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Functional Validation of Maize Phosphate Transporters Using Overexpression Lines

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Abstract Phosphate transport is essential for maize growth, development, and yield, as it regulates nutrient uptake, allocation, and signaling pathways critical for plant productivity. In this study, we conducted a comprehensive functional validation of maize phosphate transporters through overexpression strategies, focusing on key members of the PHT1, PHT2, and PHT3 families. We characterized the genomic organization, expression patterns, and regulatory features of these genes, followed by Agrobacterium-mediated transformation to generate targeted overexpression lines under constitutive and tissue-specific promoters. Phenotypic assessments revealed enhanced phosphate uptake efficiency, improved root architecture, increased biomass, and greater stress tolerance in transgenic lines. Molecular analyses, including transcriptomic profiling, protein localization studies, and metabolic flux measurements, confirmed the functional enhancement of phosphate transport and related metabolic pathways. A case study on a specific PHT1 transporter demonstrated significant agronomic benefits, including improved yield under phosphate-limited conditions. These findings provide critical insights into the roles of phosphate transporters in maize physiology and highlight their potential for breeding phosphate-efficient cultivars, contributing to sustainable agriculture and phosphorus resource conservation.

Keywords Maize; Phosphate transporter; Overexpression; Nutrient absorption; Sustainable agriculture

1 Introduction

Phosphorus is very important for plants, especially in high-yield crops like corn. Phosphorus is involved in both leaf growth and grain filling. But the problem is that although the soil may contain a considerable amount of phosphorus, the portion that plants can actually absorb is very limited (Zhang and Xu, 2024). In reality, this often leads farmers to have to rely on excessive fertilization to maintain yields (Wang et al., 2020a). Interestingly, some corn can still grow normally when there is insufficient phosphorus, which brings up a key topic: How do they manage to efficiently absorb and rationally allocate phosphorus resources? (Liu et al., 2016).

Behind this, there is something called a "phosphate transporter", especially the proteins of the PHT1 family. They act like porters, transporting phosphorus from the soil into plants and completing its redistribution within the plants (Nagy et al., 2006). In corn, there are more than one type of such proteins. Some are actively expressed in the root system, while others work in the leaves. Moreover, certain transporter proteins become particularly "active" once phosphorus deficiency occurs or when symbiosis occurs with arbuscular mycorrhizal fungi (Liu et al., 2018). They can even help transport phosphorus from the old leaves to the newly grown parts, while participating in some phosphorus-related regulatory mechanisms and signal feedback (Hu et al., 2024).

Although researchers have identified multiple maize genes involved in phosphorus transport, their respective roles and the regulatory details behind them are still not clear enough. To further understand the functions of these genes, a common approach is to conduct overexpression experiments to see what effects increasing the "working intensity" of a certain protein will have on phosphorus uptake, phosphorus regulation and overall development of plants (Xu et al., 2020). For instance, research has found that enhancing the expression levels of ZmPT7 or ZmPt9 not only boosts absorption but also improves phosphorus distribution within the body, and can even influence traits such as plant height and root systems. This approach is actually quite intuitive and effective.

Therefore, this study intends to use overexpression materials to focus on verifying the functions of several key phosphate transporters and see exactly how they affect the absorption and internal distribution of phosphorus in corn. During the research process, molecular analysis, physiological data and phenotypic observations will be combined to identify those "star transporters" that may have the potential to improve phosphorus utilization efficiency. The ultimate goal is to provide ideas for future breeding and reducing reliance on chemical fertilizers, and to promote the greener and more efficient development of corn production.

2 Phosphate Transporter Families in Maize: Classification, Genomics, and Expression

2.1 Classification and diversity of phosphate transporter gene families

When it comes to phosphate transporters in corn, the PHT1 family is almost the most thoroughly studied. In B73 corn, researchers identified 13 *PHT1* genes (from *ZmPHT1;1* to *ZmPHT1;13*), they are mainly responsible for absorbing phosphorus from the soil into plants (Liu et al., 2016). However, it is not only PHT1 that exists in corn; PHT2 and PHT3 also occupy positions in the system - PHT2 is more commonly found in plastids, while PHT3 is related to mitochondria. This indicates that these proteins each have their own functions. For instance, the SPX family, although not the main force in translucency, also plays its own role in phosphorus signaling and homeostasis. Studies have identified as many as 33 *SPX* genes (Xiao et al., 2021), which is actually not a small number.

