



REVIEW ARTICLE

Targeted genetic manipulation of phytic acid biosynthesis in cereal crops

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Abstract

Phytic acid or myo-inositol hexakisphosphate (IP6) is the predominant storage form of phosphorus in cereal grains. It is considered a major anti-nutritional factor as it chelates essential nutrients such as iron, zinc, calcium and magnesium, thereby reducing their bioavailability in the human diet. This reduction in mineral uptake contributes to micronutrient deficiencies, commonly referred to as “hidden hunger”, which has become a major concern in many developing countries, despite significant efforts to enhance the nutritional quality of cereals. Elevated levels of phytic acid can exacerbate micronutrient malnutrition in populations heavily dependent on cereal-based diet. Traditionally, post-harvest processing techniques, such as soaking, fermentation, milling and conventional breeding approaches such as germplasm screening and the use of low phytate mutants were used to develop low phytic acid levels cereal varieties. Nevertheless, the agronomic and economic trade-offs of these methods created an urgent need to shift to genetically safe approaches. Genomic manipulation techniques, including marker-assisted breeding and genome editing tools such as CRISPR/Cas systems, offer significant potential to precisely target genes involved in phytic acid biosynthesis and transport. These techniques enable the development of low-phytic-acid cereal varieties with improved mineral bioavailability and enhanced nutritional quality. Recent studies have demonstrated the efficiency of these techniques in manipulation of *MRP* and *IPK* gene family resulting up to 85 % and 60 % phytic acid reduction in wheat and rice respectively. Since cereals play a significant role in food system transformation, this review emphasises the scope of these techniques in breeding low-phytic-acid cereals for global nutritional sustainability.

Keywords: biofortification; cereals; genome editing; nutritional security; phytic acid

Abbreviations: ANF: Anti nutritional factor; CRISPR: Clustered regularly interspaced short palindromic repeats; DEGs: Differentially-expressed genes; DNA: Deoxyribonucleic acid; DSBs: Double-stranded breaks; GM: Genetic modification; GMOs: Genetically modified organisms; HDR: Homology dependent repair; LCMS: Liquid chromatography mass spectroscopy; LPA: Low phytic acid; MAB: Marker assisted breeding; MABB: Marker assisted backcross breeding; MIKs: Myo-inositol kinases; mRNAs: MicroRNAs; MRPs: Multi-drug resistance associated proteins; NHEJ: Non-homologous end joining; PLC: Phospholipase C; PLD: Phospholipase D; QTLs: Quantitative trait loci; RILs: Recombinant inbred lines; RISC: RNA-induced silencing complex; RNA: Ribonucleic acid; RNAi: RNA interference; SNP: Single nucleotide polymorphism; TILLING: Target induced local lesions in genome; TALENS: Transcription activator-like effector nucleases; WHO: World health organization; ZFN: Zinc finger nucleases.

Introduction

Cereals such as rice, wheat, maize, barley, sorghum and millets constitute a fundamental component of the global diet, providing essential calories and nutrients. The top 3 cereals, wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.), are the major and staple food crops of most people across the world (1). They play a significant role in food system transformation owing to their vital role in the human diet (2). Beyond food sufficiency, the nutritional quality and bioavailability of essential nutrients in staple crops are critical determinants of dietary quality (3). Although the

consumption of healthy food increases nutritional uptake, the bioavailability of nutrients and their absorption from foods also play an essential role in curbing malnutrition. For example, inadequacy of zinc and iron bioavailability in the human system causes restricted growth, immune disorders, high mortality and reduced working efficiency in pregnant women and children being highly affected (4). According to the World Health Organization (WHO, 2022) reports, nearly 2 billion, i.e. 30 % of the world population are suffering from micronutrient malnutrition (5). The consumption of healthy food, to strengthen the immune system, is now a major focus worldwide and is essential to tackle the emerging pandemic concerns. However, the

presence of phytic acid (PA) (myo-inositol hexakisphosphate, IP6) in significant quantities within cereal grains presents a considerable anti-nutritional challenge, as it chelates essential micronutrients, thereby reducing their bioavailability (6). This chelation activity, particularly with phosphorus, iron, calcium and zinc, impedes nutrient absorption in monogastric animals and humans alike, negatively impacting the nutritional quality of these staple foods (7). The PA chelates micronutrients, forming insoluble complexes that resist digestion, thereby lowering mineral absorption in the human gut (8). Thereby, populations relying heavily on cereal-based diets are at higher risk of micronutrient deficiencies such as iron-deficiency anemia and zinc deficiency, contributing to widespread “hidden hunger” (9). Consequently, genetic manipulation strategies aimed at reducing PA content in cereals have emerged as a critical area of research to enhance the nutritional value of these crops and address micronutrient deficiencies globally (10). A deeper understanding of the PA biosynthetic pathway, including the identification of key enzymes and regulatory genes, is crucial for developing effective strategies to manipulate its content in cereal grains (11). In this context, this review provides an elaborate understanding of the dual role, mechanism of PA accumulation and the genetic advancements in PA reduction, while retaining the agronomic performance intact.

Anti-nutrients' role in shaping nutritional quality

Although considerable efforts are being made to enhance the micronutrient levels in crops, the bioavailability of nutrients in the human body often remains limited. A primary constraint on nutrient bioavailability is the presence of compounds known as anti-nutritional factors (ANFs) in plants. The plant produces certain secondary metabolite compounds for its self-defense against excessive grazing and protection from external factors such as animals and pests, called “anti-nutrients”. They are the compounds that bind to the nutrients in foods, making them less available for absorption by the human body (12). Major anti-nutrients in cereal grains include phytates, tannins, oxalates, polyphenols, saponins and trypsin inhibitors. Anti-nutritional factors mainly function by forming chelates with mineral ions like Fe^{2+} , Zn^{2+} , Mg^{2+} and Ca^{2+} and by reducing protein digestibility. Besides their ability to reduce various minerals and nutrients, these anti-nutrients can cause toxicity when present in higher amounts in the diet (13). They serve as a part of the defense mechanism in plants, protecting them against fungal and microbial attacks. However, in humans, they play a dual role, depending on the type and quantity of anti-nutrients consumed. Considering the negative effects of anti-nutrients on the bioavailability of various essential nutrients, there is a surging need to discover ways to reduce their levels to become non-limiting factors. However, the challenge is to reduce their levels without hampering the growth and development of plants due to their non-availability (14).

