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Stacking of Multiple Resistance Genes in Wheat via Transgenic Approaches

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Abstract Wheat diseases continue to threaten global food security, leading to yield losses and reduced grain quality. Consequently, effective and sustainable disease resistance strategies are urgently needed. This study explores the stacking of multiple resistance genes in wheat through transgenic approaches as a promising solution to these challenges. We first outline the principles of gene stacking, including the necessity to overcome pathogen evolution, ensure broad and durable resistance, and meet environmental and agricultural needs. We then discuss various transgenic strategies, such as direct genetic transformation, synthetic multigene constructs, and CRISPR/Cas-mediated genome editing, highlighting their potential for assembling and integrating multiple resistance genes. We also detail specific resistance genes commonly used in transgenic wheat, including those targeting rusts (e.g., *Lr34*, *Sr22*, *Yr36*), fungal and viral pathogens, and genes involved in broad-spectrum defense (e.g., pathogenesis-related proteins). Using the case study of transgenic stacking for rust resistance, specifically against Ug99 rust, we illustrate the practical applicability and global impact of this approach. We also explore the technical challenges, biosafety regulations, and genetic complexity that hinder its implementation. Looking ahead, we explore innovations in synthetic biology, precision gene editing, and breeding for climate resilience. Finally, we summarize recent advances in gene stacking, identify key gaps, and highlight the future potential of transgenic technology for enhancing durable disease resistance in wheat.

Keywords Transgenic wheat; Gene stacking; Disease resistance; CRISPR/Cas genome editing; Rust resistance genes

1 Introduction

In recent years, the problem of frequent wheat diseases has shown no sign of abating. The "familiar faces" such as stem rust and powdery mildew still cause considerable production losses worldwide. Although people had high hopes for the single resistance gene (*R* gene) in the past, reality soon poured cold water on it-pathogens are not herbivores either; they evolve, and resistance thus becomes ineffective. Thus, "multi-gene combination" has become a more reliable option. However, it's easier said than done. Traditional gene stacking breeding is not only cumbersome but also often makes the breeding process complex and time-consuming because the resistance loci are not closely adjacent (Athiyannan et al., 2022).

The development of technology has indeed brought about a turning point. Nowadays, plant genomics and transformation technologies enable us to clone multiple resistance genes at a single site and then transfer them into the wheat genome. This is known as transgenic resistance stacking (RTGS). Compared with the traditional way, this method not only saves time but is also more efficient. In addition to expanding the range and duration of resistance, it is also possible to discover high-quality disease-resistant genes in wild or distant species that cannot be obtained through traditional means. For instance, there have been studies that have successfully cultivated wheat strains carrying up to five resistance genes through this approach, which still perform strongly against various pathogenic bacteria (Koller et al., 2023).

So the focus of this study lies here-we want to see how far the current research on achieving the superposition of disease-resistant genes through genetic modification in wheat has come. In addition to summarizing the technical principles and application effects, the problems encountered will also be discussed. Of course, recent cases and related genomic resource integration work will not be absent. Finally, some ideas and prospects on how to achieve more durable resistance in breeding in the future will also be put forward.

2 Rationale for Gene Stacking in Wheat

2.1 Overcoming pathogen evolution and resistance breakdown

In the wheat fields, rust and powdery mildew are almost always present. The key issue is not just that they "come frequently", but that they "become faster". Pathogenic bacteria can constantly evolve into stronger new strains. Once a certain resistance gene is identified, the original disease-resistant variety will be quickly breached, and yield loss will follow. Relying on a single gene is often just a temporary matter. Then, is there any way to prevent pathogenic bacteria from making a move? Genetically modified superposition technology offers a direction. It is not simply adding defense lines one by one, but concentrating multiple resistance genes at one site to build a composite barrier. In this way, it becomes much more difficult for pathogens to bypass all resistance mechanisms at once. Some studies have pointed out that this strategy can indeed enable wheat strains to maintain stable resistance to a variety of highly virulent pathogenic bacteria, and the situation of resistance failure is also reduced (Aravindh et al., 2020; Jost et al., 2023; Yu et al., 2023).

