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Engineering C4 Photosynthetic Pathway into Wheat: Progress and Prospects

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Abstract Under conditions of high temperature and drought, C3 crops such as wheat experience increased photorespiration, reduced photosynthetic efficiency, and limited yield. The C4 photosynthetic pathway has a high efficiency in carbon dioxide concentration and good utilization rates of water and nutrients, and is regarded as an important direction for increasing the yield of C3 crops. Recent studies have found that there are C4-related genes in wheat and they are expressed in tissues such as grains. In plants such as rice, the exogenous expression of some C4 enzymes has been achieved. However, there are still challenges in constructing the C4 pathway in wheat, such as cell-specific expression, Kranz structure reconstruction, and complex gene regulatory networks. By integrating methods such as systems biology and gene editing, this study aims to promote the realization of the C4 mechanism in wheat, providing solutions for food security and climate change.

Keywords C4 Photosynthetic pathway; Wheat (*Triticum aestivum* L.); Genetic engineering; Photosynthetic efficiency; Systems biology

1 Introduction

Global food security is now facing significant challenges. The world's population is constantly increasing, arable land is decreasing, and climate change is becoming more and more serious. To ensure food supply, it is necessary to increase crop yields and the efficiency of resource utilization. This has become an important task in agricultural science. The breeding methods of the past "Green Revolution" have nearly reached the limit of yield increase on major food crops and are difficult to meet the future demand for food, fiber and fuel (Dehigaspitiya et al., 2019; Pradhan et al., 2022; Nadipalli et al., 2024). Therefore, cultivating new crops that are high-yielding, efficient and stress-resistant is the key to the sustainable development of global agriculture.

Wheat is one of the most important C3 crops in the world. However, its photosynthetic efficiency is limited by the C3 pathway itself. In high-temperature, low-CO_2 and arid environments, photorespiration will increase, carbon fixation efficiency will decline, and the utilization rates of water and nitrogen will also decrease. All these limit the yield potential of wheat (Perdomo et al., 2017; Juric et al., 2019; Prasanna et al., 2025). In addition, C3 crops have poor adaptability to environmental stress, which makes food security risks greater (Perdomo et al., 2017; Sonmez et al., 2022).

C4 photosynthesis has its unique biochemical advantages and leaf structure advantages. Under conditions such as high temperature, strong light and drought, the photosynthetic efficiency and resource utilization rate of C4 photosynthesis are higher. C4 plants reduce photorespiration and increase carbon assimilation rate through CO_2 concentration mechanism (Wang et al., 2014; Schuler et al., 2016; Prasanna et al., 2025). Introducing the C4 photosynthesis mechanism into C3 crops such as wheat holds promise for breaking through yield limitations and addressing food security issues (Cui, 2021; Pradhan et al., 2022; Mukundan et al., 2024; Prasanna et al., 2025). Current studies have shown that introducing C4-related genes into C3 crops can enhance their photosynthetic efficiency, stress resistance and yield, etc. (Furbank et al., 2023; Nadipalli et al., 2024; Prasanna et al., 2025).

2 The C4 Photosynthetic Pathway: An Overview

2.1 Biochemical mechanisms

The C4 photosynthetic pathway is an efficient way to concentrate carbon. It reduces photorespiration and enhances the efficiency of photosynthesis by concentrating CO_2 at the site of Rubisco enzyme through the division of labor between mesophyll cells and vascular sheath cells.

There are three main types of C4 pathways, which are classified according to the different decarboxylases used as: NADP-malate type (NADP-ME), NAD-malate type (NAD-ME), and phosphoenolpyruvate carboxykinase type (PCK). They all differ in metabolic processes, enzyme activity regulation, substrate transport and energy requirements, etc. For instance, the decarboxylation reaction of NADP-ME type mainly takes place in chloroplasts, while that of NAD-ME type relies on mitochondria. The degree of dependence of different types on the REDOX state of chloroplasts and mitochondria also varies.