2.2 Genomic organization and evolutionary relationships

From the perspective of chromosome distribution, the *PHT1* gene is not concentrated together. In corn, these 13 genes are distributed on chromosomes 1, 2, 5, 7, 8 and 10. They are not randomly present - many of them can be traced back to gene replication that occurred at different times. Some replication events occurred in modern times, such as ZmPHT1; 1 and ZmPHT1; 9; And ZmPHT1; 1 and ZmPHT1; The relationship between 13 might be even earlier. These replication activities illustrate the process of genome expansion. Phylogenetic studies have found that these genes of corn are closely related to the corresponding genes of rice, sorghum and short-stemmed grass, indicating that their functions are likely conserved in the Poaceae family (Su et al., 2013). However, one point cannot be ignored: whether monocotyledonous or dicotyledonous, many plants have undergone polygenic replication of the transporter family, which is obviously a trace left by earlier evolutionary events (Takabatake et al., 1999).

2.3 Known expression patterns and regulation

When and where the corn phosphate transporter plays a role is not fixed. Many *PHT1* genes, such as *ZmPHT1; 2, 4, 6, 7, 9, 11* are activated at low phosphorus levels, and some also show elevated expression when cooperating with arbuscular mycorrhizal fungi (Liu et al., 2018). However, there are exceptions, such as ZmPHT1; Its regulatory methods under certain conditions are slightly different from those of other members. The expression of these genes also varies in different tissues. Some are highly expressed only in pollen or mycorrhizal tissues, while others are related to the re-transport of phosphorus in buds (Nagy et al., 2006). In addition, promoter sequence analysis has discovered many cis-regulatory elements that respond to phosphorus starvation or mycorrhizal signals (Li et al., 2020), which further indicates that the regulatory system is very complex. The behavior of the *SPX* gene is also typical. They are also easily induced under phosphorus deficiency conditions, demonstrating the high regulatory nature of corn in phosphorus management (Figure 1).

3 Functional Roles of Maize Phosphate Transporters

3.1 Uptake of phosphate from soil under varying environmental conditions

Sometimes, there is not much phosphorus in the soil, and at such times, plants have to find ways to absorb it more efficiently. Corn can achieve this mainly through some phosphate transporters, especially members of the PHT1 family like ZmPT7 and ZmPt9. Proteins like ZmPT7 are expressed in both roots and leaves, especially when there is a phosphorus deficiency, they are significantly activated, enhancing the plant's ability to absorb phosphorus. And for instance, ZmPt9 can only take effect after partnering with arbuscular mycorrhizal fungi. This type of fungus has a higher colonization rate in low-phosphorus environments. They can help corn absorb the phosphorus that was otherwise difficult to obtain. Therefore, these transport proteins not only have strong adaptability but also can "adjust strategies" according to soil conditions, helping plants improve nutrient utilization efficiency.

