T-DNA borders to allow unlinked integration of the two genes. We analysed the transformants for co-transformation efficiency, increased phytase activities in the grain, integration of the kanamycin resistance gene of the vector-backbone and segregation between the *HvPAPhy_a* insert and the hygromycin resistance gene. The frequencies of the four parameters imply that it should be possible to select 11 potentially *cisgenic* T_1-lines out of the 72 T_0-lines obtained, indicating that the generation of *cisgenic* barley is possible at reasonable frequencies with present methods. We selected two potential *cisgenic* lines with a single extra copy of the *HvPAPhy_a* insert for further analysis. Seeds from plants homozygous for the insert showed 2.6- and 2.8-fold increases in phytase activities and the activity levels were stable over the three generations analysed. In one of the selected lines, the flanking sequences from both the left and right T-DNA borders were analysed. These sequences confirmed the absence of truncated vector-backbone sequences linked to the borders. The described line should therefore be classified as *cisgenic*.

Keywords: *Agrobacterium*, barley, *cisgenesis*, *HvPAPhy_a*, grain phytase activity, marker-free transformants.

Introduction

Genetic modification is an important breeding tool in the development of crops with improved quality. However, in Europe in particular, the technology has been met with substantial scepticism among the general public and in consequence thereof also by the growers, the agro industry and the retailers. A number of studies have described the major concerns of the general public (Gaskell and Bauer, 2001; Bauer and Gaskell, 2002; Lassen *et al.*, 2002). One concern about transgenic crops is the mingling of genetic materials between species that cannot hybridize by natural means. This reservation is often linked to ethical considerations and fears for potential health risks and for the spreading of new gene combinations in the environment. Also, it is apparent that the public perception of GM crops is highly dependent on whether the traits introduced are considered useful and of benefit to the public.

With the aim of meeting the concerns about mingling of genetic material between species, two new transformation concepts have been proposed, that is, *intragenesis* (Nielsen, 2003; Rommens, 2004) and *cisgenesis* (Schouten *et al.*, 2006). In contrast to *transgenesis* where genes and DNA sequences can be moved between any species, both concepts imply that the plants are transformed only with their own genetic materials or genetic materials from closely related species capable of sexual hybridization. Furthermore, foreign genes such as selection marker genes and vector-backbone genes should be absent or eliminated from the primary transformants or its progeny. The gene pool exploited by *intragenesis* and *cisgenesis* is accordingly identical to the gene pool available for traditional breeding. There are two major differences between the two concepts. *Intragenesis* allows for the utilization of new gene combinations created by *in vitro* rearrangement of functional genetic elements such as promoter regions, coding regions with or without introns and terminal regions. In *cisgenesis*, such rearrangements are not permitted and the 'cisgene' is accordingly a complete copy of the endogenous gene including the promoter, introns and the terminator in the normal-sense orientation. Furthermore, when using *Agrobacterium*-mediated transformation, *intragenesis* requires T-DNA border sequences isolated from the sexual compatible gene pool (P-borders) while *cisgenesis* uses the T-DNA borders from *Agrobacterium tumefaciens*.

The implementation of the *intragenesis* and *cisgenesis* concepts may imply that crops genetically modified according to these concepts are more acceptable for the public. In February 2010, a Eurobarometer survey was conducted based on representative samples from 32 European countries on the public opinion of *cisgenesis* as compared to *transgenesis* using apple resistant to canker, scab and mildew as case studies (http://ec.europa.eu/public_opinion/archives/ebs/ebs_341_winds_en.pdf; Gaskell *et al.*, 2011). The survey showed that across the European Union, 55% of the interviewed people supported *cisgenic* apples while only 33% supported transgenic apples. The interviewed people expressed the opinion that they found that *cisgenic* apples were more natural than transgenic apples, less problematic for the environment and more useful.

The implementation of the *intragenesis* and *cisgenesis* concepts also carries the anticipation that crops developed according to these guidelines eventually can be deregulated to a level where a minimum of risk assessment is required (Schouten *et al.*, 2006; Rommens, 2010). At present, the very high cost associated with regulatory approval, in particular in the EU area, is a major impediment to the introduction of genetically modified crops. In consequence, many genetically modified crops and traits are currently not included in breeding programmes although they may be considered useful by the general public.

Recently, the first *cisgenic* plant was reported, a *cisgenic* apple plant resistant to scab (Vanblaere *et al.*, 2011). In the present study, the challenges associated with implementing *cisgenesis* were assessed in a major cereal crop, barley. Besides, we wanted to study whether it was possible to improve a particular quality trait by the insertion of extra gene copies from the species itself. As our candidate gene for *cisgenesis*, we chose a barley phytase (*HvPAPhy_a*, Dionisio *et al.*, 2011), preferentially synthesized during seed development and thereby responsible for most of the phytase activity deposited in the mature grain.

Phytases are the only phosphatases that can initiate the sequential liberation of phosphate groups from phytic acid. They are accordingly very important for the production of bioavailable phosphate from the stored phytic acid that accounts for around 70% of the total phosphate in the seeds (Brinch-Pedersen *et al.*, 2002). Monogastric animals like pigs and poultry have no phytase activity in their digestive tract, and the phytase level of the mature barley grain is inadequate. In consequence, most of the phytic acid of the barley grain is secreted and spread with the manure to the agricultural soils and eventually to the aquatic environment causing algal growth and eutrophication. Large-scale supplementation with rock phosphate, a non-renewable resource, is generally undertaken to meet optimal growth requirements in the monogastric livestock (Steen, 1998). Furthermore, microbial-derived phytase is commonly added to the feed in areas with intensive livestock production to improve the poor bioavailability of phosphate (Brinch-Pedersen *et al.*, 2002). However, many farmers grow their own cereals for feed and do not add phytase. There is accordingly a great need for improving the phytase potential and thereby the phosphate bioavailability in feed crops like barley.

The natural allelic variation for phytase activity in known barley cultivars and wild barley is limited to maximum phytase activities of around 1300 FTU/kg flour (Dai *et al.*, 2010). Classical breeding for higher phytase activity is therefore difficult. Genetic modification has previously been used to generate plants with increased phytase activity in seeds of wheat, maize, soybean and canola using transgenic approaches with phytase transgenes isolated from microorganisms and promoters originating from other genes (Denbow *et al.*, 1998; Brinch-Pedersen *et al.*, 2000, 2003, 2006; Zhang *et al.*, 2000; Chen *et al.*, 2008). These studies have shown that transformation with a microbial phytase encoding gene is as efficient for ensuring phytic acid degradation as addition of microbial phytase to the feed.

In the present study, we show that genetic modification according to the *cisgenesis* concept is possible in barley at reasonable frequencies. We further show that through the insertion of a genomic clone for *HvPAPhy_a*, it is possible to increase the activity of phytase.

Results

Generation of transgenic barley

The sequencing of the *HvPAPhy_a* genomic clone revealed that the coding sequence of 2266 bp consisted of five exons. In addition to this, we decided to include around 2000 bp of the flanking promoter region and 800 bp of the terminator region (Figure 1b). The genomic *PAPhy_a* was inserted into pClean-G185 with the promoter region at the left T-DNA border site and the terminator region at the right T-DNA border site (pClean-G185-*PAPhy_a*). This orientation was chosen because smaller deletions at the left border region might not affect the regulatory properties of the promoter region owing to its large size, while deletions of the short terminator region might be more problematic.