Some key enzymes in C4 plants, such as PEPC, MDH, and PPDK, usually have higher catalytic efficiency and special cellular localization. The genes of these enzymes originated from the homologous genes of the C3 ancestor, but through functional changes and adjustment of expression patterns, they acquired the function of C4 (Furbank et al., 2000; Rao and Dixon, 2016; Brautigam et al., 2018; Fan et al., 2021; Alvarez and Maurino, 2023; Chen et al., 2023).

2.2 Anatomical requirements

C4 photosynthesis requires special leaf structures, the most typical of which is the Kranz structure. Its characteristic is that mesophyll cells and vascular bundle sheath cells are closely arranged, and Rubisco enzyme is mainly concentrated in vascular bundle sheath cells. CO_2 is first fixed in mesophyll cells and then transported to vascular sheath cells for further reactions.

C4 plants generally have a high vein density, well-developed vascular sheath tissue, low gas diffusivity, and also have a special chloroplast distribution and morphology. The anatomical details of different C4 lineages are not exactly the same, such as the size, quantity, distribution of vascular bundle sheath cells and the way they connect with mesophyll cells.

Some C3 plants also have structures similar to C4, which indicates that the anatomical features of C4 may only require a small amount of genetic changes to form (Westhoff and Gowik, 2010; Ludwig, 2013; Lundgren et al., 2014; Ermakova et al., 2019; Alenazi et al., 2023; Alvarez and Maurino, 2023).

2.3 Natural diversity of C4 crops

According to existing research, we can find that the C4 photosynthetic pathway has independently occurred in angiosperms over 60 times, involving 19 families, which is a typical phenomenon of convergent evolution. C4 plants are mainly distributed in tropical and subtropical regions, including food and energy crops such as corn (*Zea mays*), sorghum (*Glycine max*) and sugarcane (*Saccharum officinarum*). Different C4 lineages have distinct biochemical types, anatomical features, gene regulation and adaptability, etc. The C4 gene families in different crops are diverse, and the selective pressures they face during survival also vary. Wild species generally retain more allele resources. Compared with C3 plants, C4 plants have higher utilization rates of water and nitrogen and are more likely to survive in adverse conditions (Sage, 2004; Rao and Dixon, 2016; Tao et al., 2019; Upadhyay et al., 2020; Chen et al., 2023).

3 Rationale for C4 Engineering in C3 Crops

3.1 Expected benefits for wheat

Wheat is a globally important staple food crop, but its photosynthetic efficiency is limited by the C3 pathway, and its yield is prone to damage under conditions of high temperature and drought. C4 engineering may bring the following benefits to wheat:

Improving photosynthetic efficiency and yield: The C4 pathway can reduce photorespiration loss and increase CO_2 utilization, thereby significantly enhancing photosynthetic rate and yield (Rangan et al., 2016; Bachir et al., 2017; Daoura et al., 2018; Prasanna et al., 2025).

Enhancing water and nitrogen utilization efficiency: C4 crops are more efficient in water and nitrogen utilization, which helps wheat maintain higher yields when resources are insufficient or the environment is harsh (Matsuoka et al., 2003; Jia et al., 2015; Prasanna et al., 2025).

Adapting to climate change: C4 characteristics can help wheat adapt to potentially hotter and drier climates in the future and ensure food security (Jia et al., 2015; Rangan et al., 2016).

Enhancing stress resistance: Increasing the expression and activity of C4 enzyme in wheat, especially in panicles and different organs, can enhance drought resistance and delay senescence (Jia et al., 2015; Bachir et al., 2017; Zhang et al., 2019).

3.2 Lessons from past C4 engineering attempts in rice

Rice is the object with the most in-depth C4 engineering research among C3 crops, and these studies provide important references for wheat:

The effect of introducing a single enzyme is limited: Although adding only one C4 enzyme (such as PEPC) can affect carbon metabolism, it has little effect on improving photosynthetic efficiency and requires the combination of multiple enzymes (Matsuoka et al., 2003; Miyao, 2003; Schuler et al., 2016; Prasanna et al., 2025).

Anatomical structure limitations: The C4 pathway requires spatial separation between mesophyll cells and vascular bundle sheath cells (Kranz structure), which is lacking in C3 crops and is a major obstacle to engineering (Matsuoka et al., 2003; Schuler et al., 2016; Cui, 2021) (Figure 1).

