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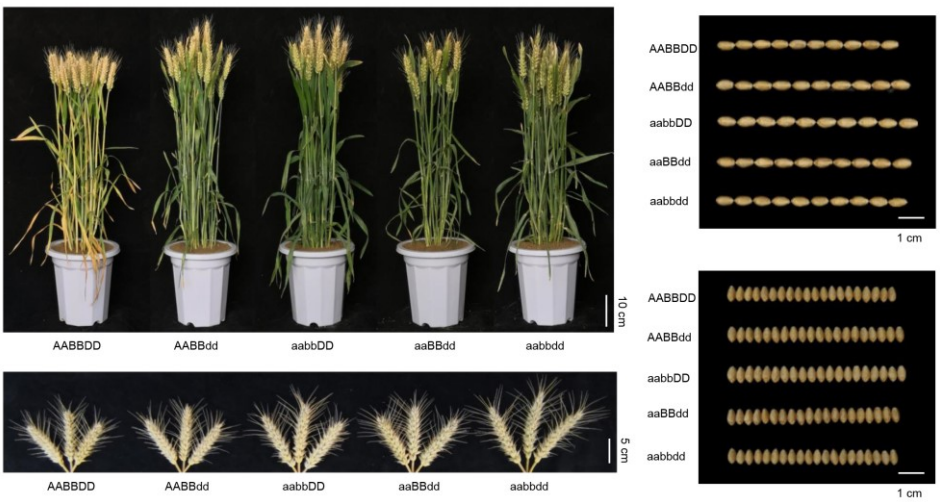
Engineering novel types of wheat plants through RNA interference and genome editing



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Year: 2022

COMMUNAUTÉ FRANÇAISE DE BELGIQUE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Engineering novel types of wheat plants through RNA interference and genome editing

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Dissertation originale présentée en vue de l'obtention du grade de docteur en **s**ciences agronomiques et ingénierie biologique

Promoteurs: Prof. Frédéric Francis
Prof. Lanqin Xia

Année civile: 2022

Abstract

Jiahui Zhang (2022). "Engineering novel types of wheat plants through RNA interference and genome editing" (PhD Dissertation in English).

Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liege.

182 pages, 13 figures, 4 tables.

Crop plants suffer severe yield losses due to significant damages caused by aphids. The level of nitrogen fertilization has a major impact on the population of aphids (nymphs and adults). A high-level nitrogen fertilizer application produces the greatest aphid population. The objectives of this thesis are to develop effective and environmentally friendly pest management strategies for aphid control and to increase nitrogen use efficiency (NUE) for the development of sustainable agriculture. The main results are as following:

RNA interference (RNAi) technology is a versatile and environmentally friendly method for pest management in crop protection. Transgenic plants expressing siRNA/dsRNA and non-transformative methods, such as microinjection, feeding, and nanocarrier-delivered mediated RNAi approach, have been successfully applied in agricultural practice for insect pest control. Grain aphid (*Sitobion miscanthi*) is one of the most dominant and devastating insect pests in wheat, which causes substantial losses to wheat production each year. Engineering transgenic plants expressing double strand RNA (dsRNA) targeting an insect-specific gene has been demonstrated to provide an alternative environmentally friendly strategy for aphid management through plant-mediated RNA interference (RNAi). Here we identified and characterized a novel potential RNAi target gene (*SmDSR33*) which was a gene encoding a putative salivary protein. Stable transgenic wheat lines expressing dsRNA for targeted silencing of *SmDSR33* in grain aphids through plant-mediated RNAi were generated. After feeding on transgenic wheat, the attenuated expression levels of *SmDSR33* in aphids were observed when compared to aphids feeding on wild-type plants. The decreased *SmDSR33* expression levels reduced significantly aphid fecundity and survival. We also observed altered aphid feeding behaviors such as longer duration of intercellular stylet pathway and shorter duration of passive ingestion in electropenetrography assays. Furthermore, both surviving aphids and their offspring exhibited decreased survival rates and fecundity, indicating that the silencing effect could be persistent and transgenerational in grain aphids. *SmDSR33* can be selected as an effective RNAi target for wheat aphid control. Silencing of an essential salivary protein gene involved in ingestion through plant-mediated RNAi was a second target and could be exploited as an effective strategy for aphid control in wheat.