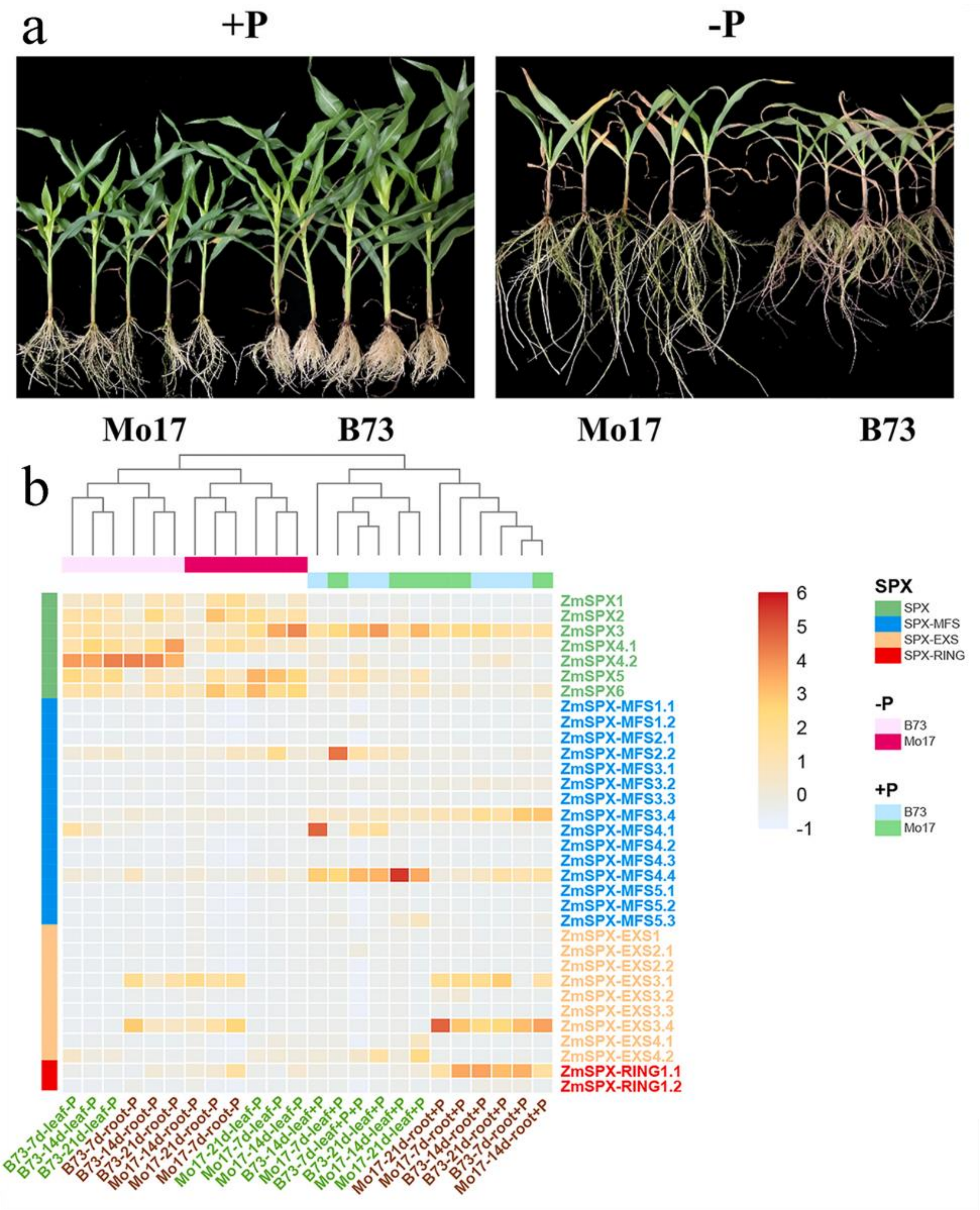

Figure 1 a Phenotypes of B73 and Mo17 on the 21st day after normal P and P deficiency stress treatments. B Expression profiles of *ZmSPX* genes reveal their different responses to deficient P (Adapted from Xiao et al., 2021)

Image caption: Heat map analysis of the expression patterns of 33 *ZmSPX* genes in B73 and Mo17 samples collected at different times, from different tissues and under different P treatment conditions. Four subfamilies are represented by different colours. Leaf samples and root samples are highlighted in green and brown, respectively. The data were normalized to log2 values, and the colour scale represents the relative expression level (Adapted from Xiao et al., 2021)

3.2 Intracellular phosphate distribution and storage

Phosphorus is not just absorbed and that's it. How it is used and distributed is also very crucial. For instance, the ZmPT7 is also responsible for transporting phosphorus from old leaves to new ones, especially when there is a phosphorus deficiency, this function becomes even more crucial. And for the storage part, vacuoles play a significant role. The ZmVPE transporter on the vacuole membrane can release the phosphorus stored inside again for the newly grown parts. Experiments have proved that once ZmVPE is overexpressed, the redistribution ability of plants is enhanced. On the contrary, after the mutation, phosphorus accumulates in the old leaves but cannot be supplied by the new leaves, which indicates that it plays a significant role in the dynamic balance of phosphorus.

3.3 Involvement in phosphate signaling pathways and stress adaptation

Light absorption and transportation are not enough. Plants also need to "sense" whether there is enough phosphorus and then decide what adjustments to make. The transporter is also involved in this link. Transcription factors like NIGT1.2 and ZmPHR1/2 regulate the expression of PHT1-like proteins and other related genes, and can also affect the way nitrogen is absorbed, and even change the appearance of roots. The regulatory means are not limited to one type. There are protein phosphorylation (such as ZmPT7), microRNA intervention (miR399 is one of them), and non-coding long Rnas (such as PILNCR2) that enhance the ability to resist phosphorus deficiency by stabilizing mRNA. In conclusion, this set of mechanisms enables corn to respond to the external environment, maintain the stability of phosphorus in its body, and at the same time enhance its adaptability to adverse conditions.

