Advances in Genetic Strategies for Reducing Phytic Acid Content in Maize: From Traditional Mutants to CRISPR/Cas9 Innovations

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Abstract

Maize is a globally significant crop with diverse uses ranging from food and feed to fuel. However, its high phytic acid content possesses nutritional and environmental challenges. Phytic acid sequesters essential nutrients like phosphorus, iron, and zinc, rendering them unavailable to non-ruminant animals and humans, and necessitating increased phosphorus fertilizer use. This article explores the biosynthesis of phytic acid in maize, highlighting lipid-dependent and lipid-independent pathways, both requiring myo-inositol and also genetic approaches to reducing phytic acid content, including the use of low phytic acid (lpa) mutants and CRISPR/Cas9 gene editing, are discussed. Mutations in lipid-independent phytic acid biosynthesis genes have shown promise in reducing phytic acid levels. The stacking of these mutants has led to significant reductions in phytic acid content in maize.

Low Phytate Maize

Maize is called the queen of cereals due to its high yield potential and worldwide adoption. It is cultivated for feed, fodder, oil, fuel, etc. The major concern in maize is the high phytic acid content in seed. The phytic acid is the reservoir of phosphorus and if we reduce the phytic acid content in plants we can also minimize the phosphorus usage in agriculture. Further, the phytic acid in crop plants acts as an antinutritional factors by sequestering phosphorus as well as micronutrients such as iron, zinc, etc. Among cereals, phytic acid content is high in maize and according to an estimate, dry-weight maize germ contains 6.39% phytic acid (Kasim and Edwards 1998). Tropical maize was analyzed for phytic acid content and most of the maize genotypes contain seed phytic acid in the range of 9-9.9% (Pramitha et al., 2020). As maize is a major cereal crop and is consumed by large human populations and domesticated animals, it is high time to reduce phytic acid content to improve the nutritional value of maize and to reduce malnutrition in humans as well as in domesticated animals. The phosphorus and myoinositol present in phytic acid remain non-available in non-ruminants due to a lack of phytase enzyme. The reduction of phytic acid in maize remains a challenge for researchers. A breeding approach of reducing phytic acid was attempted but by reducing phytic acid an undesirable agronomical trait also inculcated in reduced phytic acid maize lines. Genetic engineering approaches are also being utilized for reducing phytic acid content. The overexpression of phytase-encoding genes leads to the reduction of phytic acid in transgenic crops. Another recently adopted approach, gene editing using CRISPR/Cas9 could be utilized for reducing phytic acid with minimal/null pleiotropic effect.

Phytic acid biosynthesis

There is the existence of two pathways for phytic acid synthesis in crop plants including maize. One biosynthesis pathway is lipid dependent and the other one is lipid independent. each pathway requires the supply of myo-inositol (Figure 1).

The lipid-dependent intermediate pathway is involved in basic cellular signalling and other processes. The knocking out gene (s) involved in the intermediate step of lipid- dependent pathway can affect the plant metabolism two pathways differ in early phosphorylation steps and the way sequential phosphorylation takes place. The phytic acid synthesis involves three processes (a) Initiation of the synthesis pathway (b) Successive phosphorylation (c) Transport and storage of phytic acid into vacuoles.

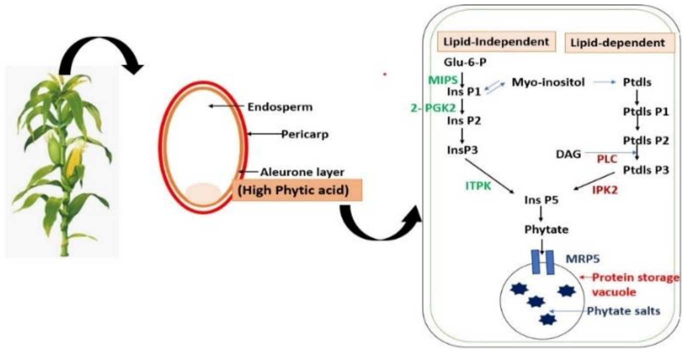

Fig 1 Phytic acid biosynthesis pathway

Phytic acid is present in excess amounts as required by plants for basic metabolism. For reducing phytate content in crops, the targeted approaches could be manifested such that reduced phytate levels in maize exhibit minimal or no effect on metabolism.