2.2 Enhancing durable and broad-spectrum resistance

It's not that the superposition of resistance genes is merely a quantitative accumulation; what matters is the combination. Some genes offer early recognition, while others act as a buffer layer for delayed onset. Whether it is the *R* gene or the resistance gene at the mature plant stage, their combination not only prolongs the resistance period but also broadens the disease resistance spectrum. Not just one disease can be prevented, such as stem rust, leaf rust and powdery mildew. Multiple "enemies" can also be caught in one net. Sometimes, when several genes come together, they can produce a stronger synergistic effect than when used alone (Figure 1).

Under high disease stress conditions, the resistance performance of this combination is particularly stable. Even if the climate or region changes, the effect will not be compromised. What is even more worth mentioning is that some "rare genes" in wild relatives can also be utilized in this model to enrich the resistance resources (Singla et al., 2016; Dinglasan et al., 2022; Li et al., 2024).

2.3 Addressing environmental and agricultural demands

In the past, people might have been more concerned about "whether there were disease-resistant genes", but now the question has become "whether less medicine can be taken". Climate change is becoming increasingly difficult to predict, the pressure for agricultural intensification is also growing, and chemical control is facing more and more restrictions. Against this backdrop, the superimposition of resistance becomes particularly realistic. It reduces the reliance on fungicides and also makes the breeding of disease-resistant varieties more efficient. The traditional breeding process is complex and time-consuming. However, gene superposition technology can accelerate this step and also alleviate some ecological and regulatory pressures. For farmers, this is not only related to the stability of output, but also a means to deal with the challenges of future agriculture. To some extent, it is also laying the foundation for the resilience of agricultural ecosystems (Hafeez et al., 2021; Saintenac et al., 2021; Zhao et al., 2024a).

3 Transgenic Strategies for Resistance Gene Stacking in Wheat

3.1 Direct genetic transformation techniques

The Agrobacterium-mediated method and gene gun technology, these two seemingly "old-fashioned" transformation approaches, have instead played a key role in the superposition of resistance genes. In the past, people thought they were rather crude, but in fact, nowadays this kind of direct transformation method can precisely introduce multiple resistance genes at the same locus, even bringing in genes from distant species, thus bypassing the common pairing problem in traditional breeding. Especially in the application of multi-gene boxes, recent studies have shown that they can be stably transferred into wheat and exhibit good resistance to highly toxic pathogens (Camenzind et al., 2024). Moreover, the efficiency is not low. Sometimes, based on the prevalence of diseases in different regions, specific gene combinations can be selected for transformation, which is more in line with the actual planting needs.