Immature embryos of the barley cultivar Golden Promise were transformed with the *Agrobacterium* strain AGL0 containing the pClean-G185-*PAPhy_a* and the pClean-S166 vector (Figure 1a). The vector pClean-S166 contains the hygromycin resistance gene with the NOS promoter and NOS terminator (Thole *et al.*, 2007). We used AGL0 which is recA+ enabling homologous recombination within *Agrobacterium* instead of the recA AGL1strain (Lazo *et al.*, 1991). It is, however, our experience that AGL0 confer higher transformation efficiencies in barley. Moreover, homologous sequences present in both pGreen and pSoup (Hellens *et al.*, 2000) have been removed from the pClean vector-system, minimizing putative homologous recombination between the two binary vectors (Thole *et al.*, 2007). A total of 1500 immature embryos were infected with *Agrobacterium*. Seventy-two plants survived the hygromycin selection and were transferred to the greenhouse. As expected, this transformation efficiency of 4.8% is lower than what we usually obtain with vectors containing a hygromycin resistance gene with the 35S-CMV promoter (15%), because the NOS promoter is around 30 times weaker than the 35S-CMV promoter (van der Krol *et al.*, 1988).

PCR analysis of the T_0-plants

Four primer pairs were used to analyse the T_0-plants transferred to the greenhouse for the presence of the T-DNA inserts and the vector-backbone of pClean-G185. The primers amplified PCR products for the hygromycin resistance gene, the kanamycin resistance gene, the promoter-end region (PE-primer pairs) and the terminator-end region (TE-primer pairs) of the genomic *PAPhy_a* insert. The forward and reverse primers of the PE- and TE-primer pairs, respectively, included the cloning sites of the sequences between the T-DNA borders and the *PAPhy_a* insert as these sequences are the only sequences inside the T-DNA borders that are different from the endogenous gene. These primer pairs do not give any information about the number of inserts in each transformant, but they were essential for the initial screening of the material and the later identification of marker-free plants. An example of the PCR analysis in T_0-plants is shown in Figure 2. As *Agrobacterium* can still be present in the regenerated T_0-transformants and give rise to PCR false positives, the T_0-plants showing all four PCR products from the pClean-S166/pClean-G185-*PAPhy_a* vector-system could be PCR false positive. Additional PCR analysis was therefore performed on T_1-plants from T_0-plants showing all four PCR products to confirm the presence of the kanamycin resistance gene of the pClean-G185-*PAPhy_a* vector-backbone.

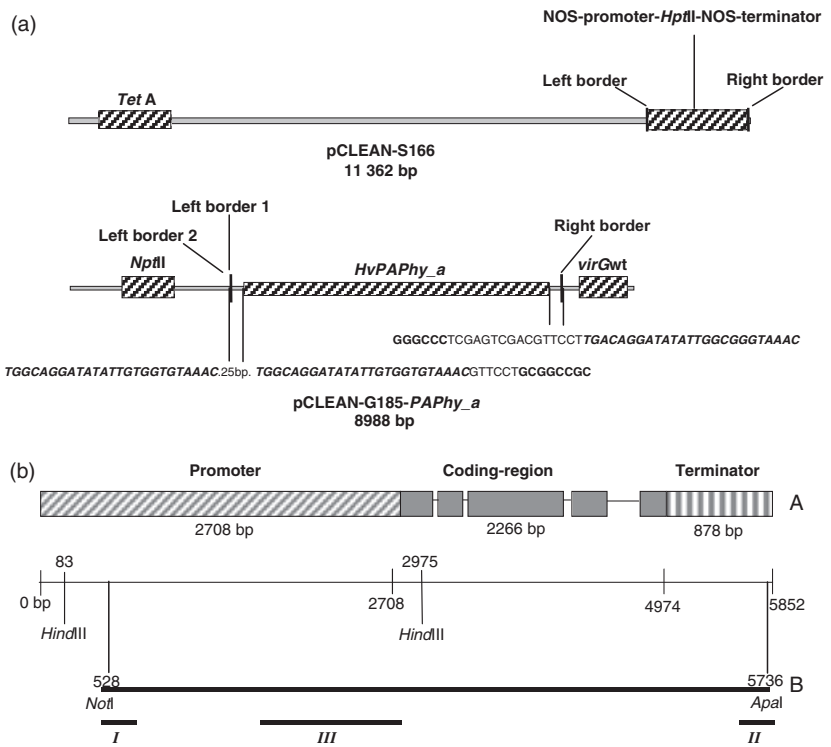


Figure 1 Schematic presentation of pClean-S166 and pClean-G185-*PAPhy_a* (a) and diagram of the *HvPAPhy_a* genomic clone (b). The sequences of the two left borders, the right border and the synthetic sequences of the multiple cloning sites left in the T-DNA including the *Not*I- and *Apa*I-cloning sites used to insert *HvPAPhy_a* into pClean-G185 are shown. The sequences of the T-DNA borders are in bold italics. The sequences of the *Not*I- and *Apa*I-cloning sites are in bold. *Tet*A, tetracycline resistance gene; *Hpt*II, hygromycin resistance gene; *Npt*II, kanamycin resistance gene. (A) The genomic clone isolated from the barley genomic lambda library. (B) The PCR product of *PAPhy_a* used for vector insertion. The *Hind*III restriction sites of the genomic clone are indicated. The PCR products for the promoter-end primers (I), the terminator-end primers (II) and the PCR product used for Southern blot analysis (III) are underlined.

Based on the PCR analysis, all 72 T_0-plants obtained in this study contained the hygromycin resistance gene. The PCR analysis revealed that 73.6% of the transformants also contained *PAPhy_a* insert(s) (Table 1). In 38.9% of the co-transformed plants, we could detect both the PE- and the TE-primer pair products of *PAPhy_a*, indicating that some of the *PAPhy_a* inserts in these plants contained intact integration of the *PAPhy_a* T-DNA. In 33.3% of these plants, we could only detect the TE-primer product, indicating deletions at left T-DNA border of all the *PAPhy_a* inserts. In one transformant (1.4%), only the PCR product of the PE-primers was detected, indicating a deletion at the right T-DNA border of all the *PAPhy_a* inserts.

The test for vector-backbone integration using the primer pairs for the kanamycin resistance gene was also performed on the 72 T_0-plants (Table 1). Here, a vector-backbone frequency of 57.1% was found for plants showing intact integration of the *PAPhy_a* inserts. Verification of the presence of the kanamycin resistance gene and further analysis of the transformants required T_0-plants setting seeds. Fifty-five T_0-plants produced enough seeds for further analysis (Table 1). PCR analysis of T_1-progenies confirmed the presence of the kanamycin resistance gene in the 12 T_0-plants setting seeds and showing all four PCR products from the pClean-S166/pClean-G185-*PAPhy_a* vector-system. Thus, based on the 23 T_0-plants setting seeds and showing intact integration of the *PAPhy_a* inserts, the vector-backbone integration frequency was 52.2% (Table 1). As expected, none of the plants showing deletion at the left T-DNA border region had vector-backbone integration. The transformant with deletion at the right T-DNA border region also showed vector-backbone integration (Table 1).