Phytic acid: Role in the plant system and human health

Phytic acid is considered the major anti-nutrient in most of the cereal grains due to its negative impact on micronutrient bioavailability in grains, especially iron and zinc. Phytate (Inositol hexaphosphate, IP6) is the salt form of PA and is found in animals, humans, plants and soil. Phytate or PA is formed either from myoinositol-1-phosphate or from phosphatidyl inositol in most plant species. It accounts for 50–80 % of the total phosphorus in different cereals (15). An experimental evidence was provided by previous researchers (16) through their comprehensive study that 65–90 % of the total P is stored as PA in cereals and oilseeds and

that it can be up to 1000 times more concentrated in seeds than in vegetative tissues like roots, stems and tubers. All of the major millets, including sorghum (900–2200 mg per 100 g of uncooked jowar), pearl millet (about 700 mg per 100 g of bajra) and finger millet (570 mg per 100 g of ragi), are regrettably high in PA (17). It accumulates mainly during the ripening period in the seeds. Due to its high negative charge at physiological pH (~6–7), PA easily precipitates in the form of phytate salts binding cations, such as iron, zinc, potassium, calcium, magnesium, some of them (mainly iron and zinc) important from a nutritional point of view, in this way reducing their bioavailability (9). The chemical structure of PA has an affinity for binding six free ions simultaneously, making them unavailable for absorption in the human body. The contents of these depend significantly on the type of grain (in order of decreasing magnitude of the ‘highest in range’ values: sorghum > wild rice > maize > rye > wheat > oat, barley (18). The human body lacks sufficient endogenous phytase activity to effectively hydrolyse phytate, resulting in reduced bioavailability of essential minerals. Generally, PA affects the bioavailability of minerals and has a strong effect on infants, pregnant and lactating women when large portions of cereal-based foods are consumed (19). The recommended safe consumption range for PA is approximately 250–800 mg per day (20). Latest references on PA intake in human diets confirm ranges of 200–2600 mg/day in Asia/African countries, with high-grain/vegetarian diets at 1000–2000+ mg, while it ranges 600–800 mg in western diets (21–23). Studies report that low-phytate genotypes significantly improve the bioavailability of key minerals, including iron, zinc and calcium, with increases of 30 % to 50 % compared with standard phytate genotypes (24). Nevertheless, it is necessary to reduce PA in foods to improve nutrient absorption and support normal growth and metabolism.

Mechanism of phytic acid biosynthesis and accumulation in cereal grains

Phytic acid is the most abundant and nutritionally significant anti-nutrient in cereal grains. Phytic acid accumulates mostly in the aleurone layer of monocotyledonous seeds (rice, millet, wheat, etc.). The PA is stored inside the vacuoles where it is transported by specific PA protein transporters, such as the MRPs (9). During the development of seeds, especially cereals, the cells become filled with starch grains and protein bodies present in aleurone layer, which contain PA that accumulates as the seed develops (15). Contrastingly, corn differs from other cereals as more than 80 % of PA is concentrated in the germ (25). The literature provides evidence that PA biosynthesis occurs mainly in the seed via lipid-dependent and lipid-independent pathways, which involve various enzymes at every step (26). Major differences between lipid-dependent and lipid-independent pathways of PA biosynthesis are elaborated in Table 1 (27).

In plants, the lipid-dependent pathway of PA biosynthesis is common in plant tissues and the lipid-independent pathway predominates in the seeds of cereals. A previous study on wheat testified that cereal grains predominantly synthesise PA by the lipid-independent pathway during seed development (28). This pathway initiates with myo-inositol-3-phosphate synthase (MIPS or INO1) converting glucose-6-phosphate to myo-inositol-3-phosphate ($Ins3P_1$), followed by dephosphorylation to free myo-inositol and sequential phosphorylations (28). The enzymes catalysing these phosphorylation reactions include inositol monophosphatase (IMP), inositol tris/tetraphosphate kinase (ITPK), IPK2 and inositol-pentakisphosphate 2-kinase (IPK1) (29). The Inositol pentakisphosphate kinase (IPK1) is

Table 1. Major differences between lipid-dependent and -independent pathways of phytic acid biosynthesis (27)

Event	Lipid-dependent pathway	Lipid-independent pathway
Occurrence	Operates in all the plant tissues (predominant during stress conditions)	Predominant in seeds (accumulated in aleurone/embryo)
Liquid involvement	Requires lipid synthesis	Does not involve lipids
Inositol source	Ins is converted to phosphatidylinositol (PtdIns) by a phosphatidylinositol synthase (PtdIS)	Independent of inositol lipid synthesis Involves sequential phosphorylation of Ins to InsP ₆ (by kinase activity)
Initial step	Myo-inositol 3-phosphate synthase (MIPS) converts glucose-6-phosphate into myo-inositol 3-phosphate (Ins3P ₁)	Ins is converted to INSP ₁ , catalysed by myo-inositol kinase (MIK)
Key enzymes involved	Phospholipase C (PLC), PtdIS and IPK2	MIPS, MIK, IPK1 and ITPK
Pathway route	Cytosolic (linear phosphorylation to InsP ₆)	ER/plasma membrane-associated, branches into early InsP3 intermediates
Impact of accumulation	Directs the main InsP ₆ pool in globoid crystals during grain filling	Supplements under P-deficiency; less InsP ₆ contribution

involved in the final step in PA biosynthesis, which is common to both lipid-dependent and independent pathways (30). Key genes controlling PA biosynthesis in cereals includes *MIPS/INO1* (myo-inositol -3-phosphate synthase): Initiates the pathway by converting glucose-6 -phosphate to myo-inositol-3-phosphate; IMP (inositol monophosphatase): Dephosphorylates myo-inositol-3-phosphate to free myo-inositol; ITPK (inositol tris/tetraphosphate kinase, e.g., TalTPK1/3/4): Adds early phosphate groups (e.g., to positions 5/6); IPK2 (inositol polyphosphate kinase/multikinase, e.g., TalPK2, OsIPK2); *IPK1* (inositol pentakisphosphate 2-kinase, e.g., TalPK1, ZmIPK1A/B); Final step converting Ins(1,3,4,5,6)P5 to InsP6 (Fig. 1). The key role of these genes in cereal crops is detailed below in Table 2. A different set of genes involved in PA transport such as MRP (e.g., TdMRP3/TaMRP3), PLC (TaPLC1) and PLD (phospholipase D, e.g., OsPLDα1).