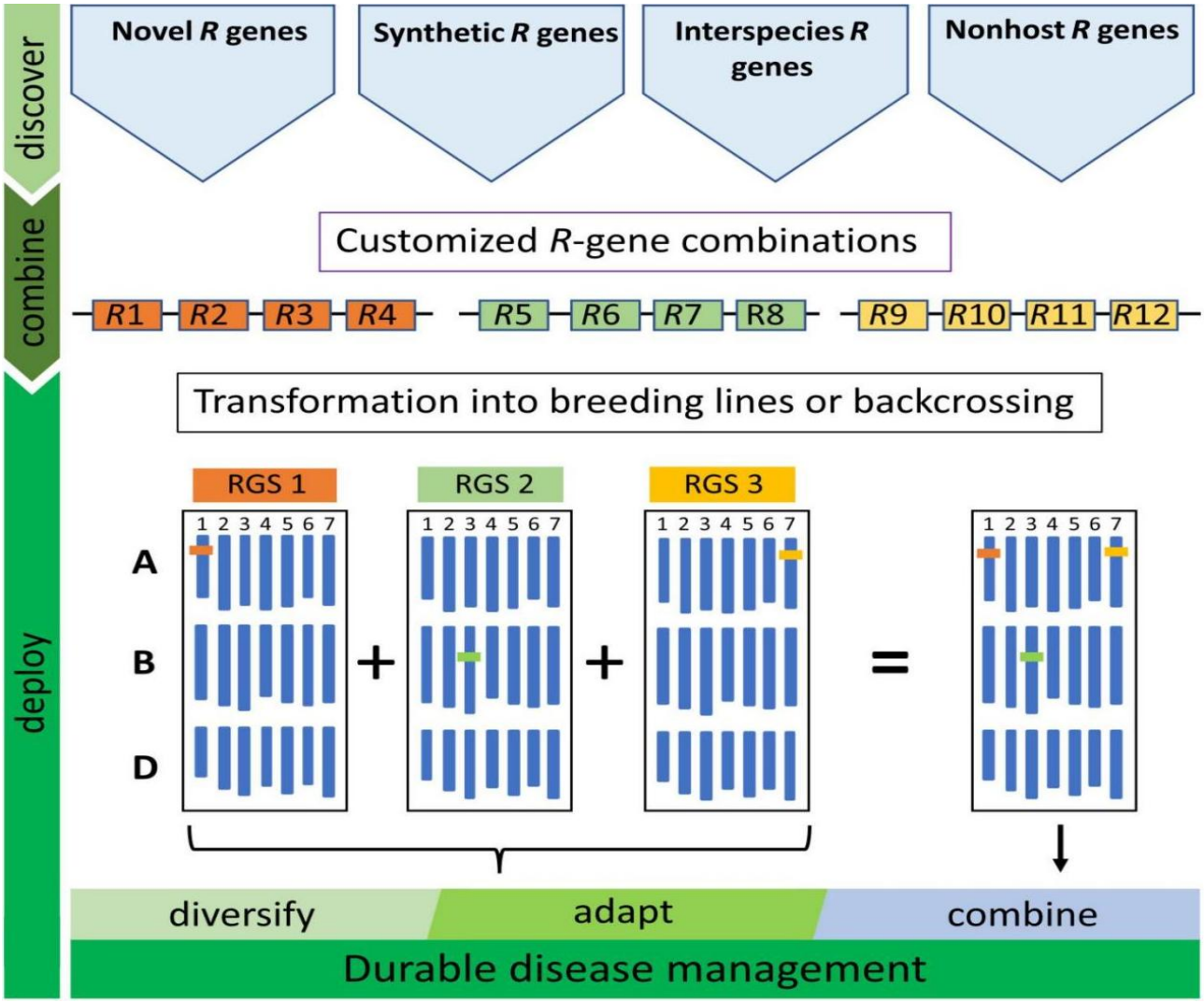

Figure 1 Deployment strategy of resistance gene stacks (RTGS). A range of resistance genes (*R*-genes) are needed to diversify RTGS for durable, broad-spectrum resistance. In addition to wheat *R* genes, genes can be sourced from land races, wild relatives, nonhost species or novel engineered genes (synthetic *R*-genes). Customized RTGS for regional or seasonal disease management can be rapidly assembled and integrated into breeding programs to adapt and react to newly evolving pathogen strains. The single locus inheritance of RTGS enables single or multiple cassettes to be used in breeding programs by backcrossing or alternatively direct transformation of elite wheat cultivars (Adopted from Jost et al., 2023)

3.2 Synthetic gene constructs and multigene vectors

Putting several resistance genes together is actually not a simple package. The design of synthetic constructs and multi-gene vectors should be ingenious. Each gene not only needs to have its own promoter, but also must ensure that their expressions do not "steal the show" from each other. The commonly used approach at present is to synthesize these genomes into a whole as a single transgenic event and introduce them into wheat, which not only ensures co-inheritance but also avoids expression conflicts. There is a rather typical example: After a construct containing five resistance genes was successfully introduced into wheat, the variety acquired broad-spectrum resistance to powdery mildew and rust. By designing in this way, researchers can also avoid those combinations that are prone to interfere with each other or cause chain burdens, reducing subsequent unstable factors from the source.

3.3 CRISPR/Cas and genome editing for gene integration

If we talk about which tool best fits the "modern feel", CRISPR/Cas definitely deserves the nomination. In the past, hybridization and backcrossing were exhausting to superimpose several genes. Now, with gene editing, multiple resistance genes can be precisely inserted into a target site in the wheat genome, and the entire process is

neat and efficient. Even better, it can perform surgery on susceptibility genes (*S* genes) or simply "create" new resistance alleles, immediately broadening the disease resistance spectrum. Although this method is still being continuously optimized, its efficiency has already been impressive (Huang et al., 2024). It not only saves time but also helps maintain the original agronomic traits without being damaged (Waites et al., 2025). In the future, CRISPR multi-gene integration technology is highly likely to become the new normal in wheat disease-resistant breeding.