The best putative target could be the gene (s) involved in the intermediate step of lipid independent pathway. By targeting the intermediate gene/paralog of lipid independent pathway, the phytic acid synthesis inside the plant will be reduced, and these low-phytate maize with minimum pleiotropic effects possess higher nutritional value.

Genetic approaches for accomplishing low phytate maize

Reduction of phytic acid content in maize is an important area of research due to its effect on both nutritional quality and environmental sustainability. However, different types of genetic approaches have been discovered that can use both conventional breeding methods and genetic engineering tools to address this problem. Below, are some key genetic strategies used to achieve low phytate maize.

1. **Use of low phytic acid (lpa) mutants**: several naturally occurring mutants with lower phytic acid contents have been found in maize and some other crops.
   - **lpa2**: The maize low phytic acid mutant, lpa2 is caused by a mutation in an inositol phosphate kinase gene. The mutant maize line contains less phytic acid as compared to wild-type plants (Shi et al., 2003).
   - **lpa3**: Maize lpa3 mutant caused by mutation in myo-inositol kinase (*MIK*) gene. When both lpa3 and lpa2 mutants combined through the crossing, the hybrid line shows a drastic reduction in phytic acid content (Shi et al., 2005). The various natural mutants in soybean, maize, Arabidopsis, and rice are available. These mutant genes can be potentially targeted to produce low phytic acid.
   - **CRISPR/Cas9-Mediated Gene Editing**: Gene editing techniques, specifically CRISPR\Cas9 have been used to target and alter genes linked to the synthesis of phytic acid and its intermediates that finally lead to the reduction in phytic acid. For instance, it has been demonstrated that lo wering phytic acid levels without changing plant metabolism as a whole is possi ble by targeting genes related to the lipid-independent process of phytic acid formation (Figure 2).
   - **Silencing Genes Related to Phytic Acid Biosynthesis:** It has been found that, gene silencing techniques can be used to reduce phytic acid contents by silencing genes involved in its biosynthesis.

Embryo-specific silencing: The embryo-specific silencing of ABC transporter results in the reduction of phytic acid and enhancement in inorganic phosphorus in maize and soybean seed (Shi et al., 2007).

Downregulation of genes: Among Phytic acid biosynthesis genes, *IPK1* and *ITPK1* are involved in maintaining involved in phosphate homeostasis. The inositol hexakisphosphate (IP6) content was reduced in *IPK1* mutant and transgenic lines expressing point mutation in *IPK1*. The IP6 content was also reduced in the *ITPK1* mutant as compared to the wild type (Kuo et al., 2018). Subsequently, the knockdown of the *ITPK* gene in rice results in reduced phytic acid content and enhancement in inorganic phosphorus (Pi), and mineral ions mobilize and present as free mineral ions in endosperm in transgenic seeds and in wild-type seeds, these mineral ions chelated as phytate globules in (Karmakar et al., 2020). Also, the seed-specific knockdown of myo-inositol-3-phosphate synthase (*MIPS*) in soybean results in a reduction in phytic acid and bioavailability of iron, zinc, and calcium got enhanced in knockdown transgenic lines (Kumar et al., 2019). The seed-specific silencing of inositol polyphosphate 6-/3-/5-kinase gene (*IPK-2*) in soybeans results in a reduction in phytic acid as compared to wild-type plants (Punjabi et al., 2018). Also, the disruption of *IPK2* leads to a drastic reduction in seed phytate in Arabidopsis (Stevenson-Paulik et al., 2005).