4 Overexpression Strategies for Functional Validation in Maize

4.1 Genetic transformation techniques in maize

To verify the function of phosphate transporters in corn through overexpression, the issue of transgenic introduction must first be addressed (Chen et al., 2024). Not all methods are applicable to all materials. Currently, there are two commonly used ones: the Agrobacterium method and the gene gun method. Agrobacterium has been chosen by many because it can produce stable, single-copy transgenic insertions and is effective for many maize genotypes, especially when using stem tips or immature embryos as explants (Wang et al., 2018). Of course, Agrobacterium can not be used in all situations. Sometimes its efficiency is low, and then a gene gun is the only option. Although the gene gun method is more "hardcore" and the number of inserted copies may be large and fragmented, it can be applied to a wider range of corn varieties, which is very important. Both of these two methods have been well used in the construction of overexpression materials (Canas et al., 2020), each with its advantages and disadvantages. Which one to choose depends on the experimental objective and the type of material.

4.2 Selection of promoter systems for targeted or constitutive overexpression

Whether the transgenic expression is good or not, the selection of the promoter is very crucial. Many people would directly use the maize ubiquitin promoter or the rice actin promoter. These promoters are of the "universal type" - basically highly expressed in any tissue. Doing so can help researchers quickly observe the influence of genes on the entire plant (Liu et al., 2015; 2020). However, sometimes it is not desirable to have genes "flying all over" the entire plant. For instance, if one wants to observe the performance of a certain organ or under specific stress conditions, more precise control is required. At this point, stress promoters like Arabidopsis rd29B or those specifically expressed in floral organs would be more appropriate (Nuccio et al., 2015). So, whether it is a broad expression or a targeted approach depends entirely on whether you are studying "global functions" or "fixed-point analysis".

4.3 Advantages and limitations of overexpression compared to knockout and knockdown approaches

Overexpression sounds all-powerful. Indeed, it can directly cause genes to "amplify their actions", especially for those genes with multiple homologous copies that are difficult to study through knockout. Sometimes, the function of a certain gene can even be directly determined through an obvious phenotype. For example, the strains produced by the FOX-hunting system have achieved this (Abe and Ichikawa, 2016). For genes whose function loss can be fatal, overexpression is often the only option. But then again, it also has "side effects" - for instance, some overly expressed traits do not necessarily reflect the true physiological state; instead, they mask the detailed regulation. In contrast, knockout or knockdown (such as T-DNA insertion or RNAi) is more suitable for "subtraction" verification. However, these methods also have blind spots, especially when it comes to redundant or essential genes, obvious phenotypes are often not observed. Overall, it is difficult to exhaust all functional information by relying on a single approach. Combining overexpression and knockout strategies is the truly effective path for functional genomics.

5 Phenotypic and Physiological Effects of Overexpression

5.1 Changes in phosphate uptake efficiency and accumulation

Not all genes are so "efficient", but when phosphate transporters like ZmPT7 or ZmVPEs are overexpressed, the absorption and accumulation of phosphorus by plants do indeed increase significantly. For instance, the transgenic strain of ZmPT7 can absorb phosphorus more effectively than the wild type and has a significantly higher total phosphorus content in its body. This extent of enhancement is actually directly related to the intensity of transgenic expression. As for ZmVPEs, its effect is a bit different - mainly more effective in transferring phosphorus from old leaves to new ones. This internal adjustment, especially when there is a phosphorus deficiency, will enable the plants to maintain a better phosphorus balance and utilization efficiency.

5.2 Alterations in root architecture and growth dynamics

The changes in the fundamental structure are sometimes more intuitive than the absorption of nutrients themselves. Regulatory factors like ZmPHR1 and ZmPHR2, after overexpression, significantly increase the number of lateral roots in corn and make the total root weight larger. This is equivalent to enhancing the ability to "find phosphorus" from the source. Interestingly, there are also some genes like *ZmARF1* and *ZmPP2AA1* that not only regulate roots but also incidentally control the expression of phosphorus transporters. As a result, more root branches and a larger root system surface area emerged. Overall, these changes in the roots are often accompanied by alterations in the above-ground parts, such as more vigorous buds and longer leaves.