4 Key Resistance Genes Used in Transgenic Wheat

4.1 Rust resistance genes (e.g., *Lr34*, *Sr22*, *Yr36*)

The "destructive power" of rust disease on wheat is no longer news, so the related resistance genes have become the first choice for transgenic superimposition. Genes like *Lr34* are often mentioned, not only because they have a wide range of disease resistance, but also because they "resist for a long time". It encodes an ATP-binding cassette transporter protein, which enables wheat to simultaneously resist leaf rust, stripe rust and even powdery mildew. Although it is not completely immune, the long-term effect is good. This gene can function at both the seedling stage and the mature plant stage, but the effect depends on the coordination of the expression level and the background genome (Risk et al., 2012; Chauhan et al., 2015). Some other anti-rust "experts" like *Sr22*, *Sr21*, *Sr13*, *Sr43* and *Sr62*, mostly from wild wheat relatives, have been successfully cloned and integrated into the five-gene box. For instance, combinations containing *Sr22* demonstrated very strong field resistance against a variety of highly toxic stem rust fungi (Chen et al., 2018). As for stripe rust, there have also been new developments recently. For instance, genes like *YrNAM* and *Yr36* have been verified to effectively combat the mainstream stripe rust populations, providing new ideas for subsequent combination strategies (Ni et al., 2023).

4.2 Fungal and viral resistance genes

Research on the resistance of wheat does not stop at rust disease. Anti-powdery mildew genes like *Pm3*, *Pm17* and *Pm57* have also long been incorporated into the transgenic superposition system. Among them, *Pm57* originated from *Aegilops searsii* and is a typical example of "borrowing genes" from wild species. Multiple *Pm3* alleles or combinations like *Pm17+Pm3b* can bring about a stronger field resistance effect (Zhao et al., 2024b). Furthermore, *Fhb7* is a very special gene. It originally originated from fungi and was "mixed" into the plant genome through horizontal gene transfer. This gene can produce a glutathione S-transferase, which is specifically used to detoxify the toxins of Fusarium, and has inhibitory effects on both scab and crown rot. The key point is that it does not affect the yield (Wang et al., 2020). There is another rather interesting case from Damai. After the *chi26* (chitinase) gene of barley is transferred into wheat, it enhances the wheat's defense against rust and powdery mildew by breaking down the cell walls of fungi. The effect of this gene has remained relatively stable over multiple generations, indicating that its inheritance is also quite reliable.

4.3 Broad-spectrum defense genes (e.g., PR proteins, antimicrobial peptides)

Not all resistance strategies rely on "point-to-point" specific genes; some activate the wheat's own defense system. For instance, *PR* genes can encode proteins such as chitinase and glucanase, which specifically attack the cell walls of pathogens and are an indispensable part of the acquired resistance (SAR) system in wheat (Eissa et al., 2017). *NPR1* is another "dispatch center" type gene. It does not directly resist diseases, but it can regulate the expression of a series of defense-related genes, thereby indirectly enhancing broad-spectrum resistance. Such genes are often a "safety net" strategy under the threat of multiple pathogens. As for antimicrobial peptides, although there are not many application cases in wheat yet, based on the experience of other crops, they have considerable potential. In the future, if multiple antimicrobial peptide genes can be effectively integrated into the wheat genome, it may become an important supplement for building multi-disease resistance.

5 Case Study

5.1 Background: the Ug99 threat and the push for durable resistance

Many researchers frown upon hearing the name "East African wheat stalk Rust Pathogen (Pgt) Ug99". This strain of wheat stalk rust disease from East Africa did not suddenly "drop in", but its appearance was like a heavy blow, making people realize that the resistance genes they originally thought were "sufficient" were actually far from

enough. The resistance measures that were previously relied on quickly became ineffective in the face of it, especially the defense line that relied on only one gene, which almost collapsed at the slightest touch. The spread rate and mutation ability of this strain have forced people to reconsider the breeding approach-broad-spectrum and persistence are no longer "options" but "must" (Zhang et al., 2017; Yu et al., 2022).

5.2 Review of the stacked gene construct and its field-ready potential

The real turning point actually does not lie in "a certain magical gene", but in the construction of a five-gene box. This combination of multiple stem rust resistance genes, when placed at the same site, can form an effective barrier for wheat from the seedling stage to the mature plant stage. This build contains both ASR and APR, among which four have been confirmed to have effects. More importantly, such a single locus superposition greatly simplifies the genetic process. What originally required generations of hybridization to achieve can now be accomplished with a single transfer. Field trials also provided positive feedback: in the face of highly invasive isolates including Ug99, these strains demonstrated significant high resistance (Luo et al., 2021; Jost et al., 2023). However, to be honest, some Pgt isolates have already been able to break through these combinations, which also reminds us that we cannot "put it once and for all". Continuous monitoring and dynamic adjustment of the portfolio strategy are the keys to going down this path.