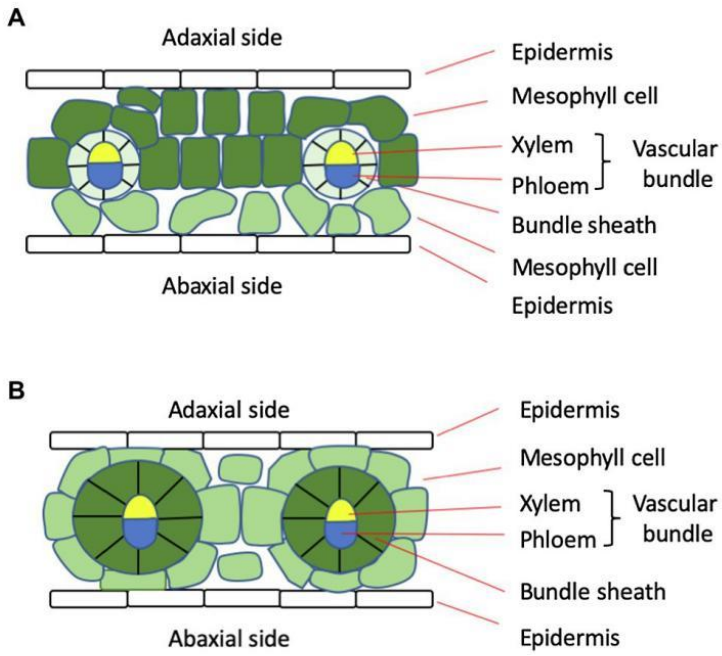

Figure 1 Diagram of the leaf anatomy of typical C3 (A) and C4 (B) plants (Adopted from Cui, 2021)

Note: In C4 plants, there are two mesophyll cells between neighboring vascular bundles, whereas in C3 plants, there are more than two. Also, the bundle sheath cells in C4 plants are much larger and contain more chloroplasts, as indicated by color intensity (Adopted from Cui, 2021)

Complex gene regulation: The high-level and cell-specific expression of the C4 gene requires precise control, which involves a complex gene regulatory network (Schuler et al., 2016; Cui, 2021; Chen et al., 2023).

Systematic understanding and multi-disciplinary collaboration are required: To successfully achieve C4 engineering, it is necessary to have a deep understanding of the photosynthetic mechanisms of C3 and C4, and combine multiple techniques such as synthetic biology, gene editing, and computational modeling (Schuler et al., 2016; Cui, 2021; Prasanna et al., 2025).

The Importance of International Cooperation: International cooperation such as the C4 Rice project provides platforms and resources, promoting theoretical research and technological progress (Schuler et al., 2016; Prasanna et al., 2025).

4 Progress in C4 Engineering in *Triticum aestivum*

4.1 Genomic resources

Wheat (*Triticum aestivum* L.) is a C3 plant, but many homologous genes related to the C4 photosynthetic pathway have been found in its genome. For instance, the homologous genes of key C4 enzymes such as PEPC, NADP-ME, MDH, and PPDK are distributed on different chromosome arms of the three subgenomes A, B, and D of wheat. These genes are all expressed in the flag leaves of wheat and can be detected at different growth stages. The expression levels of these genes vary greatly among different varieties. This provides a rich genetic resource and genetic diversity basis for the C4 engineering of wheat (Rangan et al., 2016; Bachir et al., 2017).

4.2 Gene discovery and functional characterization

Researchers have identified C4 photosynthetically related genes in the wheat genome and confirmed that these genes are highly expressed during grain development, especially in the photosynthetic tissues of the caryopsis, such as the cross cell and tubular cell layers. Enzyme activity detection indicated that PEPC, NADP-ME, MDH and PPDK were all active in wheat flag leaves, and their expression levels were closely related to the photosynthetic rate. The unique C4 photosynthetic pathway in wheat grains also provides a molecular basis for its adaptation in high-temperature and arid environments (Rangan et al., 2016; Bachir et al., 2017).