5.3 Impact on yield, biomass, and stress tolerance

Ultimately, whether overexpression treatment can lead to an increase in output is the most concerning issue for everyone. The answer is: In a low-phosphorus environment, it is indeed possible. Like ZmPT7 and ZmPTF1, after overexpression, the dry weight of the buds increased, the grains became plumper, and the quantity also increased (Li et al., 2011). Some genes, such as *ZmNF-YC1* and *ZmAPRG*, not only affect yield but also enhance photosynthesis under low phosphorus conditions. More importantly, these transgenic lines usually perform well under adverse conditions as well - not only phosphorus deficiency, but also under drought, saline-alkali and even osmotic stress, they are more resilient than the control (Li et al., 2022). Stable nutritional status and strong root systems are the core elements that support this tolerance.

6 Molecular and Biochemical Characterization of Overexpression Lines

6.1 Transcriptomic profiling to assess gene expression changes

Not all transcriptome analyses are so complex that they are hard to understand. Methods like RNA sequencing and qRT-PCR have long been used to study the expression changes of phosphate transporters after overexpression. Once the regulatory factors ZmPHR1 and ZmPHR2 are overexpressed, the expression of many genes will be mobilized in a low-phosphorus environment, such as those involved in phosphorus signaling, hormone pathways, metal ion transport, and photosynthesis (Hu et al., 2024). However, this is not the only example. The overexpression of ZmNF-YC1 and ZmAPRG can also affect the expression of a batch of lipid metabolism and photosynthetic genes, and ultimately enhance the tolerance of plants to low phosphorus. In conclusion, this type of analysis can help us clearly understand the direct or indirect impact of transporters at the gene expression level.

6.2 Protein localization studies to confirm transporter targeting

Although some proteins have been expressed, it is also necessary to confirm exactly where they function. At this point, the GFP fusion protein comes in handy. Like ZmPT7, it is ultimately located on the plasma membrane in corn cells, a fact that can be replicated in different systems, which also indicates that it is indeed involved in the absorption and transport of phosphorus. Another transport protein called ZmVPE has a different location and has been confirmed to be on the vacuole membrane, which precisely matches its function of "mobilizing" phosphorus from the vacuole (Guo et al., 2024b). So, in essence, this kind of localization experiment is to confirm whether these proteins have "gone to where they should".

6.3 Enzyme activity and metabolic flux analyses for phosphate-related pathways

From a biochemical perspective, the changes brought about by overexpression of transporters are also considerable. The Pi absorption capacity of the ZmPT7 overexpression strain is stronger, and it can also transport phosphorus from old leaves to new leaves. Of course, this transport activity is also affected by phosphorylation (Wang et al., 2020a). The situation of ZmVPE is slightly different. It enhances the release of phosphorus in the vacuoles, that is, it enables the internal Pi to re-participate in the flow, thereby helping plants maintain phosphorus balance. Another example is that after overexpression of ZmAPRG, not only did the phosphorus-related fluxes change, but also the lipid structure and photosynthetically related metabolites changed, indicating that its role under low-phosphorus conditions may be more complex than imagined.

7 Case Study

7.1 Background and selection of the target transporter gene for overexpression

Not all transporters in corn are worthy of in-depth study, but ZmPT7 is an exception. This gene belonging to the PHT1 family was noticed because it has a particularly strong response when there is a phosphorus deficiency. It expresses itself both in the roots and leaves, and the reaction is very obvious. Similar genes can also be found in other plants, and their functions are quite close as well. Taking these characteristics into account, researchers selected ZmPT7 as a candidate for overexpression validation to see exactly how much it could play in phosphorus absorption and distribution.

7.2 Transformation process and verification of transgenic lines

Not all experiments go smoothly, but the transformation process this time is relatively mature. Researchers used Agrobacterium-mediated methods to introduce ZmPT7 into corn and established overexpressed plants. Next, it was necessary to verify whether the conversion was successful, so PCR detection and expression analysis and other methods were used. To further confirm its function, this gene was also placed in yeast and Arabidopsis mutants. The results showed that it could indeed make up for the missing phosphorus transport function (Wang et al., 2020a).

7.3 Key findings: enhanced phosphate uptake, altered physiological traits, and potential agronomic benefits

When phosphorus is insufficient, these overexpressed maize lines show particularly obvious symptoms - their phosphorus absorption capacity is much stronger than that of the control (Figure 2). What's more interesting is that they can effectively transfer the phosphorus from the old leaves to the new ones. Overall, the total phosphorus content of the plants is also higher. Its growth condition is also much better than that of the wild type. However, the matter is not that simple. The research also found that the activity of ZmPT7 is regulated by phosphorylation, indicating that its working mechanism may be more complex than expected. In summary, the overexpression of ZmPT7 is indeed helpful in enhancing phosphorus absorption and internal redistribution, and it also provides a promising direction for cultivating efficient corn varieties under low-phosphorus conditions in the future.