5.3 Global implications and lessons learned

What does this case prove? It is demonstrated that modern genomic tools and transgenic strategies can indeed be useful when dealing with complex agricultural diseases. But this does not mean that the problem has been solved. The pathogen of rust disease is still evolving, and the regulatory threshold for genetically modified crops is not low either. In different countries and regions, how to implement these gene combinations and how to carry out personalized deployment based on the distribution of diseases still rely on long-term strategies for support. There is experience: the combination can be strong, but not rigid. It has to be used in conjunction with agricultural management methods and flexibly adjusted according to regional differences. Otherwise, even the best genes will one day be broken through (Zhang et al., 2021).

6 Challenges and Limitations of Gene Stacking in Wheat

6.1 Technical challenges in multigene engineering

Although the idea of superimposing multiple genes on a crop like wheat, which has a large and complex genome, is good, it is not so smooth to implement. Not only do multiple resistance genes have to be inserted into a specific site, but they also need to be expressed stably. This in itself is quite "tormenting". Although there have been successful cases of five gene superpositions, larger constructs, such as those with eight genes and a size of 63 kb, have not yet been thoroughly tested. No one can guarantee exactly how much can be contained in wheat. Moreover, can a set of combinations perform well in all varieties and various climates? This is also a question mark. Sometimes, a certain gene that can resist diseases in one variety may not work in another. Whether the resistance is strong or not depends not only on the genes themselves but also on whether the "compatibility" with the wheat variety and the "personality" of the local pathogenic bacteria match.

6.2 Regulatory and biosafety concerns

Technically, it can be advanced, but whether it can be widely promoted is another matter. Especially in the field of genetically modified organisms, wheat has not gone as smoothly as some other crops. The review process for genetically modified organisms in many countries is quite strict, especially when it comes to new varieties with multiple gene superpositions, the approval process is even more lengthy. Even if it has been scientifically verified to be safe, the regulatory environment in some regions will still slow down the deployment progress. Moreover, the issue of gene loss is not an unfounded worry. Some people are worried that the genes transferred in might be accidentally passed on to wild relatives or affect non-target organisms. Therefore, it is fundamentally difficult to obtain approval without conducting long-term monitoring and a comprehensive risk assessment. In addition, issues such as the ownership of intellectual property rights and public acceptance, which are "beyond technology", also make commercial promotion not so easy.