4.3 Transgenic and genome-editing approaches

Scientists have adopted transgenic methods on C3 crops such as rice to express C4-related enzymes, thereby enhancing their photosynthetic efficiency and nitrogen utilization efficiency. However, the C4 project in wheat crops is still in its infancy. At present, research on wheat crops mainly focuses on the cloning and expression regulation of the C4 key enzyme gene, as well as the functional verification in different tissues and at different developmental stages. Synthetic biology and gene editing techniques (such as CRISPR/Cas9) can achieve precise regulation and expression of multiple gene modules. However, at the current stage, completely reconstructing the functional pathway of C4 in wheat remains a considerable challenge (Matsuoka et al., 2003; Cui, 2021; Pradhan et al., 2022; Talukder et al., 2024; Prasanna et al., 2025).

4.4 Anatomical engineering

To achieve C4 photosynthesis, the expression of corresponding enzymes is required, as well as specific leaf structures (such as Kranz structures), so that the photosynthetic process can be spatially separated. Currently, researchers have observed chloroplast duality similar to that of mesophyll cells and vascular sheath cells in C4 plant leaves in the caryopsis tissue of wheat grains, providing a natural reference for anatomical engineering. However, reforming the Kranz structure in major photosynthetic organs such as wheat leaves remains a major challenge in the C4 project. Future research needs to delve into the molecular mechanisms that control cell differentiation and spatial expression, and combine methods such as directed evolution and artificial selection to promote new breakthroughs in wheat C4 anatomical engineering (Rangan et al., 2016; Schuler et al., 2016; Li et al., 2017; Cui, 2021; Talukder et al., 2024).

5 Technical Challenges

5.1 Genetic complexity

In C4 photosynthesis, multiple key enzymes and regulatory elements need to be mobilized, and the genes of these enzymes and regulatory elements are distributed on different chromosomes and subgenomes. In wheat, C4-related genes (*PEPC*, *NADP-ME*, *MDH*, *PPDK*) are located in different chromosome arms. The expression of these genes varies greatly at different growth stages of wheat plants and among different wheat varieties (Bachir et al., 2017; Daoura et al., 2018; Chen et al., 2023). The evolutionary process of C4 photosynthesis involves multiple procedures such as gene replication, functional differentiation, and changes in expression patterns, which increases the difficulty of gene manipulation. To achieve the engineering of C4, it is necessary to comprehensively understand and precisely regulate these gene networks in advance (Chen et al., 2023; Raturi et al., 2024).

5.2 Coordinated expression of multi-gene networks

The C4 photosynthetic mechanism requires the coordinated expression of multiple genes in time and space, especially the division of labor between mesophyll cells and vascular bundle sheath cells (Schuler et al., 2016; Cui, 2021; Chen et al., 2023; Prasanna et al., 2025). At present, although some C4 enzymes can be efficiently expressed in C3 crops, it is still difficult to simultaneously express all key enzymes in the correct cell type and subcellular location (Schuler et al., 2016; Ermakova et al., 2020; Cui, 2021). Factors such as the selection of gene promoters, the combination of regulatory elements, the control of transgenic insertion positions and expression levels can also affect whether the C4 pathway can function normally (Ermakova et al., 2020; Chen et al., 2023).

5.3 Anatomical constraints

The efficiency of C4 photosynthesis is very high, mainly relying on the "Kranz" structure. This special structure enables mesophyll cells and vascular bundle sheath cells to be closely arranged and functionally differentiated (Schuler et al., 2016; Cui, 2021; Mukundan et al., 2024; Prasanna et al., 2025) (Figure 2). As wheat is a C3 crop, it naturally lacks this structure in its body, making it difficult to form an effective CO_2 concentration mechanism. Although anatomical and functional differentiation structures similar to C4 (such as the "Bose" structure) have emerged during wheat grain development, it remains difficult to reconstruct the Kranz structure in leaves (Rangan et al., 2016; Cui, 2021; Rangan et al., 2024). At present, scientists have not yet fully grasped the genetic regulatory mechanism of the Kranz structure, which to some extent restricts the progress of C4 engineering.