Genetic manipulation of cereals for low phytic acid (lpa) levels

Several conventional post-harvest processing methods such as soaking, fermentation, malting and milling have historically been employed to reduce PA levels in cereal grains. A comprehensive overview of these processing techniques and their effects on anti-nutrient reduction in cereals was provided by earlier researchers (33). However, extensive human intervention trials conducted in recent years have generated strong evidence that consumption of genetically low-phytate grain-based foods results in significantly higher micronutrient absorption-particularly iron, zinc and calcium-compared to diets based on high-phytate grains (34–36). The critical outcomes of these studies clearly demonstrate that breeding low-phytate crop varieties represents the most effective and sustainable strategy for combating mineral deficiencies (35). Furthermore, recent advances in breeding and biotechnological approaches, including mutation breeding, RNA interference (RNAi), Clustered regularly interspaced short palindromic repeats (CRISPR-Cas9)-mediated gene editing, gene introgression through marker-assisted backcross (MABC) breeding and phytase enzymes have greatly facilitated the development of low-phytate cereal cultivars. An overview of current research progress in the development of low-PA cereal varieties is presented below.

Employing mutation breeding and chemical mutagenesis for low phytic acid cereals

Mutation breeding has been successfully applied to generate low PA mutants in crops like maize, rice and *Arabidopsis* (37). Chemical mutagens such as ethyl methane sulfonate are frequently employed to induce these genetic variations, as demonstrated by the identification of novel mutants like Pusa LPA Mutant 11 and the lpa5, lpa9 and lpa59 lines in rice (38, 35). Chemical mutagens, such as ethyl methanesulfonate, facilitate the induction of point mutations for single base changes within the DNA sequence (39). These induced mutations can affect genes critical to the PA biosynthetic pathway, leading to altered enzyme activity or expression, which subsequently reduces PA accumulation. For example, in rice, a novel allelic variant, lpa1-3, at the OsLpa1 locus was identified through genetic and molecular analysis of a low PA mutant variety developed via chemical mutagenesis. This variant harbored a point mutation (C623T) resulting in a significant reduction in seed PA content (40). Mutation breeding offers the advantage of creating novel genetic variations that may not naturally occur in existing germplasm collections (41). Furthermore, regulatory considerations favour mutation breeding over transgenic approaches, as chemically induced mutants are generally exempt from the stringent biosafety

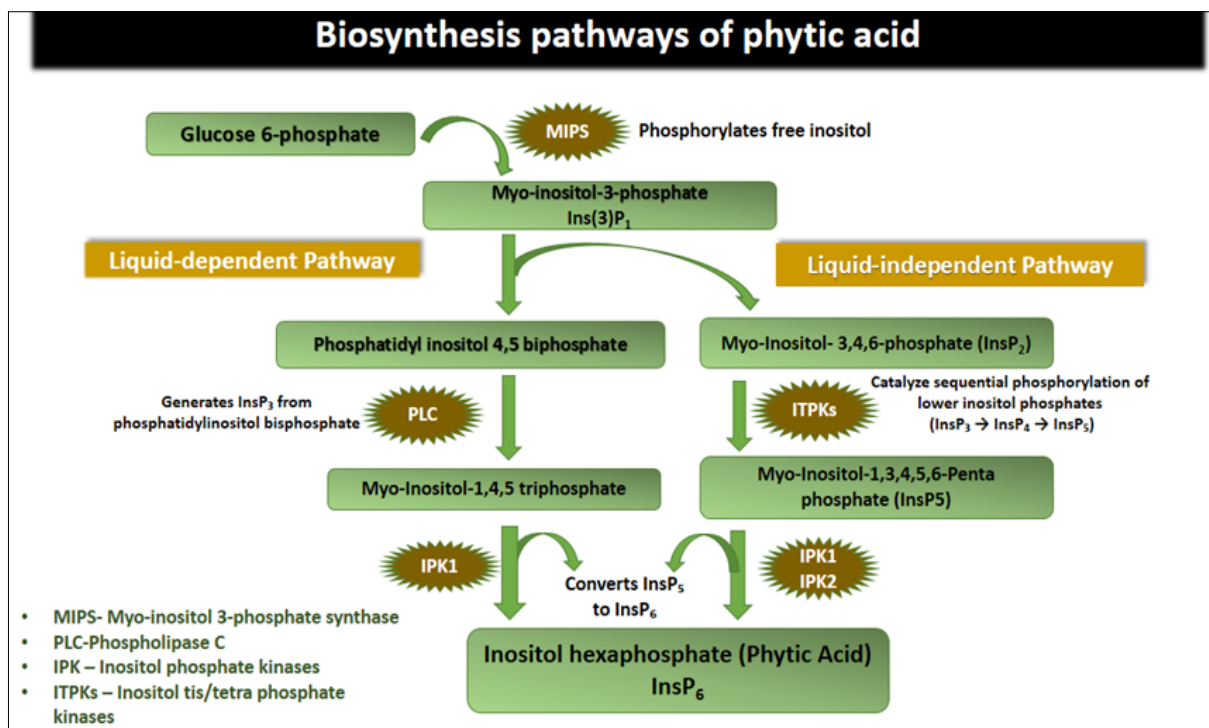


Fig. 1. Phytic acid biosynthesis pathways.