Phytase activity in seeds of the transformed To-plants

The phytase activity was determined in the T_1-seeds of the 55 seed setting T_0-plants. The T_0-plants will all be hemizygous for the *PAPhy_a* inserts, and seed samples from each plant will be segregating for the inserts and therefore contain a mixture of seeds without any inserts and seeds hemizygous and/or homozygous for the inserts. The average *PAPhy_a* gene dosage in the samples should therefore be the same as in seeds hemizygous for *PAPhy_a* inserts.

The results were divided into three groups: (i) phytase activities in seeds of plants where the PCR product of the PE- and TE-primer pairs were not detected (ii) phytase activities in seeds of plants only showing the PCR product of the TE-primer pairs and (iii) phytase activities in seeds of plants showing the PCR product of both the PE- and TE-primers pairs (Figure 3).

The phytase activity in seeds of the non-transformed Golden Promise was 1350 FTU/kg flour. However, seeds of the 11 transformants not showing the PCR products of the PE- or TE-primer pairs had phytase activities ranging from 950 to 2280 FTU/kg flour (Figure 3a) with an average activity of 1841 FTU/kg flour and a SD of 442. The reason for this variation is not known, but T_0-plants regenerated from *in vitro* cultures are often weaker than seed-derived plants, and T_1-seeds

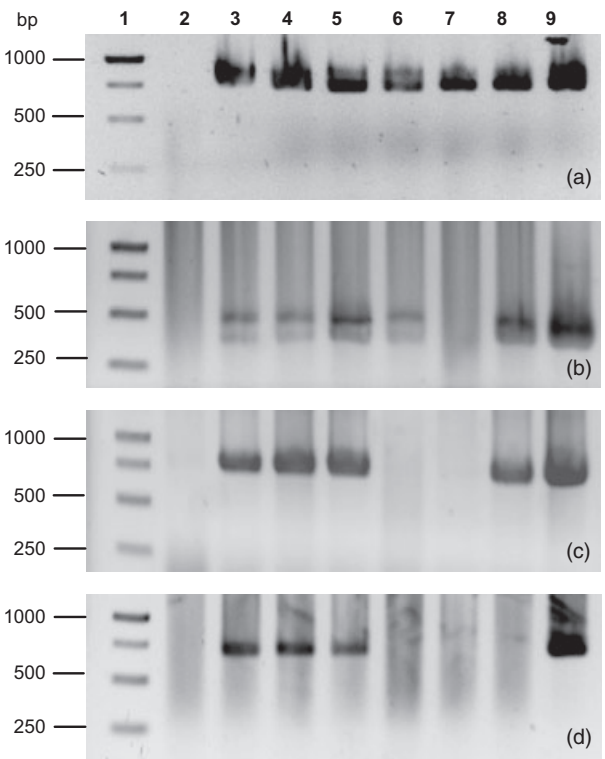


Figure 2 PCR analysis of T_0-lines. Examples of the PCR analysis with primer sets for (a) the hygromycin resistance gene, (b) the terminator-end (TE) of the *PAPhy_a* insert, (c) the promoter-end (PE) of the *PAPhy_a* and (d) the kanamycin resistance gene. Lane 1: 1-kb ladder, lane 2: non-transformed Golden Promise, lanes 3–8: T_0-plants, lane 9: vector controls (a: pClean-S166; b–d: pClean-G185-*PAPhy_a*). Lane 3–5: T_0-plants showing all four PCR products, indicating intact integration of the *PAPhy_a* inserts including vector-backbone. Lane 6: T_0-plants only showing the TE-primer PCR product of the *PAPhy_a* insert at the right T-DNA border, indicating deletions at the left T-DNA border. Lane 7: T_0-plants only showing the hygromycin resistance gene PCR product, indicating that the T-DNA of pClean-G185-*PAPhya* was not integrated into the genome of this plant. Lane 8: T_0-plants showing both PCR products of the *PAPhy_a* insert but no PCR product of the kanamycin resistance gene, indicating intact integration of the *PAPhy_a* insert but no vector-backbone integration. Lane 8 represents the plant PAPhy07 from which marker-free progeny were later identified.

of many of these plants showed slightly poorer grain filling than normally observed in the greenhouse. In wheat, it was shown that phytases of the endogenous *TaPAPhy* genes are deposited in the aleuronic layer (Dionisio *et al.*, 2011). The phytase level per kg flour will therefore vary according to the degree of grain filling. The phytase level in this group of plants was used as a second control for the phytase level in the other two groups.

The phytase level in seeds of the 20 transformants where only the TE-primer pair product was detected ranged between 1250 and 5500 FTU/kg flour (Figure 3b). The SD of 442 FTU/kg flour observed between the T_1-seeds of plants without *PAPhy_a* inserts accounts for some of this variation. However, the phytase activities of 2400–5500 FTU/kg flour observed in seeds of ten of these plants indicate that these transformants contain one or more *PAPhy_a* inserts where the deletions at the promoter region did not influence the expression of the insert. The remaining ten T_0-plants in this group did not show higher expression levels in the seeds than seeds from T_0-plants where the *PAPhy_a* insert was not detected (Figure 3a). These transformants most likely contain inserts where deletions at the promoter-end region abolish the expression of the insert(s).

Table 1 PCR analysis of T_0-plants for the presence of the *PAPhy_a* insert and the kanamycin resistance gene of the pClean-G185 vector-backbone

PCR amplification product	T_0-plants transferred to the greenhouse	T_0-plants setting seeds
No. of T_0-plants PCR-positive for the hygromycin resistance gene	72	55
Percentage of T_0-plants PCR-positive for the TE- and/or PE-primer pairs of *PAPhy_a*	73.6 (53/72)	80.0 (45/55)
Percentage of T_0-plants PCR-positive for both the TE- and PE-primer pairs of *PAPhy_a*	38.9 (28/72)	41.8 (23/55)
Percentage of these T_0-plants also PCR-positive for the kanamycin resistance primer pairs	57.1 (16/28)	52.2 (12/23)
Percentage of T_0-plants PCR-positive for only the TE-primer pairs of *PAPhy_a*	33.3 (24/72)	36.4 (20/55)
Percentage of these T_0-plants also PCR-positive for the kanamycin resistance primer pairs	0 (0/24)	0 (0/20)
Percentage of T_0-plants PCR-positive for only PE-primer pairs of *PAPhy_a*	1.4 (1/72)	1.8 (1/55)
Percentage of these T_0-plants also PCR-positive for the kanamycin resistance primer pairs	100 (1/1)	100 (1/1)

PE, promoter-end; TE, terminator-end.

The phytase activities in seeds of the 23 transformants showing the PCR products of both *PAPhy_a* primer pairs ranged from 1800 to 7500 FTU/kg flour (Figure 3c). Here, seeds of 21 out of 23 transformants showed phytase activities of 2400 FTU/kg flour or more. The higher phytase activity levels in this group are probably due to a higher number of intact *PAPhy_a* inserts integrated into these T_0-plants.