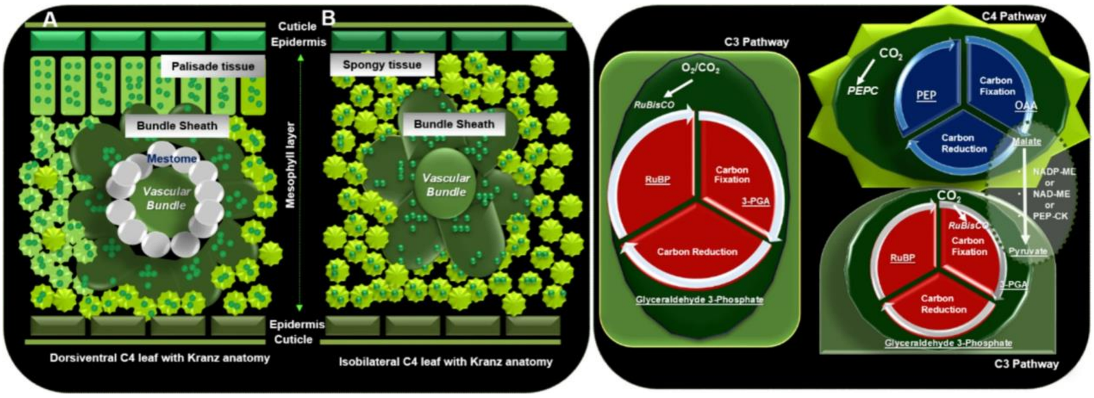

Figure 2 Anatomy and physiology of C3 and C4 plants (Adopted from Mukundan et al., 2024)

Note: A Histology of NAD-ME dorsiventral C4 leaf showing the mestome (a non-chlorophyllous layer of tissue between the bundle sheath and vascular bundle) along with Kranz features B Histology of isobilateral C4 leaf showing the Kranz features C C3 pathway (Calvin cycle) showing the enzyme, substrate and carbon assimilation D C4 pathway (Hatch and Slack pathway) showing the enzyme, substrate and carbon assimilation along with decarboxylation enzymes (Adopted from Mukundan et al., 2024)

5.4 Metabolic balance and trade-offs

Introducing the C4 pathway into C3 crops will have a significant impact on the original C3 metabolic network, which may bring about new metabolic bottlenecks or side effects (Cui, 2021; Prasanna et al., 2025). For instance, the high-level expression of C4 enzymes needs to be matched with energy supply, reducing power distribution, and carbon flow balance; otherwise, it may inhibit growth or waste resources (Ermakova et al., 2020; Cui, 2021; Prasanna et al., 2025). Furthermore, the synergistic patterns of the C4 and C3 pathways in different tissues and at different developmental stages remain unclear and require further optimization through systems biology and metabolic modeling (Schuler et al., 2016; Cui, 2021; Prasanna et al., 2025).

6 Emerging Tools and Strategies

6.1 Synthetic biology approaches

Synthetic biology provides a technical and theoretical basis for the modular reconstruction of the C4 photosynthetic pathway. By delving into the mechanism of action and evolutionary process of key enzymes in C4 metabolism, researchers have proposed a simplified engineering model based on known C4 metabolic components. These models have promoted the expression and functional verification of C4-related genes in C3 crops such as wheat. Furthermore, synthetic biology also supports methods such as targeted mutagenesis and directed evolution to optimize the activity and position of C4 enzymes in cells, thereby accelerating the engineering process of C4 traits (Schuler et al., 2016; Prasanna et al., 2025).

6.2 Advanced genome editing

Advanced genome editing technologies such as CRISPR/Cas provide powerful tools for precisely regulating the functions of C4-related genes. Through gene knock-in, knockout and site-directed mutagenesis, the key C4 enzymes can be efficiently expressed in wheat leaves and localized to appropriate subcellular locations. These techniques can also be used to regulate factors related to the anatomical structure of Kranz, laying the foundation for the spatial partitioning required for C4 photosynthesis (Schuler et al., 2016; Cui, 2021; Prasanna et al., 2025).