Table 2. Phytic acid (PA) biosynthesis and transporter genes in cereals

Gene family	Enzyme employed	Role of the enzyme in pathway	Cereal crop and the enzyme	Mutational effect	Reference
<i>MRP</i>	Multidrug resistance-associated protein (ABC transporter)	Accumulates InsP ₆ into globoids	TdMRP3/TaMRP3 (wheat)	Suppression of gene effect lowers PA in aleurone	(31)
<i>MIPS/INO1</i>	Myo-inositol-3-phosphate synthase	Glucose-6-P to Ins(3)P ₁	TaINO1 (wheat), OsINO1 (rice)	High expression induces PA; the variation is linked to single nucleotide repeats (SNPs)	(32)
<i>IPK1</i>	Inositol pentakisphosphate 2-kinase	Ins(1,3,4,5,6) P ₅ to InsP ₆ (final step)	TaIPK1 (wheat), OsIPK1 (rice), ZmIPK1A/B (maize)	Gene silencing results in PA reduction by 28-83 %	(28)
<i>PLC/PLD</i>	Phospholipase C/D	Lipid-dependent	TaPLC1 (wheat), OsPLDα1 (rice)	Alters the gene expression and directs into an alternative route	(20)
<i>IPK2</i>	Inositol pentakisphosphate 2-kinase	InsP ₃ to Ins(1,3,4,6)P ₄ or P ₅	TaIPK2 (wheat), OsIPK2 (rice), ZmIPK2 (maize)	Lowered InsP accumulation in mutants	(30)
<i>IMP</i>	Inositol monophosphatase	Ins(3)P ₁ to free myo-inositol	TaIMP (wheat)	Down-regulation of PA biosynthesis genes when exposed to stress	(30)
<i>ITPK</i>	Inositol tris/tetraphosphate kinase	Ins(1)P to Ins(1,4,5)P ₃ or Ins(1,3,4)P ₃ <i>i.e.</i> , early phosphorylation	TaITPK1/3/4 (wheat), OsITPK2 (rice)	Higher fold up-regulation of <i>TaITPK1</i> in grains; which results in early phosphorylation	(30)

regulations that govern genetically modified organisms (GMOs) in many jurisdictions (42).

Applications of mutation breeding in reducing phytic acid content in cereals

Mutation breeding has been successfully applied across various cereal crops, including maize, rice, barley and wheat, leading to the development of several low PA (*lpa*) lines (33, 37). Notably, the first low phytate genotypes were isolated in maize from chemically-mutagenised populations, which exhibited decreased PA phosphorus with a concomitant rise in inorganic phosphate. In rice, non-lethal *lpa* mutants have been obtained with PA content ranging from 42.1 % to 94.1 % relative to their original cultivars (37). Target induced local lesions in genome (TILLING)-induced complete null mutations in TdMRP3 homeo-alleles (A1/B1) of 2 durum wheat mutants (Kronos 3179/4443) reduce PA by 85 % in grains, boosting micronutrient accumulation (Fe +186 %, Zn +78 %, Mn +36 %) while

maintaining viable agronomics (31). This stands as the first non-GMO *lpa durum* wheat with superior mineral biofortification and TdMRP3 (ABCC13) confirmed as a key PA vacuolar transporter (conserved in cereals). In a recent study reported a novel mutation in the OsMRP5 gene (*LOC_Os03g04920*) in the Q3 (*lpa14*) mutant derived from the Super Basmati rice cultivar (24). They developed a CAPS marker that differentiates the Q3 mutants from the parent line, laying a solid foundation for marker-assisted breeding of low-phytate rice varieties. Furthermore, previous researchers successfully developed a double mutant of maize with *lpa1/lpa2* genes (43). The PA level was found to be reduced more in the double mutants (41 %) compared to individual mutants *lpa1* (36%) and *lpa2* (30%).

Despite its effectiveness in phytate reduction, mutation breeding presents certain challenges. The random nature of mutagenesis often necessitates extensive screening of a large plant population to identify individuals exhibiting the desired *lpa* mutation

(34, 44). Although exploring genetic variability offers opportunities to identify new phytate-reducing mechanisms, certain *lpa* mutations have been linked to significantly reduced germination and yield losses (27, 45). Therefore, careful selection is essential to eliminate negative side effects on the plant performance. A recent study in soybean suggests that moderately decreasing PA by targeting single genes, rather than pursuing ultra-low PA through multiplex mutagenesis, might be an optimal strategy to minimise trade-offs with yield performance (46). In essence, mutation breeding by randomly targeting and disrupting genes involved in the PA biosynthetic pathway offers a viable strategy for improving the nutritional profile of cereals.

RNAi-induced gene silencing for low phytic acid cereals

RNAi is a powerful and precise gene silencing tool employed to "suppress" or "silence" specific gene expression. This genetic tool offers great potential for changing gene expression in cereal plants for improving their nutritional quality and disease resistance (47). The fundamental principle of RNAi involves introducing double-stranded RNA (dsRNA) as a transgene into a target cell (48). Then the process typically initiates with the generation of small interfering RNAs (siRNAs), usually 20 to 25 base pairs in length, from genetic constructs that encode hairpin RNAs (hpRNA) (49). These siRNAs guide a protein complex, known as the RNA-induced silencing complex (RISC), to target and cleave target mRNA sequences, leading to stable, efficient and precise suppression of corresponding gene expression (48).

Applications of RNAi in reducing phytic acid content in cereals

RNAi technology has been effectively utilised to manipulate the PA biosynthesis pathway in various cereals. Research studies have successfully employed RNAi to target key genes in wheat. For instance, RNAi-mediated suppression of the inositol pentakisphosphate kinase (*IPK1*) gene in wheat, which is crucial in the PA biosynthesis pathway, led to a 40–65 % reduction in *TalPK1* transcript expression (28). The study also revealed a 56 % reduction in PA accumulation in wheat, as well as an elevation in the levels of grain Fe and Zn. Similarly, RNAi-mediated down-regulation of inositol triphosphate kinase genes *OsITP5/6K/1* and *OsIPK1* resulted in a significant 42 % reduction in PA content in rice grains (50). The *IPK1* gene is a frequent target for achieving phytate reduction because it encodes the most downstream enzyme in the PA formation pathway, catalysing the phosphorylation of inositol phosphates to higher phosphorylated forms like IP6 (phytic acid) (51). Mutations in the *MIPS1* and *IPK1* genes have shown to dramatically decrease PA concentration by disrupting the biosynthesis pathway (52). Overall, RNAi continues to remain a valuable and effective tool in the genetic manipulation of cereals for developing low PA varieties, thereby contributing significantly to enhancing the nutritional value of these critical food crops (53, 54).