8 Implications for Crop Improvement and Phosphate Management

8.1 Potential applications in breeding phosphate-efficient maize varieties

Not every plot of land is suitable for heavy application of phosphorus fertilizer, especially in phosphorus-deficient or alkaline soil, where conventional methods are difficult to maintain high yields. Against this backdrop, it has become particularly crucial to breed corn varieties that can efficiently utilize phosphorus. Researchers have identified many candidate genes related to phosphorus absorption and utilization (Guo et al., 2024a). If these genes can be integrated into the breeding program, it is possible to produce varieties that can have good harvests even with less fertilization. Of course, genetic improvement alone is not enough. Sometimes, using phosphorus-solubizing bacteria or mycorrhizal bacteria together, especially in problem soils, may yield better results (Adnan et al., 2020; Wahid et al., 2020; Beltran-Medina et al., 2023).

8.2 Integration with sustainable agricultural practices to reduce fertilizer dependency

Excessive use of chemical fertilizers does not necessarily mean they are effective. It wastes resources and may even pollute the environment. In contrast, more precise and economical fertilization methods, such as strip fertilization, foliar spraying or local application, are more worthy of promotion (Rafiullah et al., 2020; Mussarat et al., 2021; Wang et al., 2023a). In addition, intercropping, planting cover crops, and using phosphorus-releasing microorganisms, although traditional, have obvious benefits in enhancing phosphorus efficiency and improving soil quality (Pavinato et al., 2017; Song et al., 2021). Interestingly, some studies conducted simulation tests, and the results showed that if the fertilization method was chosen correctly and the planting method kept up, the yield of corn could not only increase by 20%, but also reduce the environmental burden by about one-third (Luo et al., 2024).