6.3 Cell-type specific promoters

The operation of the C4 photosynthetic pathway depends on the rational distribution of enzymes between mesophyll cells and vascular bundle sheath cells. In C4 plants, these functional genes have acquired new tissue expression patterns. Therefore, identifying and utilizing specific promoters has become a key step in achieving cell type-specific expression. By comparing genomes and analyzing promoter sequences, researchers have identified a variety of ccis elements that affect C4 gene expression and subcellular localization. These elements provide important molecular tools for wheat C4 engineering (Chen et al., 2023; Raturi et al., 2024).

6.4 Comparative systems biology

Systems biology combines genomic, transcriptomic, proteomic and metabolomic data to help reveal the regulatory networks and evolutionary patterns of the C3 and C4 photosynthetic pathways. Comparative analysis reveals that C4-related genes already exist in C3 crops, but their expression patterns and localization still need to be optimized. Systems biology can also be used to simulate the effects of C4 engineering on the overall metabolism and growth of wheat, thereby providing references for multi-gene synergistic regulation and yield trait optimization (Schuler et al., 2016; Cui, 2021; Chen et al., 2023; Raturi et al., 2024).

7 Potential Impacts of Successful C4 Engineering in Wheat

7.1 Yield gains under different climates

The engineering of the C4 photosynthetic pathway into wheat is expected to significantly increase yield under adverse conditions such as high temperature and drought. C4 plants (such as corn) have higher photosynthetic efficiency and more biomass accumulation under conditions of high temperature and water shortage. One important reason for this is that their CO_2 fixation mechanism is more efficient (Rangan et al., 2016; Wang et al., 2022; Rezaei et al., 2023). Studies have found that wheat expresses some C4 photosynthetically related genes during the grain development stage. This indicates that optimizing the photosynthetic contributions of C3 and C4 through genetic improvement may enable wheat to have higher yield and more stable performance under extreme climates (Rangan et al., 2016). Simulation and field trial results show that if the wheat genotype has higher radiation utilization efficiency, earlier flowering time and larger grain weight, the yield can increase by 6% to 69% in the case of more frequent extreme weather in the future (Yan et al., 2021). In addition, if the source reservoir characteristics of wheat are improved, the yield can theoretically increase by 16% to 52% under current and future climatic conditions, but sufficient nitrogen fertilizer input is required (Martre et al., 2024).

7.2 Adaptation to climate change

The C4 photosynthetic mechanism makes crops more heat-tolerant and drought-tolerant, which can alleviate the adverse effects brought about by climate change. Under the current climate change conditions, the yields of C3 crops (such as wheat) have decreased significantly in high temperatures and droughts. Moreover, the benefits of CO_2 fertilization are limited and are easily offset by high temperatures (Asseng et al., 2018; Makowski et al., 2020; Pequeno et al., 2021; Rezaei et al., 2023). In contrast, C4 crops perform better under drought and high temperatures. If wheat can achieve C4 engineering, it will enhance its ability to cope with climate change, especially in low latitudes and arid regions (Rangan et al., 2016; Pequeno et al., 2021; Wang et al., 2022). At the same time, climate-smart measures (such as variety improvement and management optimization) can further reduce yield loss and even lead to net yield growth in some places (Pequeno et al., 2021; Sedebo et al., 2022; Abramoff et al., 2023).

7.3 Economic and social implications

If the C4 project wheat is successfully promoted, it may bring about multiple economic and social benefits. Firstly, increased production and enhanced climate adaptability contribute to ensuring global food security, especially in developing countries that are highly threatened by climate change (Rangan et al., 2016; Pequeno et al., 2021; Martre et al., 2024). However, to reach the maximum yield potential, a large amount of nitrogen fertilizer input is required, which may increase environmental pressure and production costs (Jobe et al., 2020; Martre et al., 2024). Farmers adopting climate-smart measures (such as planting new varieties and optimizing management) can increase production by more than 34%, thereby increasing income and promoting rural economic development (Sedebo et al., 2022). In terms of policy-making, it is also necessary to consider the knowledge and practical experience of small-scale farmers to promote the popularization of technology and sustainable development.