The phytase activities in the seeds of the transformant only showing the PCR product of the PE-primer pair are not included in Figure 3. The seeds of this plant showed a phytase activity of 1800 FTU/kg flour, indicating that the *PAPhy_a* insert was not expressed because of damaging deletions at the right T-DNA border.

Identification of progeny with segregation between *PAPhy* and hygromycin inserts

The segregation study was performed in the progeny of five T_0-plants obtained from the first transformation experiment. The five T_0-plants showed PCR products of both *PAPhy_a* primer pairs. The plants were named PAPhy01, PAPhy02, PAPhy03, PAPhy05 and PAPhy07 and showed seed phytase activities ranging from 2500 to 3400 FTU/kg. Three of these T_0-plants showed the amplification product of the kanamycin primer pairs revealing that at least one of the *PAPhy_a* inserts

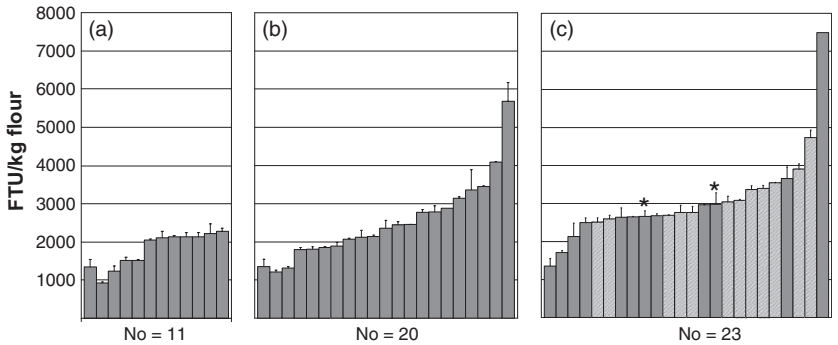


Figure 3 Phytase activities in seeds of T_0-plants. The T_0-plants were divided into three groups: (a) T_0-plants not showing the PCR products of *PAPhy_a* insert, (b) T_0-plants showing only the PCR product of the terminator-end (TE) primer pairs at the right T-DNA border of *PAPhy_a* inserts, (c) T_0-plants showing the PCR products of both the TE and promoter-end (PE) primer pairs of *PAPhy_a* inserts. FTU: phytase units. The first column of each figure represents the phytase activity of non-transformed Golden Promise seeds. (▩) Plants not showing the PCR product of the kanamycin resistance gene of the pClean-G185 vector-backbone. (▨) Plants showing the PCR product of the kanamycin resistance gene of the pClean-G185 vector-backbone. Bars represent SE. Asterisks indicate the two plants PAPhy05 and PAPhy07 from which maker-free progeny were later identified. The phytase activity of 1800 FTU/kg flour found in the seeds of the transformant only showing the PCR product of the PE primer pair is not included in this figure.

Table 2 Segregation between the *PAPhy_a* inserts and the hygromycin resistance inserts in progeny of five T_0-plants

Transformants	No. of progeny	Observed segregation			
		H^+P^+*	H^+P^-	H^-P^+	H^-P^-
PAPhy01	50	34	14	0	2
PAPhy02	29	28	0	0	1
PAPhy03	25	17	3	5	0
PAPhy05	40	24	9	5	2
PAPhy07	56	41	13	2	0

*P, *PAPhy_a* insert; H, hygromycin resistance gene.

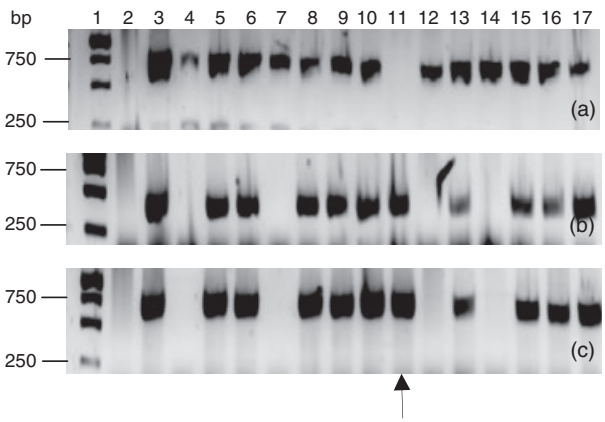


Figure 4 Segregation between the hygromycin resistance gene and the *PAPhy_a* insert in T_1-progeny of the plant PAPhy07. The PCR products of the hygromycin resistance gene (a), the terminator-end (b) and the promoter-end (c) primer pairs of the *PAPhy_a* insert were used for the analysis. Lane 1: 1-kb ladder, lane 2: non-transformed Golden Promise, lane 3: PAPhy07-T_0, lanes 4–17: T_1-progeny from PAPhy07. The arrow indicates a T_1-plant without the PCR product of the hygromycin resistance gene but with both PCR products of *PAPhy_a*.

from pClean-G185 in each of these T_0-plants had vector-backbone integration. In two of the T_0-plants (PAPhy01, PAPhy02), no progeny containing the *PAPhy_a* insert without the hygromycin resistance insert was identified (Table 2). In the remaining three T_0-plants (PAPhy03, PAPhy05 and PAPhy07), progeny were identified that contained *PAPhy_a* inserts but no hygromycin resistance inserts. PAPhy03 showed 22 progeny with *PAPhy_a* inserts and three progeny without *PAPhy_a* inserts and thus did not follow the 3 : 1 segregation for a single insert. PAPhy05 and PAPhy07 both almost exactly followed the 3 : 1 segregation for a single *PAPhy_a* insert, indicating a single *PAPhy_a* insert in both plants. PAPhy05 and PAPhy07 also lacked the kanamycin resistance gene of the pClean-G185-*PAPhy_a* vector-backbone as judged by the PCR analysis. An example of the PCR segregation analysis in the progeny of PAPhy07 is shown in Figure 4.

Identification of marker-free plants homozygous for the *PAPhy_a* insert and measurement of the phytase activity

The marker-free T_1-plants of PAPhy05 and PAPhy07 were further propagated, and plants homozygous for the *PAPhy_a* insert were identified. These homozygous plants were tested once more by PCR for the absence of the hygromycin resistance gene, the kanamycin resistance gene and the tetracycline resistance gene (Figure S1). Moreover, Southern blot analysis was performed to confirm the presence of a single *PAPhy_a* insert in each transformant and to document that the *PAPhy_a* inserts of the two transformants were not inserted in tandem configurations (Figure S2).

The phytase activities were measured in seeds of the PAPhy05 and PAPhy07 plants with no *PAPhy_a* insert, with a hemizygous *PAPhy_a* insert and with a homozygous *PAPhy_a* insert (Figure 5). The phytase activity in the seeds of the hemizygous and homozygous plants of PAPhy05 and PAPhy07 was almost the same. The 1 : 2 : 1 mixture of the *PAPhy_a* segregating seeds from the hemizygous plants showed a twofold increase in phytase activity. Seeds from the homozygous plants of PAPhy05 and PAPhy07 showed a 2.6- and 2.8-fold increase in phytase activity, respectively.