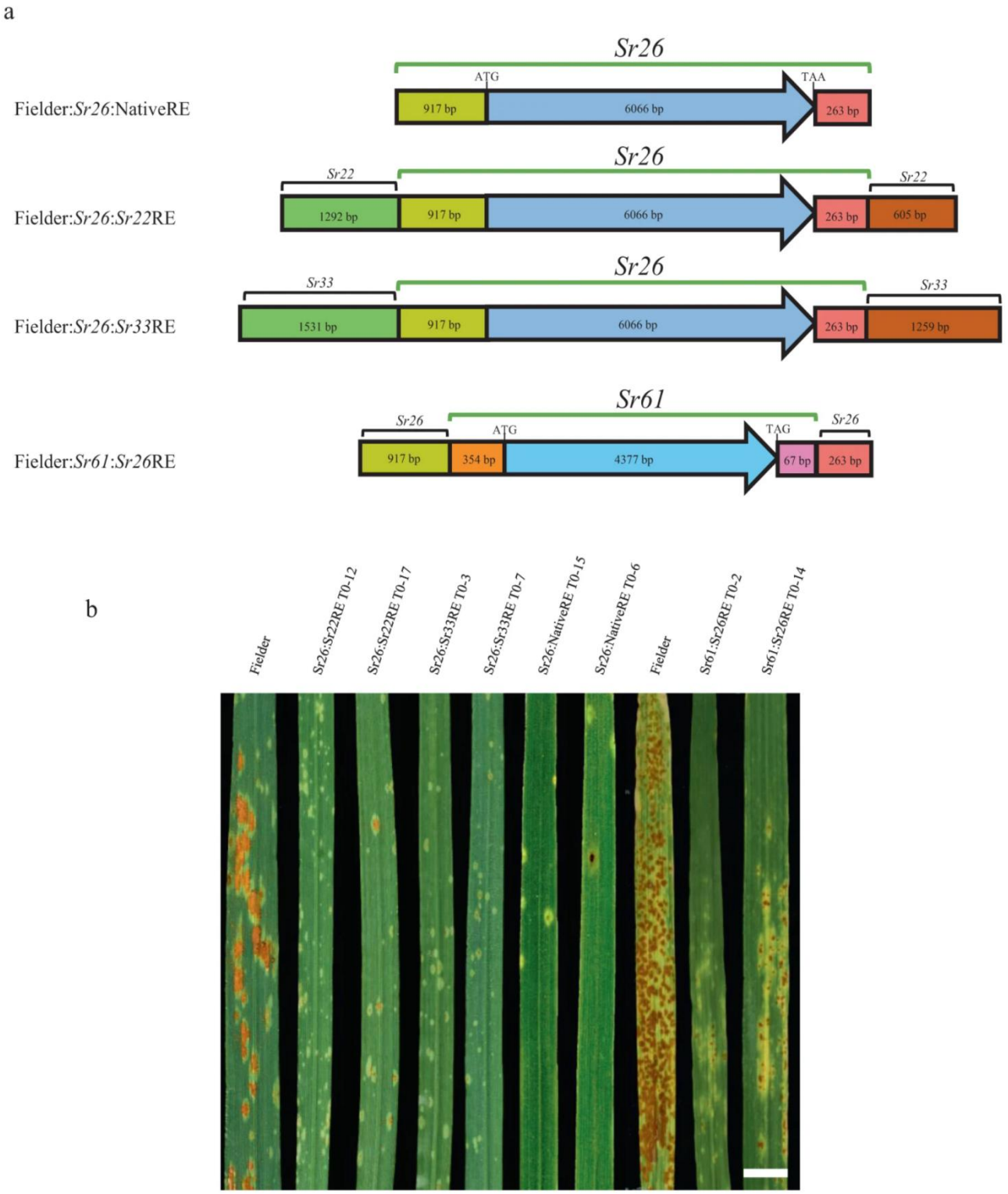

Figure 2 Validation of the *Sr26* and *Sr61* candidate genes by transformation (Adopted from Zhang et al., 2021)

Image caption: a Four constructs used for wheat transformation. The three *Sr26* constructs encoded *the Sr26* gene candidate and either its native regulatory sequences (*Sr26*:*NativeRE*), regulatory sequences from *Sr22* (*Sr26*:*Sr22RE*) or regulatory sequences from *Sr33* (*Sr26*:*Sr33*RE). A single construct was used for the *Sr61* gene candidate under the regulatory control of *Sr26* *5′* and *3′* regulatory elements (REs). *5′* REs and *3′* REs are indicated by black brackets with sizes in bp indicated for all four constructs. *Sr26* and *Sr61* intron/exon coding regions are shown as light and dark blue arrows. b Phenotypic responses of representative T_0 plants produced for all four constructs and inoculated with *Pgt* race 98-1,2,3,5,6+Sr50 along with non-transgenic Fielder control lines. All lines except the susceptible Fielder control showed low infection types. Photographed 12 days of post-inoculation. Bar shows 1 cm (Adopted from Zhang et al., 2021)

6.3 Genetic and epigenetic interactions

Ideally, when multiple resistance genes work together, the effect should be better. But in reality, sometimes there is a situation where "you suppress me and I interfere with you" instead. Cross-inhibition may occur among genes, which ultimately leads not to a stronger effect but to a weakened overall effect. Epigenetic interventions cannot be ignored either-invisible changes such as DNA methylation and histone modification can secretly alter gene expression, resulting in fluctuations in resistance (Ramirez-Gonzalez et al., 2018). What makes it more complicated is that wheat itself is a polyploid crop. When multiple homologous genes are copied together, it is very easy for them to "compete with each other". This uncertainty forces breeders to be more cautious when pairing genomes. Which genes to select, how to combine them, and what the performance will be after the combination all need to be verified one by one. Decisions cannot be made based on experience and intuition. Otherwise, no matter how much is piled up, it may still backfire.