8 Future Prospects and Research Priorities

8.1 Multi-institutional collaborations

The C4 photosynthesis project is very complex and requires multi-institutional cooperation on a global scale. International projects like the C4 Rice Project have proved that cooperation plays an important role in resource integration, technology sharing and standard setting. In the future, if interdisciplinary and transnational cooperation can be promoted, breakthroughs in fields such as genomics, systems biology and synthetic biology can be accelerated, and the feasibility and efficiency of C4 engineering can be improved (Sage and Zhu, 2011; Schuler et al., 2016; Pradhan et al., 2022; Prasanna et al., 2025).

8.2 Bridging basic and applied research

For the successful engineering of C4, it is essential to have a thorough understanding of the photosynthetic mechanisms, gene regulatory networks, and structures like the Kranz anatomy of C3 and C4. At present, basic research has identified many key genes and regulatory elements and studied their expression patterns. But more efforts are needed to transform these findings into engineering strategies that can be applied to wheat. In the future, priority should be given to closely integrating basic research with applied research, forming a complete innovation chain from molecular mechanisms to field performance (Matsuoka et al., 2003; Cui, 2021; Pradhan et al., 2022; Chen et al., 2023; Raturi et al., 2024).

8.3 Integrating C4 traits with other innovations

Combining C4 traits with other innovative traits, such as stress resistance and efficient resource utilization, is an important direction for enhancing the adaptability and yield of wheat. The overexpression of the C4 gene not only enhances photosynthetic efficiency but also makes crops more drought-tolerant and heat-resistant. Future research can explore the combined effect of C4 traits with new technologies such as climate-smart crops and enhanced rhizosphere carbon sinks, to achieve comprehensive improvement of multiple traits (ansson et al., 2018; Yadav and Mishra, 2020; JMukundan et al., 2024; Talukder et al., 2024; Prasanna et al., 2025).

9 Conclusion

In recent years, significant progress has been made in the research of C4 photosynthetic pathway engineering in wheat. Research has found that there are C4-related genes in the wheat genome, and the activity and expression of C4 enzymes can be detected in specific parts such as grains and panicles. These enzymes have a positive impact on carbon assimilation and yield under conditions such as high temperature and drought. Molecular evolution and comparative genomics studies have shown that C3 crops (such as wheat) themselves have the basis of C4 pathway genes. These genes have formed new tissue expression patterns during evolution, providing genetic resources for the engineering of C4 traits.

In wheat, C4 enzymes such as PEPC, NADP-ME, MDH and PPDK have been identified and their activities have been detected at different growth stages and in tissues. The high expression of some genes is related to the improvement of photosynthetic efficiency. Wheat grains and panicles exhibit stronger C4 enzyme activity under stress such as drought, indicating that the C4 pathway plays a significant role in stress resistance and carbon utilization efficiency in non-lobar organs. With the development of synthetic biology and genetic engineering technology, progress has been made in the expression and localization of C4-related genes in C3 crops. However, to achieve a complete C4 pathway, it is still necessary to solve the problems of spatial division and the reconstruction of specific anatomical structures (such as Kranz structures).

To achieve C4 wheat, multi-disciplinary collaboration is required, including molecular biology, genetics, plant physiology, synthetic biology and computational modeling. In-depth research on systems biology and gene regulatory networks can help break through the limitations of single enzyme engineering, promote multi-gene expression, tissue-specific regulation and anatomical structure remodeling. International cooperation projects (such as the C4 Rice Project) have provided references for this kind of cross-disciplinary cooperation.

In the future, the C4 wheat project can be advanced in phases. The first step is to deeply analyze the expression regulation and function of C4-related genes in wheat; The second step is to achieve modular introduction of multiple genes and tissue-specific expression using synthetic biology methods. The third step is to explore and reshape the Kranz structure or a structure with similar functions; The fourth step is to optimize the C4 trait through field trials and molecular breeding to enhance yield and resource utilization efficiency. The ultimate goal is to cultivate C4 wheat with high light efficiency, strong stress resistance and sustainable development, providing feasible solutions for global food security and climate-resilient agriculture.

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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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