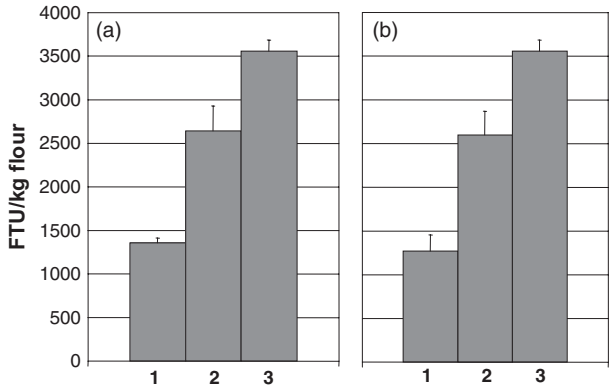


Figure 5 Phytase activities in seeds of plants segregating for the *PAPhy_a* insert. The phytase activities were measured in seeds derived from (a) PAPhy05 marker-free plants and (b) PAPhy07 marker-free plants without the *PAPhy_a* insert (1), hemizygous for the insert (2) and homozygous for the insert (3). FTU: phytase units. Bars represent SE.

Flanking regions of the *PAPhy_a* insert in the PAPhy07 marker-free line

The marker-free PAPhy05 and PAPhy07 plant lines homozygous for the *PAPhy_a* insert were further analysed for flanking sequences. Unfortunately, we were only able to isolate the right border sequences of the *PAPhy_a* insert in PAPhy05 (data not shown). For the PAPhy07 plant line, the nucleotides of the junctions between the T-DNA and the flanking genomic regions are shown in Figure 6a. At the right T-DNA border, the border was nicked between the third and the fourth nucleotide of the 25-nucleotide border sequence leaving three nucleotides of the right border in the plant genome. At the left T-DNA border, the border was nicked between the 16th and 17th nucleotide of the border closest to the insert leaving 16 nucleotides of the left border in the plant genome. Additionally, 14 and 22 synthetic nucleotides of the multiple cloning site in pClean-G185-*PAPhy_a* were integrated in the genome of PAPhy07 at the left and right T-DNA borders, respectively (Figure 1a). Thus, a total of 36 synthetic and 19 T-DNA border nucleotides out of the 36 synthetic and the 101 T-DNA border nucleotides present in the pClean-G185-*PAPhy_a* were integrated in the genome of PAPhy07. Importantly, the DNA flanking neither the left nor the right T-DNA border nucleotides showed homology to the DNA of the vector-backbone.

We were able to isolate 1056 bp of the right-border-flanking region and 734 bp of the left-border-flanking region (Figure 6b). The sequences were blasted against the NCBI database nucleotide collection and the EST collection. At *E*-values of 1e−20 or smaller, two regions included all the alignments with *Hordeum vulgare* in the nucleotide collection. The region from 628 to 989 bp of the 361-bp showed 87% homology to the 375-bp repetitive element named Xumet_AY661558-1. The region from 1129 to 1298 bp of 169-bp showed 76% homology to the 162-bp miniature inverted repeat transposable element named Thalos_AY661558-1. These elements were first identified in the Hv-eI4E sequence (AY661558.1) by Wicker *et al.* (2005). The regions also included the majority of the alignments with the EST collection accessions of *H. vulgare*, indicating that both elements are sometimes transcribed in barley (Figure 6b). The last 151 bp of the left-border-flanking sequences showed 100% homology to the 5′ end of an EST of 480 bp (AJ464005.1). This EST has been isolated from barley shoots 2, 3 and 4 days after germination and has no known function.

Primer pairs designed within both the left and the right flanking areas of the *PAPhy_a* insert were used for PCR analysis of the wheat–barley addition lines to identify the chromosome number and arm in which the *PAPhy_a* insert had integrated. The results show that the flanking region of both the left and the right sites of the insert are located on the short arm of chromosome 3H (Figure 6c).

The amplified and sequenced pre-insertion target sequence from the non-transgenic parent plant showed that the integration of the *PAPhy_a* insert had caused a deletion of 22 nucleotides at the insertion site and a deletion of one nucleotide 5 bp from the left border (Figure 6d). A five-nucleotide overlap between the left T-DNA border and the plant DNA was observed. Sequence similarities at the left border have been suggested to facilitate the T-DNA integration (Tinland, 1996).

Discussion

In the present study, we have explored the possibility of generating genetically modified barley plants according to the *cisgenesis* concept (Schouten *et al.*, 2006). In *cisgenic* plants, the 'gene of interest' should originate from the same species or a closely related species with which it can intercross. Moreover, marker genes and vector-backbone sequences should be absent or eliminated from the primary transformants or its progeny. As our 'gene of interest' we used a barley genomic clone for the *HvPAPhy_a* gene, a gene that encodes a phytase enzyme synthesized during grain filling and responsible for most of the phytase activity deposited in the mature grain.

The genomic clone proved to be fully functional when inserted into the barley genome. Transformed T_0-plants showed up to sixfold increases in grain phytase activity as compared to the wild type. The increase in phytase activity monitored in two marker-free plant lines with a single-copy *PAPhy_a* insert was found to be stable over the three generations analysed. Seeds of plants homozygous for the single-copy *PAPhy_a* insert showed 2.6- to 2.8-fold increases in phytase activity, revealing a positive correlation between gene dosage and gene expression.

We did not observe reduction in the phytase activity as compared to the wild type in any of the plants containing extra copies of the endogenous *PAPhy_a*. Several studies have shown that suppression of gene expression can occur when the number of homologues to an endogenous host gene is increased (van der Krol *et al.*, 1990; Napoli *et al.*, 1990; Vaucheret *et al.*, 1997; De Paoli *et al.*, 2009). This phenomenon termed co-suppression is regulated post-transcriptionally and is considered to be induced by high transcription levels of the gene (Vaucheret *et al.*, 1997; De Paoli *et al.*, 2009). The co-suppression efficiencies do, however, vary greatly from gene to gene (De Paoli *et al.*, 2009). Further studies could clarify whether *PAPhy_a* is prone to co-suppression. Crossings between different lines with several copies of *PAPhy_a* and subsequent generation of double haploids combined with quantitative gene expression analysis can allow for the generation of a linear dosage series that may establish the transcript levels required to trigger the co-suppression.