CRISPR/Cas9-mediated gene editing for targeted modifications

While RNAi has proven to be a robust approach for reducing PA content in crops, next-generation genome-editing technologies, particularly CRISPR-Cas9 editing, are increasingly being explored as efficient alternatives. The CRISPR/Cas9 (CRISPR-associated protein 9) is a transformative gene-editing technology that enables accurate, site-specific genome modifications. The system functions by creating targeted double-strand breaks (DSBs), which are subsequently repaired by endogenous cellular pathways, enabling gene disruption (knockout) or precise sequence modification (gene editing) (55). The

CRISPR-Cas9 enables multiplex genome editing, allowing simultaneous manipulation of multiple genes, making it a promising strategy to overcome the limitations of single-gene modifications, particularly for complex traits such as PA accumulation (56). Owing to its high precision, efficiency, reproducibility and engineering feasibility, CRISPR-Cas9 has become a powerful tool for gene editing in crop plants (57). Furthermore, technology made it even easier to obtain transgene-free edited lines, where the Cas9 transgene can be segregated out in subsequent generations (58).

Gene editing broadly encompasses a suite of technologies that enable the precise addition, deletion, or alteration of genetic material within an organism's DNA (59). Over the past decade, several genome-editing platforms have been developed, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas systems (60). Among these, the CRISPR/Cas type II-A system derived from *Streptococcus pyogenes* is the most widely adopted. The type II CRISPR/Cas9 construction requires only a synthetic "linker loop or scaffold" that fuses the protospacer-containing crRNA and tracrRNA into single guide RNA (sgRNA) (61). This sgRNA, combined with the Cas9 endonuclease together enables sequence-specific DNA cleavage (62). Early applications of CRISPR-mediated gene silencing targeting PA biosynthesis pathways demonstrated promising outcomes. For instance, silencing of the *IPK6* gene using a seed-specific promoter (*Oleosin-18*) resulted in reduced PA accumulation in rice grains (63). Subsequently, multiple studies successfully employed CRISPR/Cas9-based approaches to reduce PA levels by targeting various low-phytate (*lpa*)-related genes in cereal crops, including rice (64, 65) and wheat (66). More recently, CRISPR-Cas9 targeted disruption of *TalPK1* gene resulted improved Fe and Zn absorption in wheat by reducing the PA accumulation (51). Collectively, these studies underscore the immense potential of CRISPR-Cas9-mediated genome editing as a precise and effective strategy for developing low-phytate cereal varieties with improved nutritional quality.

CRISPR/Cas9 mechanism of targeted gene editing

The CRISPR/Cas9 gene editing system operates as a molecular scissor, directed by an RNA molecule to induce precise changes in the DNA sequence. The core components of this system include: Guide RNA, Cas9 nuclease. Guide RNA (gRNA) is an engineered RNA molecule, often a single-guide RNA (sgRNA), consists of a CRISPR RNA (crRNA) that recognises the target DNA sequence through complementary base pairing and a trans-activating CRISPR RNA (tracrRNA) essential for binding with the Cas9 nuclease (55, 61, 67). This Cas9-sgRNA complex binds to the target site where sgRNA locates the homologous sequence typically 3 base pairs upstream of a protospacer adjacent motif site and creates a precise DSB (68, 69). Once a DSB is introduced, the cell's natural DNA repair mechanism gets activated. In case of the CRISPR-based gene editing system, there are two primary pathways for DNA repair. The DSBs can be repaired via homologous end-joining (HDR) repair pathway, predominantly seen during the late S and G2 phase of the cell cycle as sister chromatids can be used as a repair template or by non-homologous end-joining (NHEJ) repair dominant in somatic cells (70).

Homologous end-joining repair pathway: DSB repair by HDR pathways relies on a large region of homology between the broken DNA (adjacent to the DSB) and an intact template molecule, considered as the essentially error-free pathway (71). This pathway is comparatively less efficient than NHEJ and utilises an exogenously

Table 3. Genes targeted for altering phytic acid (PA) levels in major cereals

Crop	Target gene	PA level reduced (%)	Approach	Reference
Rice (<i>Oryza sativa</i>) Super Basmati	<i>OsMRP5</i>	54–63	Novel mutation	(24)
	<i>OsITPK6</i>	32	CRISPR-Cas9	(65)
	<i>SPDT</i>	20–30	Agrobacterium-mediated transformation	(64)
	<i>OsITPK1</i>	69	RNAi-mediated silencing	(63)
Wheat (<i>Triticum aestivum</i>)	<i>TaIPK1</i>	60–70	CRISPR-Cas9	(51)
	<i>TaMRP3</i>	85	TILLING	(31)
	<i>TaABCC13</i>	22–34	RNAi-mediated silencing	(66)
Maize (<i>Zea mays</i>)	<i>lpa1/lpa2</i>	41	Double mutation	(33)
	<i>lpa2</i>	34	MABB	(81)
	<i>lpa1</i>	44–56	MABB	(82)

supplied homologous DNA template to accurately repair the broken site, allowing for the introduction of precise changes, such as specific mutations or gene insertions (55).

Non-homologous end-joining repair pathway: This is the predominant repair pathway that occurs throughout the cell cycle. The pathway can be further classified into classical (cNHEJ) and alternative (aNHEJ), with both operating in an antagonistic manner. cNHEJ pathway not only protects the broken ends of DNA from degradation but also hinders the ligation of previously unlinked broken ends. A cNHEJ repair may result in small deletions or insertions next to perfect ligations, whereas repair via the aNHEJ pathway may result in larger deletions since microhomologies, present at the break sites, are used for annealing (72). These indels can disrupt gene coding sequences or regulation elements, causing frame-shift mutations or premature stop codons, effectively knocking out the gene (73).