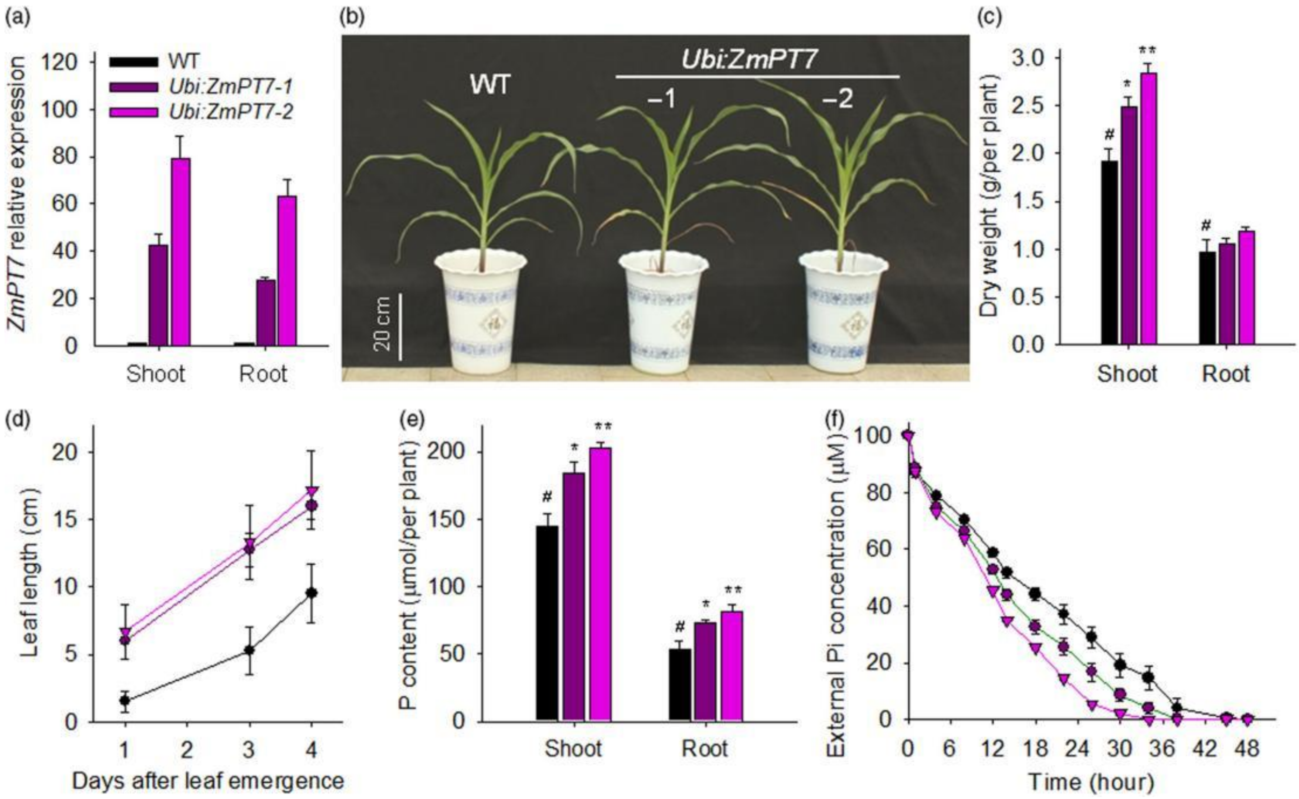

Figure 2 Overexpression of *ZmPT7* increases maize Pi uptake. (a) qRT-PCR analysis of *ZmPT7* expression in maize *ZmPT7*-overexpressing lines (*Ubi:ZmPT7-1* and *Ubi:ZmPT7-2*). Data are means±SE of three plants. (b) Phenotype comparison between the *ZmPT7*-overexpressing lines and wild-type maize, which germinated and grown for 40 days. (c) Dry weights of 40-day-old *ZmPT7*-overexpressing lines and wild-type maize. Data are means±SE of six plants. (d) Leaf elongation of the ninth leaf. Data are means±SE of six plants. (e) Total P contents of 40-day-old *ZmPT7*-overexpressing lines and wild-type maize. Data are means±SE of six plants. (f) Pi-uptake rate comparison among the 10-day-old *ZmPT7*-overexpressing lines and wild-type plants. Data are means±SE of three biological repeats, each repeat contained two plants. Asterisks in c and e indicate significant differences compared with wild-type plants (WT). $^*P < 0.05$; $^{**}P < 0.01$.$^{\#}$ (Adopted from Wang et al., 2020a)

8.3 Relevance to global phosphorus resource conservation and food security

The global phosphate rock is not inexhaustible. Resources are not unlimited. This is the reality. Long-term reliance on chemical fertilizers not only leads to a decreasing amount of phosphate rock but also brings about many environmental problems (Kumar et al., 2022). To solve this problem, it is not enough to merely reduce the amount used; it is more important to enhance the utilization efficiency. At this point, all aspects, whether from the perspective of breeding, field management, or relying on the power of microorganisms, must be utilized (Gong et al., 2023). In the long run, only by combining these approaches can we not only meet the growing food demands of the global population but also ensure a more stable ecological and resource development.

9 Concluding Remarks

Not all genes related to phosphoric acid can draw people's attention, but in corn, some transporters do play a key role. For instance, ZmPT7, ZmVPEs and ZmPt9, researchers have demonstrated through overexpression experiments that they not only participate in phosphorus absorption but also manage the redistribution and balance of phosphorus within plants. Especially in a low-phosphorus environment, overexpressed plants can significantly exhibit better growth conditions and stress resistance. Such results actually open a window for us to understand the mechanism by which corn ADAPTS to phosphorus restriction - transport proteins are not merely channels; they are also regulators.

However, merely knowing that these genes are useful is not enough. How to use it, on which varieties to use it, and when to use it are issues at another level. When viewed in the context of breeding, tools such as gene overexpression, CRISPR/Cas9 editing, and transcriptome analysis are not merely scientific research methods but key paths that can truly be transformed into agronomic traits. These methods share a common feature - they can help us understand more accurately which transport proteins are at work, which regulatory links deserve attention, and also shorten the distance from the laboratory to the field.

Of course, promoting such research ultimately cannot bypass the three words "sustainability". Phosphate fertilizer is not unlimited, and global resources are also facing the pressure of depletion. Therefore, whoever can utilize phosphorus more efficiently will take the initiative in future agriculture. This is not merely a scientific challenge, but also a choice made in practice. Continuously exploring the diversity of transport proteins and understanding how they respond to the environment and participate in regulation is a key threshold for our breeding to move forward. However, truly efficient and stress-resistant corn varieties can only be achieved through the joint promotion of this series of studies.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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