We used the marker gene elimination method to obtain marker-free plant lines. The plants were co-transformed with the genomic clone of *PAPhy_a* and the hygromycin resistance

(a)

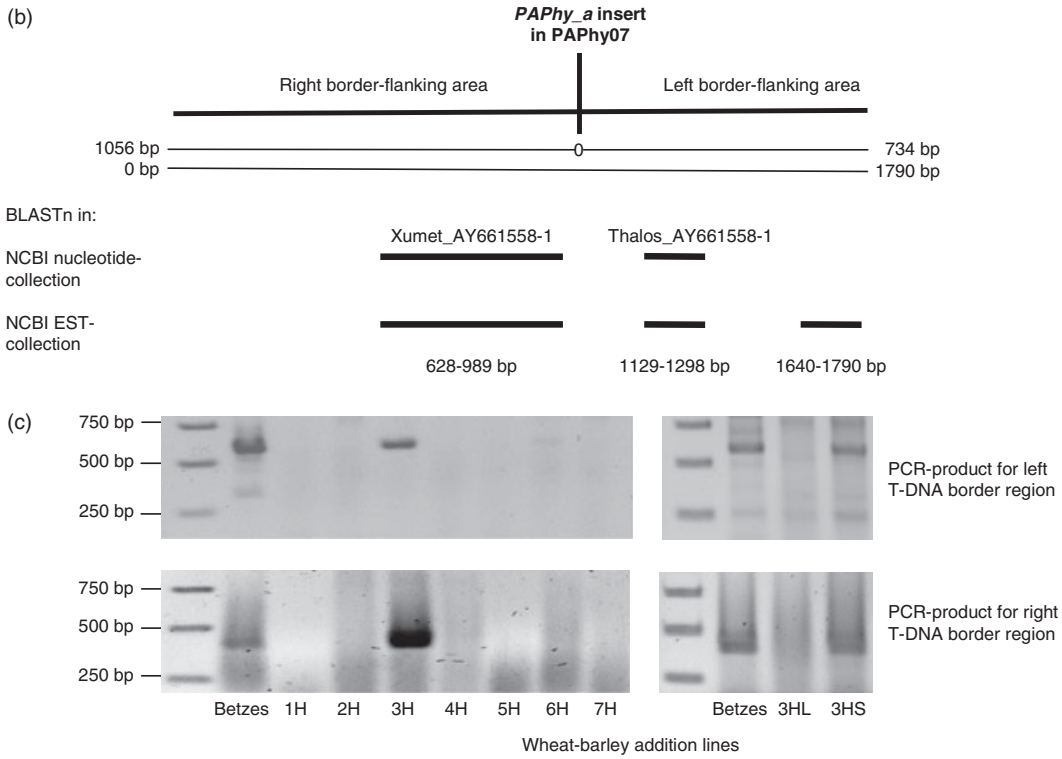
Left T-DNA border region

***Not*I-site** **Left T-DNA border** **Left T-DNA border**
GCGGCCGCAGGAAC***GTTTACACCACAATATATCCTGCCA***. (25 bp).***GTTTACACCACAATATATCCTGCCA***AGATCTTACGT
PAPhy07:
GCGGCCGCAGGAAC***GTTTACACCACAATAT***TATCGGCCATACACACACTAAACAGTACGTATTGCATTTCAAGGTTAAATTC

Right T-DNA border region

***Apa*I-site** **Right T-DNA border**
GGGCCCTCGAGTCGACGTTCCT***TGACAGGATATATTGGCGGGTAAAC***TAAGTCGCTGTATGTGTTTGTTTGAGATCCTCTA
PAPhy07:
GGGCCCTCGAGTCGACGTTCCT***TGA***ACTTTCCGGATTACTGTGCATGCTCTCTTGTAATTGTCTTCTTGATTTTTACTGATGG

(b)



(c)

(d)

TGTGTGTATGGCCCGATAATATT (ACGCGCGAGCAACAAACAACGG) ACTTTCCGGATTACTGTGCAT **Pre-insertion site**

TGTGTGTATGGCC–GATA***ATATTGTGGTGTAAAC-****PAPhy_a*-insert-***TGA***ACTTTCCGGATTACTGTGCAT **Insertion site**

Figure 6 Sequence analysis of the insertion site and the flanking regions of the *PAPhy_a* insert in the plant PAPhy07. (a) Sequence alignment of the left and right T-DNA borders of the vector pClean-S185-*PAPhy_a* and the flanking regions of the *PAPhy_a* insert. (b) BLASTn of the flanking regions of the *PAPhy_a* insert. BLASTn in NCBI nucleotide collection: *Hordeum vulgare* accessions showing alignments to the 628–989 region at *E*-values smaller than 1e−20: AY643844.1; FJ477093.1; FJ477092.1; AY661558.1; AB370200.1; AJ310994.1; AF509769.1; AF427791.1; EU593537.1; AK251967.1; AK251019.1. *Hordeum vulgare* accessions showing alignments to the 1129–1298 region at *E*-values smaller than 1e−20: FJ477093.1; FJ477092.1; AY661558.1; AF427791.1; EU812563.1; DQ249273.1; AY641411.1. BLASTn in NCBI EST collection: *H. vulgare* accessions showing alignments to the 628–989 region at *E*-values smaller than 1e−20: BJ478605.1; BU992600.1; EX595901.1; BJ464188.1; CK567670.1; BU998094.1; CB877936.1; BJ486668.1; BJ486638.1; DN159683.1. *Hordeum vulgare* accessions showing alignments to the 1129–1298 region at *E*-values smaller than 1e−20: BY864230.1. *Hordeum vulgare* accession showing alignment to the 1640–1790 region at an *E*-value of 1e−66: AJ464005.1 (c) Identification of the chromosome number and arm of the *PAPhy_a* insert using PCR analysis of DNA from the wheat–barley addition lines with primer pairs designed within the left-border-flanking sequences and the right-border-flanking sequences. (d) Alignment of the insertion site sequence of the *PAPhy_a* insert and the pre-insertion site sequence. Nucleotides in bold are the sequences from the left and right border T-DNA integrated into the genome. The 22 nucleotides in brackets are nucleotides deleted by the *PAPhy_a* insertion. The five-nucleotide overlaps between left border and plant DNA are underlined.

gene flanked by their own T-DNA borders on separate pClean vectors (pClean-G185 and pClean-S166; Thole *et al.*, 2007). In this way, unlinked integrations are possible, allowing for the segregation of the two genes in the following generations. The co-transformation frequency of 73.6% obtained in the present study is similar to frequencies in studies where marker-free

transformants have been obtained using a two-vector/one-*Agrobacterium* strain approach in rice (Afolabi *et al.*, 2004) and a one-vector/one-strain approach in barley (Matthews *et al.*, 2001). Moreover, the observed frequency of unlinked integration (60%) is similar to the one reported in rice using a one-vector/one-strain approach (Komari *et al.*, 1996) and higher than the 22%–24% obtained using a two-vector/one-strain approach in rice (Afolabi *et al.*, 2004) and a one-vector/one-strain approach in barley (Matthews *et al.*, 2001). However, it should be stressed that the 60% frequency of unlinked integration in our study is based on the segregation analysis of only five lines.

The vector pClean-G185 used for *PAPhy_a* insertion carries two left T-DNA borders to reduce read-through of the left border resulting in the transfer and integration of vector-backbone sequences (Kuraya *et al.*, 2004). Vector-backbone integration in barley has previously been investigated using a vector with the normal single left border sequence. In that study, 48% of the lines that possessed intact copies of the T-DNA also contained vector-backbone (Lange *et al.*, 2006). This frequency compared to the 52.2% found in the plants showing intact integration of the *PAPhy_a* inserts does not suggest a reduction in read-through by the two left borders of pClean-G185.