Applications of CRISPR/Cas9 in reducing phytic acid content in cereals

The CRISPR/Cas9 has been successfully used to target genes in the PA biosynthesis pathway, reducing PA content and decreasing the bioavailability of essential micronutrients in several important crops. The strategy was successfully employed to reduce PA levels in wheat by disrupting genes such as *inositol pentakisphosphate 2-kinase 1*, thereby improving the bioavailability of grain zinc and iron (53, 74). CRISPR/Cas9-mediated gene silencing of *TaIPK1* achieved a significant reduction of PA in wheat grains (51). This, in turn, enhanced the bioavailability of micronutrients (1.5 to 2.1-fold increase in Fe and a 1.6 to 1.9 fold rise in Zn) by approximately a 60–70 % PA reduction in *TaIPK1A* transcript in the mature seeds of T_2 transgenic lines of wheat. The mutant lines provide a great source for developing biofortified wheat in the future. In rice, the CRISPR/Cas9 system has been utilised to generate mutants of a phospholipase D gene (*OsPLD1*). Metabolic profiling of these *ospld1* mutants exhibited substantially reduction in phosphatidic acid production and a lower PA accumulation compared to wild-type rice, alongside changes in the expression of major phytic acid biosynthesis genes (75). Simultaneous knockout of orthologs for phytate transport and phytate synthase coding genes, such as *IPK1* and *ITPK1*, in crops like maize, rice and wheat offers a promising strategy for significantly preventing excessive PA accumulation as well as phosphorous loss in grain (76). The key genes targeted for altering PA levels in major cereal grains are detailed below in Table 3.

The precision of CRISPR/Cas9 allows for highly targeted genetic modifications (GM), minimising off-target effects and enabling the development of crop varieties with improved nutritional profiles. Its ability to introduce specific mutations or even insert new sequences precisely offers a powerful avenue for crop enhancement. However, potential challenges remain unanswered. One of the key considerations is the potential risk of pleiotropic effects, where intended modifications for PA reduction might inadvertently impact other important plant yield traits or agronomic performance (75, 46). Researchers are actively working to optimise CRISPR/Cas9 strategies to achieve desired low PA traits without compromising crucial characteristics such as yield or stress resistance. The other greatest challenge faced is the public acceptance and awareness of the edited crops (26). Engaging farmers through training programs can strengthen extension programs and region-specific release of edited lines can reduce the scope of environmental exposure to the horizontal gene flow. Overall, the future policies and research pipelines should

simultaneously focus on this concern to enhance the practical implications of these edited crops in public domains.

Candidate gene identification and introgression

Understanding the genes involved in PA biosynthesis, their functional characterisation and targeting these genes for suppression could be one of the best strategies for reducing phytate (77). Candidate gene identification for *lpa* in cereals leverages multi-omics approaches like GWAS, transcriptomics, proteomics and metabolomics to pinpoint variants in biosynthesis/transport genes. The key candidate genes targeted for developing low PA lines in major cereal crops are listed in Table 3 below. Gene introgression transfers the selective genetic material from wild relatives or donors into the background of an elite plant variety (78). Gene introgression for *lpa* lines in cereals transfers validated *lpa* alleles (e.g., *lpa1/IPK1*, *lpa2/ITPK*, *MRP/ABCC*) from mutants/donors into elite backgrounds via marker-assisted backcross breeding (MABB), minimising linkage drag. Initially successful introgression of *lpa* alleles into elite background were conducted in cereal crops such as maize (79, 80), rice (64) etc. Recently, a successful introgression of the *lpa2* from the donor parent (LPA 2) to the genetic background of the recurrent parents BML 45 and BML 6 was achieved through MABB to develop a low PA maize by previous researchers (81). Simultaneously, they validated a foreground marker, *umc2230*, closely linked to the low PA-determining *lpa2* gene located on the short arm of chromosome 1 in maize. The NILs developed from MABB progenies of maize carrying the *lpa2* gene conferred low PA levels (2.37 mg g⁻¹ in BML 6 and 2.40 mg g⁻¹ in BML 45) compared to the parental lines BML 6 (3.59 mg g⁻¹) and BML 45 (3.16 mg g⁻¹). Later, another successful introgression of the *lpa1* mutant allele conferring low PA into the parental lines of popular maize hybrid DMH 121 (i.e., BML 6 and BML 45) through MABB was done by previous researchers (82). This introgression resulted in a 44–56 % reduction in the PA content compared to the parental lines. Marker-assisted gene pyramiding of *lpa1* & *lpa2* genes from donor inbred lines, namely, LPA1 (1.30 mg g⁻¹) and LPA2 (1.72 mg g⁻¹), into elite maize lines BML6 (3.59 mg g⁻¹) and BML45 (3.16 mg g⁻¹) was successfully achieved through a series of backcrosses and intercrosses (83). The study clearly differentiated that PA levels of the IC₁F₃ lines range much lower than those of the

recurrent parents and were slightly comparable to those of the donor parents. Although useful for transferring elite traits, naturally gene introgression carries negative impacts on agronomic and ecological aspects, which makes it less acceptable for the practical implications. The risk of linkage drag is one such drawback that introduces unwanted wild genes into the elite backgrounds alongside the targeted genes, leading to a yield and quality penalty. Moreover, the wild allele introgression may also threaten biodiversity via outcrossing or superweeds etc. Use of molecular markers for foreground (target gene), background selection and flanking markers to break the unwanted linked regions during backcrossing can purge the effect of linkage drag. Alternatives such as fine-tuning of alleles introgressed, multi-environment trials, ecological risk assessment and integration with genomic selection can help overcome the ecological risks through horizontal gene flow in the environment.