7 Future Perspectives and Innovations in Wheat Gene Stacking

7.1 Integration of synthetic biology tools

If traditional genetically modified organisms are regarded as "transporters", then synthetic biology is more like "designers". It doesn't merely allow us to piece together several genes, but enables us to design a modular resistance system from scratch. The emergence of such tools has made the originally complex superimposition of wheat genes more possible. Especially after the improvement of cloning efficiency and the ease of obtaining high-quality genomic data of wheat, it is no longer difficult to screen resistance genes from wild and local varieties, or even to construct new metabolic pathways. In the past, due to the limitations of natural resistance, now some "unconventional" paths can be conceived. Even if it is not the inherent mechanism of wheat, it may be introduced through synthetic means. Coupled with the increasing strength of computing tools and databases, the speed of screening and functional verification of candidate genes has also kept pace (Li et al., 2021; Chen et al., 2024).

7.2 Precision breeding with gene editing technologies

The CRISPR/Cas gene editing technology is no longer "aloof". In precise wheat breeding, it is increasingly becoming a "standard configuration" in the laboratory. Unlike traditional genetically modified organisms that require a large building block, current editing tools allow for direct modifications at designated positions, such as deletion, alteration, replacement, and addition. You can operate however you like (Ma et al., 2024). More importantly, this type of editing method can be combined with breeding strategies such as marker-assisted selection and genomic selection. The efficiency is not only slightly improved, but multiple target traits-such as disease resistance, high yield, and quality improvement-can be advanced simultaneously (Wang et al., 2019). Of course, the continuous improvement and annotation update of the wheat reference genome are also indispensable behind this. As these foundations become increasingly solid, it is no longer an unattainable goal to build "green super wheat" that can adapt to various pressures.

7.3 Toward climate-resilient wheat varieties

What should wheat look like in the future? The answer may not merely be about having stronger disease resistance, but also being able to withstand extreme environments such as drought, high temperatures, and salinity. Under climate change, the traditional concept of "stable high yield" needs to be redefined, and the gene superposition approach in this context must also shift towards a "stress resistance + adaptation" type. Integrating genes with multiple traits such as drought resistance, heat resistance and salt resistance into one variety is not merely a technical show-off, but the foundation for maintaining global food security. To achieve this goal, relying solely on genetic technology is not enough; it also requires the coordinated advancement of multiple approaches such as phenotypic analysis, rapid breeding, and digital gene banks (Rasheed et al., 2018). The use of the pan-genome will also play a role. It provides a broader genetic background and sources of variation, offering more "tabs" for combinations. In conclusion, the wheat of the future must be capable of "adapting to the climate on its own"; otherwise, even the highest yield potential may be wasted in extreme weather (Hussain et al., 2022).

8 Concluding Remarks

The topic of disease resistance has always been unavoidable in wheat breeding. However, the combination of genetically modified organisms and resistance genes is no longer a "concept"; it does indeed offer the possibility of being able to withstand and resist for a long time. Especially when dealing with those pathogens that always "break through" in various ways, such as stem rust, some varieties have demonstrated very stable field resistance. Among them, a relatively representative case is where five resistance genes were integrated into the same locus. This "combination punch" approach not only enhances the resistance strength but also makes the genetic process simpler. In addition, new genes like *Sr26*, *Sr61* and *Sr43* have also been successfully cloned and put into use, and the gene resource library is constantly expanding.

But then again, progress is one thing, but the problems still haven't lessened. Pathogens do not stop waiting for us-new variants keep emerging, and some can even bypass existing polygenic combinations. This also makes people realize that even if multiple genes are superimposed, it is not "foolproof". Moreover, from a technical perspective, we still do not know exactly how many effective genes can be stably superimposed in wheat, and it may not be clear whether the genes will "fight" with each other. In addition, practical problems are also right before our eyes. The regulatory threshold for genetically modified organisms is high, and the public's acceptance is not necessarily so friendly. All these are influencing whether these technologies can truly enter commercial breeding. Therefore, apart from the breakthroughs in the laboratory, how to better explain and promote them to the society and regulatory authorities is equally crucial.

What needs to be done next might not be as simple as continuing to "stack a few more genes". Monitor the dynamics of pathogens and adjust the gene combination in a targeted manner; Discover new resistance resources from wild species and even non-host plants; Exploring more flexible and adaptable superimposition strategies must all be put on the agenda. The superposition of transgenic resistance genes does indeed offer a breakthrough approach. It's just that this path cannot be paved by a single technology. It needs to go hand in hand with precision breeding, synthetic biology and crop management, and be tailored to local conditions and needs. Only in this way is it possible to truly transform this progress into a practical force for disease resistance, stable production and ensuring food security.

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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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