In the present study, we were able to identify two hygromycin- and kanamycin resistance gene-free T_1-lines containing a single *PAPhy_a* insert. Although the kanamycin resistance gene was not present in the two lines, short truncated vector-backbone sequences linked to the left border and sometimes to the right border are frequently reported (Kim *et al.*, 2003; Wu *et al.*, 2006; Petty *et al.*, 2009). Isolation of flanking regions of the single *PAPhy_a* insert in the two marker-free lines was therefore attempted. However, we were only able to isolate 188 bp of the RB region of the *PAPhy_a* insert in the marker-free PAPhy05 plant line, which showed insertion in a very AT-rich area (data not shown). The left border region could not be isolated with the primers used in this study, indicating some rearrangements in the left border region. Hence, we do not know whether the plant line contains truncated sequences of vector-backbone linked to the left border. We can therefore not unequivocally classify the plant PAPhy05 as *cisgenic*.

Flanking regions were isolated from both the left and right T-DNA border sites of the *PAPhy_a* insert in the marker-free plant line PAPhy07. The flanking sequences did not show any homology to vector-backbone sequences of pClean-G185-*PAPhy_a*, revealing that there was no read-through into the vector-backbone at neither the left nor the right T-DNA borders in this plant line. The marker-free transformed plant line PAPhy07 can therefore be classified as a *cisgenic* plant line according to the definitions of Schouten *et al.* (2006).

It is apparent that it is very difficult with present methods to generate *cisgenic* plants that are without rearrangements at the T-DNA integration site. A thorough study of T-DNA insertions in *Arabidopsis* showed rearrangements in the target sequence in 97% of the integration events, the most common being deletions, but duplications of the target site sequences and even major chromosomal rearrangements such as translocations were also observed (Forsbach *et al.*, 2003). The results of our study showed that the integration of the *PAPhy_a* insert into the PAPhy07 plant line did not induce translocations because the flanking regions of both the right and the left T-DNA borders mapped to the same chromosome, that is, the short arm of chromosome 3H. However, the integration did induce a 22-bp deletion at the target site. Still, the BLASTn searches of the target site–flanking sequences in the NCBI databases did not indicate that the *PAPhy_a* insert interrupted any coding sequence and the phenotype of the plant was completely normal.

It is also apparent that it is not possible with the method used in the present study to generate plants that are totally devoid of foreign DNA. In PAPhy07, a total of 19 T-DNA border nucleotides and 36 synthetic nucleotides were integrated into genome. The *Agrobacterium*-derived T-DNA nucleotides could have been avoided by substituting the borders with P-borders isolated from a sexually compatible gene pool as is required for *intragenesis*. However, the presence of at least a few extra synthetic nucleotides for cloning is difficult to leave out. Moreover, we also cannot exclude the presence of truncated sequences of T-DNA-unlinked vector-backbone or *Agrobacterium* chromosomal sequences in the PAPhy07 genome as have been reported in some studies (Kononov *et al.*, 1997; Ülker *et al.*, 2008; Petti *et al.*, 2009). However, such T-DNA unlinked sequences could be eliminated by backcrosses to the parent or to breeding lines. Current progress in genome sequencing will enable the detection and thus facilitate the elimination of such foreign sequences.

In summary, we have documented that genetically modified barley lines can be obtained according to the *cisgenesis* concept. The introduced gene, a phytase, caused a 2.8-fold increase in the phytase activity of the grain, and the increase in activity was stable over three generations. This illustrates that increasing dosage by the insertion of extra copies of an endogenous gene might be a useful tool for crop improvement.

The increase in phytase activity from a single extra *PAPhy_a* insert amounted to around 2200 FTU/kg flour. Normally, microbial phytase is added to the feed at a concentration of 1500 FTU/kg, which makes about 60% of the phosphate bound in phytate bioavailable (Kerr *et al.*, 2010). This implies that the current approach will be commercially interesting for farmers who do not purchase commercial phytase-supplemented feed but mix their own feed from home-grown cereals. If needed, the number of *PAPhy_a* inserts can easily be increased by crossing different *cisgenic* lines followed by double haploid production until a possible threshold for increased phytase activity is reached.

Although we only selected two potentially *cisgenic* lines for further analysis in the present study, the methods used to generate *cisgenic* plants seemed to be quite efficient. In the total material of 72 T_0-lines, we obtained 19 plants that were co-transformed with both T-DNAs, expressed the *PAPhy_a* insert and did not show the PCR product of the kanamycin resistance gene from the vector-backbone of pClean-G185-*PAPhy_a*. Based on the 60% unlinked integration frequency obtained, we can predict that it should be possible to select 11 potentially *cisgenic* T_1-lines out of the 72 T_0-lines obtained.

Experimental procedures

Identification and isolation of the genomic *HvPAPhy_a*-gene

A lambda phage genomic barley library was used for the isolation of the genomic *HvPAPhy_a* clone (Stratagene no. 946104; Stratagene, Cedar Creek, TX). The DNA library was made from the barley winter cultivar Igri and contains 9- to 23-kb inserts of genomic barley DNA cloned into the Lambda FIX®II/*Xho*I partial fill-in vector kit (Stratagene). The whole library can be

represented in 2×10^6 plaque-forming units (PFU), so 40 (12 × 12 cm) plates with 50 000 PFU per plate were used for the initial screening. The probe used was the cDNA of *PAPhy_a* isolated and characterized by Dionisio *et al.* (2011). Transfer of the PFU to filters, hybridization, screening and selection of positive plaques was done according to the procedure of Sambrook *et al.* (1989). The initial PFU selection was followed by two additional selections where the selected PFU were diluted to 1000 and 50 plaques per 9-cm plates, respectively. The probe was labelled with [^{32}P] using Ready-To-Go DNA labelling beads (Amersham Biosciences, Piscataway, NJ). Two PFU-clones hybridizing with the cDNA of *PAPhy_a* in the third selection were amplified using the lambda DNA isolation kit (Qiagen Denmark, Copenhagen, Denmark). These clones were sequenced and both clones contained the genomic *PAPhy_a* gene. We sequenced 2708 bp of the promoter region, 2266 bp of the coding region and 878 bp of the terminator region (Figure 1b).

Agrobacterium transformation vectors

The vector-system pClean based on the pGreen/pSoup system was used for transformation (Hellens *et al.*, 2000; Thole *et al.*, 2007). Details of the pClean vector-systems are available on the pClean website (http://www.jic.ac.uk/staff/philippe-vain/pclean/pclean1.htm). The vectors pClean-G185 and pClean-S166 were chosen as vector-pairs (Figure 1a). The pClean-S166 vector contains a hygromycin resistance gene with a NOS promoter and a NOS terminator within its T-DNA and a tetracycline resistance gene in the vector-backbone (Thole *et al.*, 2007). The pClean-G185-vector contains two left T-DNA borders, a multiple cloning site with six different cloning restriction sites within the T-DNA and a vector-backbone with a *virG*wt gene and a kanamycin resistance gene (Thole *et al.*, 2007). The genomic *PAPhy_a* gene was amplified by PCR from the selected lambda clone using primers with flanking restriction sites corresponding to restriction sites within the T-DNA of pClean-G185. We chose the *Not*I and the *Apa*I as cloning sites. The primer at the promoter site was flanked with the *Not*I restriction site sequences, and the primer at the terminator site was flanked with the *Apa*I restriction site sequences. Both flanking restriction sites were followed by additional nucleotides to ensure the cutting of the restriction sites. The sequence of the primer pairs and the PCR conditions are shown in Table S1. The primer pairs amplified a 5208-bp product of the genomic *PAPhy_a* gene (Figure 1b). The PCR product was subsequently inserted into the *Not*I/*Apa*I-cloning site in pClean-G185 using standard procedures. The insertion was checked by sequencing. This vector is named pClean-G185-*PAPhy_a* throughout this study. The *Agrobacterium* strain AGL0 was co-transformed with the pClean-G185-*PAPhy_a* and the pClean-S166 vectors using the freeze/thaw method and selected on medium with 50 mg/L kanamycin and 7.5 mg/L tetracycline according to Thole *et al.* (2007).