Utilising phytase enzymes in phytate reduction

The eventual opposition of GMOs in the commercial sector and social norms forced the research community to switch to otherwise safer breeding strategies. One such approach is the use of bio-enzymes viz. phytases to counteract the PA biosynthesis. The use of phytases provides a reliable alternative to GMOs. Phytase enzymes, also known as myo-inositol hexa-kisphosphate phosphohydrolase, catalyse the sequential release of phosphate from PA, resulting in lower inositol phosphate esters (IP₅ to IP₁) and free inorganic phosphate (Pi) (84) (Fig. 2). Thus, phytase enzyme not only makes phosphorus available for bone growth but also release the minerals bound to the PA, thus enhancing nutrient availability from plant-based diets (85). Phytase enzymes can be classified into different types based on the position of the phosphate ester bond they preferentially hydrolyse (e.g., 3-phytase, 6-phytase). They are naturally present in varying amounts in some plant species and microorganisms (bacteria, fungi). The enzymatic degradation process has two main benefits: increased mineral bioavailability and phosphorus availability. By breaking down the PA-mineral complexes, phytases release the bound minerals, making them bioavailable for absorption in the digestive tract, enhancing the nutritional value of plant-based diets. In addition, releases inorganic phosphate (Pi), which makes phosphorus more accessible. Purple

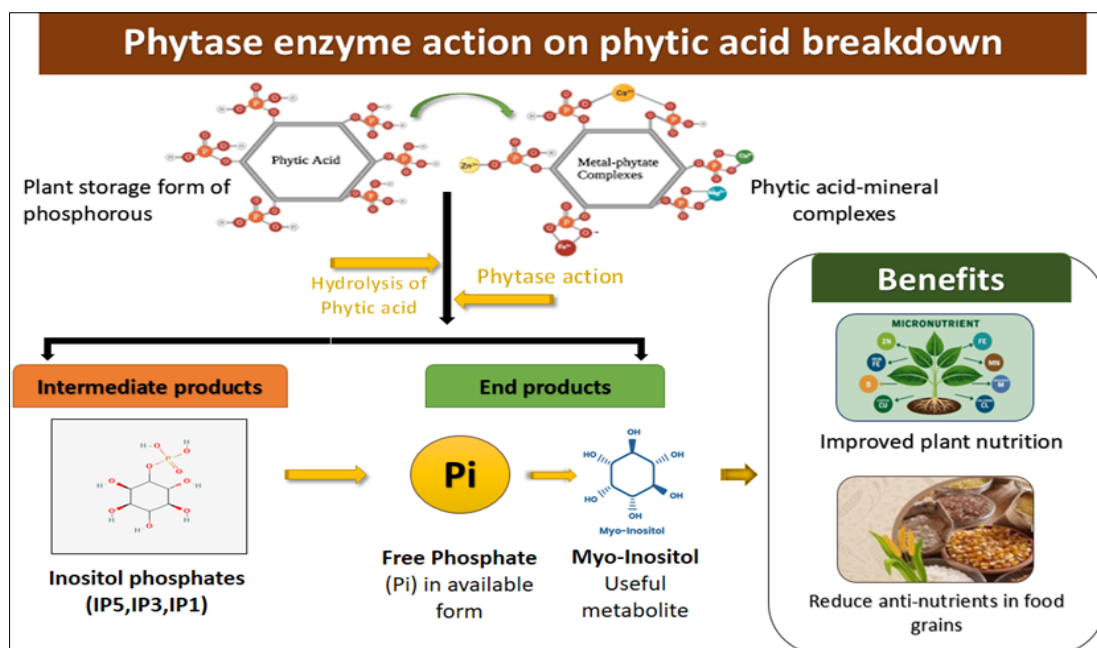


Fig. 2. Phytase action on breaking down the phytic acid-mineral complexes.

acid phosphatases (PAPs) family includes a set of phytases that can breakdown phytate activity (86). The PAPs that can hydrolyse phytate are known as PAPHy. Identified two sequence motifs (His229 in PAPHy motif 4 and Lys410 in the MPE domain) that strongly influence phytate activity (86). The study provide a substantial evidence of efficiency of engineering these motifs for enhancing phytase activity.

Applications of phytase enzymes in phytic acid reduction in cereals

The target plant can be genetically modified to express phytase genes often derived from microbial sources, enabling it to produce the enzyme internally. The major goal is for the plant to produce its own phytase enzyme, which can then act on the PA within the grain either during seed development, germination, or during digestion. The aim is to achieve internal degradation of PA, thus pre-digesting the anti-nutrient and improving the nutritional quality of the food or feed directly at the source level. This approach can also be referred as 'phytase biofortification'. This internal phytase activity can lead to a significant reduction in PA content and a corresponding increase in the bioavailability of essential minerals, making the crops more nutritious upon consumption. Adopting these enzymes in PA reduction is considered the more reliable and acceptable approach. A recent investigation also revealed the efficiency of phytase supplementation in enhancing the availability of trace elements (22).

Major challenges for phytic acid reduction in cereals

Unintended pleiotropic effects

A major challenge is mitigating the unintended pleiotropic effects of the gene modifications targeting PA biosynthesis. Genes involved in PA biosynthesis also contribute to plant growth, seed development and stress response, which can sometimes lead to undesirable outcomes. For example, while CRISPR-Cas9 can significantly reduce seed phytic acid, some higher-order mutants designed for this purpose, such as those in soybean, have been observed to exhibit poor agronomic performance (46). A CRISPR-Cas9-mediated mutation of the *OsITPK6* gene in rice has been revealed to have negative impacts on plant growth and reproduction (65). Therefore, a key challenge is to achieve a substantial reduction of PA without compromising crucial characteristics such as yield, plant vigor, or resistance to environmental stress (46). Research efforts are focused on optimising gene editing strategies to minimise these trade-offs, for instance, by targeting single genes for moderate PA reduction rather than pursuing complete elimination (46). Developing strategies that minimise unintended consequences is essential because disruption of protein-protein interaction networks often underlies the negative pleiotropic effects observed in natural or engineered mutants (51).