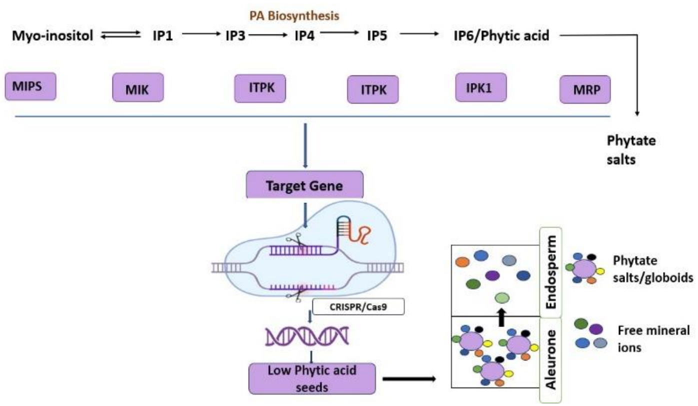

Fig 2 Gene knockout of phytic acid biosynthesis genes by using CRISPR/Cas9

There is availability of mutant lines in various crops for low phytate content and in terms of crop performance variation is found as compared to their wild-type counter partner. Likewise, the LPA barley mutant exhibits a similar grain yield as compared to the wild type at five different locations (Raboy et al., 2015). The Ethyl methanesulfonate (EMS) mediated double mutant for *MRP5* paralog showed 15% reduction in phytic acid content (Sashidhar et al., 2020). The four paralogs of Inositol 1,3,4-trisphosphate

5/6-kinase (*ITPK*) are present in *Brassica napus*. Furthermore, the triple mutant for *ITPK* in *Brassica napus* showed reduced phytic acid contents and enhanced Pi levels (Sashidhar et al., 2020).

Challenges with Low phytate crops

Developing low-phytate crops such as maize has various benefits for improving nutrition and environmental sustainability, but there are various challenges in its successful implementation.

- **Agronomic compromises**: One of the main problems with decreasing phytate levels in plants is that it also affects the growth and yield of crops. Some low phytate mutants have shown low seed viability and other growth-related issues. It is very important to develop a balance between low phytic acid and preserving the robust agronomic performance of crops (Raboy et al., 2015).
- **Pleiotropic Effects:** Genetic modifications, especially those using advanced tools like CRISPR/Cas9, can lead to unintended side effects. These unintended changes might influence other important metabolic pathways or plant processes, potentially causing unforeseen problems (Graham et al., 2020). Hence, Thorough testing and validation of these modifications are crucial to ensure they don't introduce new issues.

Conclusion

The pursuit of low-phytate maize represents a significant advancement in addressing both nutritional and environmental challenges associated with high phytic acid content. As a critical crop with widespread applications, reducing phytic acid in maize can improve the bioavailability of essential nutrients like phosphorus, iron, and zinc, thereby enhancing human and animal nutrition while reducing the reliance on phosphorus fertilizers.

Genetic approaches, including the use of low phytic acid (lpa) mutants and innovative CRISPR/Cas9 gene editing technologies, have made considerable strides in this area. These methods target specific biosynthesis pathways of phytic acid, leading to promising reductions in its content without severely compromising the maize's overall growth and yield. The combination of natural mutants, targeted gene editing, and gene silencing techniques has demonstrated the potential to create maize varieties with lower phytic acid levels and improved nutritional profiles.

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References

Graham, N., Patil, G.B., Bubeck, D.M., Dobert, R.C., Glenn, K.C., Gutsche, A.T., Kumar, S., Lindbo, J.A., Maas, L., May, G.D. and Vega-Sanchez, M.E., 2020. Plant genome editing and the relevance of off-target changes. *Plant physiology*, *183*(4), pp.1453-1471.

Karmakar, A., Bhattacharya, S., Sengupta, S., Ali, N., Sarkar, S.N., Datta, K. and Datta, S.K., 2020. RNAi-mediated silencing of ITPK gene reduces phytic acid content, alters transcripts of phytic acid biosynthetic genes, and modulates mineral distribution in rice seeds. *Rice science*, *27*(4), pp.315-328.