Barley transformation

The spring cultivar Golden Promise was grown in growth cabinets at 15 °C at day and 10 °C at night with 16-h light period at a light intensity of 350 μ E/m^2/s. Immature embryos isolated 12–14 days after pollination were used for *Agrobacterium* transformation following the procedure of Bartlett *et al.* (2008) with the exception that 500 μM acetosyringone was included in the CI medium used for co-cultivation (Hensel *et al.*, 2008), 30 mg/L hygromycin was used instead of 50 mg/L in the transition, regeneration and rooting medium, and 0.1 mg/L benzylaminopurine was added to the regeneration medium. The plants that developed roots in the rooting medium were transferred to the greenhouse.

PCR analysis of transformants

Four primer pairs were used for PCR analysis of the T_0-plants: (i) hygromycin resistance gene primers to amplify a 727-bp fragment of the *Hpt*II gene (Vain *et al.*, 2003); (ii) TE-primers (Figure 1b) to amplify a 458-bp fragment of the TE region of the *PAPhy_a* insert including the *Apa*I-cloning site in the reverse primer; (iii) PE-primers (Figure 1b) to amplify a 747-bp fragment of the promoter-end region of the *PAPhy_a* insert including the *Not*I-cloning site in the forward primer; and (iv) kanamycin resistance gene primers to amplify a 694-bp fragment of the *nptII*-gene. T_0-plants setting seeds and showing all four PCR products of the primer pairs were further analysed in the next generation to eliminate PCR false positives caused by the presence of *Agrobacterium* in T_0-plants. Four to ten T_1-progeny from each of these T_0-plants were PCR-analysed with the *PAPhy_a* insert and *npt*II-gene primers.

Segregation analysis between the hygromycin resistance genes and the *PAPhy_a* inserts was performed on progeny from five T_0-plants. T_1-progeny were PCR-analysed using the primers described above.

The two selected hygromycin- and kanamycin resistance gene-free plant lines were further propagated to obtain lines homozygous for the *PAPhy_a* insert. Ten to twenty progeny from each marker-free T_1-plants were analysed for the *PAPhy_a* insert by PCR. One homozygous T_1-plant from the PAPhy05 line was identified in the T_2-generation. For the PAPhy07-plant line, T_3-plants were analysed in the same way, leading to the identification of a homozygous T_2-plant. Plant lines homozygous for the *PAPhy_a* insert were PCR-analysed again with the four different primers described above and a primer pair amplifying a 711-bp fragment of the tetracycline resistance gene located in the vector-backbone of pClean-S166. All primer sequences and PCR conditions used are shown in Table S1.

Analysis of gene expression in transgenic plants by phytase activity assays

The phytase activities were analysed in the crude protein extracts from milled mature seeds according to Brinch-Pedersen *et al.* (2000) and Engelen *et al.* (1994). The phytase activity in seeds of all plants was determined in duplicate. Random samples of 20–25 seeds from each transformant were milled and the flour was used for phytase activity analysis.

Genomic DNA gel blot analysis of the marker-free plant lines

Genomic DNA gel blot analysis was performed to confirm the presence of a single non-tandem inserted *PAPhy_a* insert and the absence of the hygromycin resistance gene in each of the marker-free lines. Genomic DNA (15 μg) from the two marker-free plants homozygous for the *PAPhy_a* insert and a transgenic control plant containing the hygromycin resistance gene but not the *PAPhy_a* insert was digested with *Hin*dIII and separated on a 1% agarose gel. *Hin*dIII cuts the *PAPhy_a* insert into two fragments. One fragment of 2447 bp included the whole promoter region and a small part of the coding region and one fragment of 2761 bp included the rest of the coding region

and the terminator region (Figure 1b). Transfer to Hybond N+ and hybridization were performed as described by Holme *et al.* (2006). The probe used for the *PAPhy_a* insert was a ^{32}P-labelled 1358-bp PCR product from the promoter region located within the 2447-bp *Hin*dIII fragment (Figure 1b). For primer details, see Table S1. The probe should hybridize to a 2892-bp fragment of the endogenous *PAPhy_a* because a *Hin*dIII site is located 2892 bp upstream of the *Hin*dIII site in the coding region (Figure 1b). For tandem insertions of the *PAPhy_a* insert in one locus, the probe should hybridize to fragments of around 6 kb. The hygromycin resistance gene probe was a 420-bp fragment released by the digestion of pVec8GFP (Murray *et al.*, 2004) by *Bam*HI and *Pst*I labelled by Ready-To-Go DNA labelling beads (Amersham Biosciences).

Identification and analysis of the flanking areas of the *PAPhy_a* insert in the PAPhy07 marker-free plant line

Flanking regions of the *PAPhy_a* insert in the PAPhy07 marker-free plant line were amplified using the DNA Walking SpeedUp™Premix Kit (Seegene Germany, Eschborn, Germany). The kit provides a PCR master mix and primers for the unknown flanking sequences. We designed three nested primers in the known sequence for both the promoter and terminator region of the *PAPhy_a* gene (Table S1). The final PCR products were cloned into TOPO®TA® cloning kit (Invitrogen, Carlsbad, CA) and the cloned products were sequenced. Flanking sequences were blasted against the NCBI database using BLASTn, and homology to published barley sequences was analysed. The wheat–barley chromosome addition lines made by hybridization between Chinese spring wheat and barley cv Betzes (Islam *et al.*, 1981; Islam and Shepherd, 2000) were used to identify the barley chromosome in which the *PAPhy_a* insert of PAPhy07 had integrated. Primer pairs (Table S1) were designed within the left-border- and right-border-flanking regions of the *PAPhy_a* insert to amplify a 557-bp PCR product of the left border region and a 395-bp PCR product of the right border region, respectively.

Primers (Table S1) designed in the flanking regions to reach over the site of the *PAPhy_a* integration in line PAPhy07 were used to amplify the pre-insertion site in the non-transgenic parent. The primer pairs amplified a 1526-bp fragment. The amplified PCR product was cloned into the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) and the cloned product was sequenced.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 PCR analysis of the PAPhy05 and PAPhy07 plants homozygous for the *PAPhy_a* insert.

Figure S2 Southern blot analysis of the PAPhy05 and PAPhy07 plants homozygous for the *PAPhy_a* insert.

Table S1 Sequences of forward and reverse primers, PCR-product size and conditions for the PCR reactions used in this study.

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