Consumer acceptance and regulatory considerations

The deployment of GMOs, including the biofortified and low-phytate genotypes, often faces public scrutiny and complex regulatory challenges. A substantial gap exists between the scientific community and the general public, indicating that genome-edited crops may face resistance unless a clear science-based campaign is launched and the public is informed of safety concerns associated the genome-edited crops (26). In developing countries, Cartagena Protocol-based biosafety regulations pose significant operational barriers to the testing and commercialisation of GM crops, including biofortified varieties (87). An extensive survey conducted previously to explore the public perception of genetically edited crops on varied participants from U.S, Japan and Germany (88). The survey revealed

a positive perception of US participants, while the Japanese and German participants demonstrated a negative perception of the acceptance of GM crops. A recent analysis also revealed that, Japan is currently far from full social acceptance of agricultural genome editing (89). These varied consumer acceptance patterns across regions, coupled with divergent global regulations, complicate commercialisation efforts. Understanding public perception and addressing concerns regarding the safety and environmental impact of these crops is paramount for their successful adoption. A very recent survey revealed that 76 % of the respondents rejected the GMOs, presuming them to be health hazard (90). The study also underscores the importance of prioritising safety verification and information disclosure in the public domain to boost acceptance. Furthermore, strict and often divergent regulatory frameworks across countries can impede the development, testing and commercialisation of GMOs (91). Navigating these regulatory landscapes efficiently and transparently remains a significant challenge.

Prospects of anti-nutritional breeding in cereals

Multi-omics approach

Advancements in genomics and other 'omics' technologies offers a comprehensive framework for the precise reduction of PA in cereal grains. Recent advances in high-throughput sequencing and omics technologies, including transcriptomics, proteomics and metabolomics, offer deeper insights into the internal networks and metabolic pathways that regulate PA biosynthesis and accumulation in grains. Genomics and transcriptomics enable the identification of key structural genes (e.g., *MIPS*, *IPK1*, *ITPK*) and transcriptional regulators controlling PA biosynthesis pathway and accumulation mechanisms. Proteomics approach complements transcript data by revealing post-translational modifications and enzyme abundance in the PA biosynthetic pathways, whereas metabolomics provides direct quantification of inositol phosphates and associated intermediates, offering insights into metabolic flux and pathway bottlenecks (92). A comparative proteomic study conducted by previous researchers using liquid chromatography-mass spectroscopy (LC-MS) coupled with 2-dimensional gel electrophoresis (2DE) analysis of low PA rice mutant line *Os-lpa-XS110-2*, along with its wildtype parent, Xuishui 110 (93). The study however, can identify only a set of proteins with an indirect effect on phytic acid biosynthesis. This knowledge can then be leveraged to design more precise and efficient gene editing strategies, accelerate breeding programs and minimise unintended consequences. Continued research in these areas will be vital for developing biofortified genotypes with optimal nutritional profiles and agronomic performance. Future research directions however may prioritise integrating multi-omics with genome editing to bypass the pleiotropic effects, such as reduced yield and poor seed quality that are frequently associated with conventional low-phytate mutants (56, 92).

Integrative approach

While genetic manipulation tools offer precise solutions, its' effectiveness can be enhanced by considering simultaneous agronomic measures such as soil nutrient management, fertilisation strategies and crop rotation. For example, understanding how nutrient availability in the soil affects PA accumulation can inform both breeding targets and necessary cultivation measures. This synergistic approach creates a more robust and sustainable system for producing nutrient-rich cereals combining genetic manipulation alongside improved agronomic management practices. Integrative breeding strategies aim to pyramid low PA with high Fe/Zn contents

and stress resistance while maintaining agronomic performance. For instance, an integrative study on wheat for nutritional traits, disease resistance and agronomic performance employing the natural variation available in the selected parent material was conducted earlier (94). The study highlighted 9 elite wheat lines that pyramided all desired genes with low PA (2.5 mg g^{-1}), elevated Zn (up to 68.24 ppm) and Fe (up to 49.8 ppm) levels, rust resistance without any severe yield penalty. In addition, 9 elite lines demonstrated an ideal combination of nutritional traits, rust resistance and favourable agronomic performance, offering significant potential for a biofortified wheat breeding program. Moreover, future programs may also be directed towards extensive human trials to understand the pattern of micronutrient absorption graphs in humans fed on low-PA diets. Overall, an integrative approach that incorporates all the discussed necessary measures will naturally accelerate the breeding of low-PA line development in cereals, as well as understanding the impact of the same on the bioavailability of essential micronutrients in the human system.

Conclusion

Phytic acid serves as the phosphorous storage compound in cereal grains and it's a major anti-nutrient in staple-cereal grains. Phytic acid plays a dual role in plant physiology and human nutrition, precise breeding strategies are essential to maintain its level without affecting plant metabolism or human nutrient availability. The advent of advanced genetic engineering strategies, including gene silencing via RNAi and precise genome editing using CRISPR/Cas9, has revolutionised tackling PA accumulation. The RNAi has successfully been deployed to down-regulate key genes in the PA biosynthesis pathway, leading to significant reductions in PA and enhanced mineral bioavailability in crops like wheat and rice. Similarly, CRISPR/Cas9-mediated gene editing offers unprecedented precision for targeted modifications, demonstrated by its application in cereal crops to disrupt phytate biosynthesis genes. Utilisation of phytase enzymes directly within cereal grains presents another promising avenue, as these enzymes can effectively degrade PA, releasing bound minerals and improving nutrient availability. However, it is crucial to consider the potential impacts of these genetic modifications on critical agronomic traits like yield and growth performance. In conclusion, the genetic manipulation of cereals to reduce PA represents a powerful and evolving field with immense potential to combat hidden hunger and enhance global food security. Continued interdisciplinary research, careful consideration of ecological and socioeconomic factors and transparent communication will be key to translating these scientific breakthroughs into widespread nutritional benefits.

Authors' contributions

NR and IK participated in the conceptualisation of the study. NR, IK, MBN carried out the primary drafting and editing of the text content. SS, EM, GP contributed to supervision and technical assistance for the preparation of manuscript. NR and IK contributed to the validation and critically reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used Grammarly and Jenni AI for grammar corrections, English language editing and formatting of the manuscript. After using these tools, the authors precisely reviewed, edited and validated the content with necessary corrections, taking full responsibility for the accuracy, originality and integrity of the publication.

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