Kasim, A.B. and Edwards Jr, H.M., 1998. The analysis for inositol phosphate forms in feed ingredients. *Journal of the Science of Food and Agriculture*, *76*(1), pp.1-9.

Kumar, A., Kumar, V., Krishnan, V., Hada, A., Marathe, A., Jolly, M. and Sachdev, A., 2019. Seed targeted RNAi-mediated silencing of GmMIPS1 limits phytate accumulation and improves mineral bioavailability in soybean. *Scientific reports*, *9*(1), p.7744.

Kuo, H.F., Hsu, Y.Y., Lin, W.C., Chen, K.Y., Munnik, T., Brearley, C.A. and Chiou, T.J., 2018. Arabidopsis inositol phosphate kinases IPK 1 and ITPK 1 constitute a metabolic pathway in maintaining phosphate homeostasis. *The Plant Journal*, *95*(4), pp.613-630.

Perera, I., Fukushima, A., Akabane, T., Horiguchi, G., Seneweera, S. and Hirotsu, N., 2019. Expression regulation of myo-inositol 3-phosphate synthase 1 (INO1) in determination of phytic acid accumulation in rice grain. *Scientific Reports*, *9*(1), p.14866.

Pramitha, J.L., Joel, A.J., Srinivas, S., Sreeja, R., Hossain, F. and Ravikesavan, R., 2020. Enumerating the phytic acid content in maize germplasm and formulation of reference set to enhance the breeding for low phytic acid. *Physiology and Molecular Biology of Plants*, 26, pp.353-365.

Punjabi, M., Bharadvaja, N., Jolly, M., Dahuja, A. and Sachdev, A., 2018. Development and evaluation of low phytic acid soybean by siRNA triggered seed specific silencing of inositol polyphosphate 6-/3-/5-kinase gene. *Frontiers in Plant Science*, *9*, p.804.

Raboy, V., Peterson, K., Jackson, C., Marshall, J.M., Hu, G., Saneoka, H. and Bregitzer, P., 2015. A substantial fraction of barley (Hordeum vulgare L.) low phytic acid mutations has little or no effect on yield across diverse production environments. *Plants*, *4*(2), pp.225-239.

Sashidhar, N., Harloff, H.J. and Jung, C., 2020. Knockout of Multi-Drug-Resistant Protein 5 genes lead to low phytic acid contents in oilseed rape. *Frontiers in plant science*, *11*, p.603.

Sashidhar, N., Harloff, H.J., Potgieter, L. and Jung, C., 2020. Gene editing of three BnITPK genes in tetraploid oilseed rape leads to significant reduction of phytic acid in seeds. *Plant Biotechnology Journal*, *18*(11), pp.2241-2250.

Shi, J., Wang, H., Hazebroek, J., Ertl, D.S. and Harp, T., 2005. The maize low-phytic acid 3 encodes a myo-inositol kinase that plays a role in phytic acid biosynthesis in developing seeds. *The Plant Journal*, *42*(5), pp.708-719.

Shi, J., Wang, H., Schellin, K., Li, B., Faller, M., Stoop, J.M., Meeley, R.B., Ertl, D.S., Ranch, J.P. and Glassman, K., 2007. Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. *Nature biotechnology*, *25*(8), pp.930-937.

Shi, J., Wang, H., Wu, Y., Hazebroek, J., Meeley, R.B. and Ertl, D.S., 2003. The maize low-phytic acid mutant lpa2 is caused by mutation in an inositol phosphate kinase gene. *Plant physiology*, *131*(2), pp.507-515.

Stevenson-Paulik, J., Bastidas, R.J., Chiou, S.T., Frye, R.A. and York, J.D., 2005. Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. *Proceedings of the National Academy of Sciences*, *102*(35), pp.12612-12617.

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