The fast-developing clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology has been extensively applied for functional genomics studies and crop improvement. Nitrogen (N) is applied in relatively large amounts in agricultural practice to promote plant growth and development. To maximize yield, semi-dwarf crop varieties of the Green Revolution (GRV) require a high nitrogen fertilizer input. With the world's population

growing and environmental pollution increasing, enhanced crop productivity with limited N supplies is a critical barrier. Improved nitrogen use efficiency (NUE) will be necessary for agricultural sustainability in the future. Wheat (*Triticum aestivum* L.) is a staple food crop consumed by more than 30% of world population. Nitrogen (N) fertilizer has been applied broadly in agriculture practice to improve wheat yield to meet the growing demands for food production. However, undue N fertilizer application and the low N use efficiency (NUE) of modern wheat varieties are aggravating environmental pollution and ecological deterioration. Under nitrogen-limiting conditions, the rice (*Oryza sativa* L.) *abnormal cytokinin response1 repressor1* (*are1*) mutant exhibits increased NUE, delayed senescence and consequently, increased grain yield. However, the function of *ARE1* ortholog in wheat remains unknown. Here, we isolated and characterized three *TaARE1* homoeologs from the elite Chinese winter wheat cultivar ZhengMai 7698. We then used CRISPR/Cas9-mediated targeted mutagenesis to generate a series of mutant lines either with partial or triple-null *taare1* alleles. All mutant lines showed enhanced tolerance to N starvation, delayed senescence and increased grain yield in field conditions. In particular, the *AABBdd* and *aabbDD* mutant lines exhibited delayed senescence and significantly increased grain yield without growth defects compared to the wild-type control. Together, our results underscored the potential to manipulate *ARE1* orthologs through gene editing for breeding of high-yield wheat as well as other cereal crops with improved NUE.

In conclusion, we engineered novel aphid-resistant and nitrogen-use efficient wheat germplasms through plant-mediated RNAi technology and CRISPR/Cas9-mediated genome editing technology.

Keywords: Grain aphid, *Sitobion miscanthi*, RNA interference (RNAi), salivary protein, wheat, aphid control, nitrogen, nitrogen use efficiency, crop plant.

Résumé

Jiahui Zhang (2022). " Concevoir de nouveaux types de plants de blé grâce à l'interférence ARN et à l'édition du génome " (thèse de doctorat en anglais).

Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège.

182 pages, 13 figures, 4 tableaux.

Résumé:

Les plantes cultivées subissent de graves pertes de rendement en raison des dégâts importants causés par les pucerons. Le niveau de fertilisation azotée a un impact majeur sur la population de pucerons (nymphes et adultes). Une application d'engrais azoté à haut niveau produit la plus grande population de pucerons. Les objectifs de cette thèse sont de développer des stratégies de lutte antiparasitaire efficaces et respectueuses de l'environnement pour le contrôle des pucerons et d'augmenter l'efficacité d'utilisation de l'azote (NUE) pour le développement d'une agriculture durable. Les principaux résultats sont les suivants :

La technologie d'interférence ARN (ARNi) est une méthode polyvalente et respectueuse de l'environnement pour la lutte antiparasitaire dans la protection des cultures. Des plantes transgéniques exprimant des ARNsi/ARNdb et des méthodes non transformatrices, telles que la microinjection, l'alimentation et l'approche ARNi médiée par nanoporteur, ont été appliquées avec succès dans la pratique agricole pour la lutte contre les insectes nuisibles. Le puceron des céréales (*Sitobion miscanthi*) est l'un des insectes ravageurs les plus dominants et les plus dévastateurs du blé, ce qui cause des pertes substantielles à la production de blé chaque année. Il a été démontré que l'ingénierie de plantes transgéniques exprimant un ARN double brin (ARNdb) ciblant un gène spécifique à un insecte fournit une stratégie alternative respectueuse de l'environnement pour la gestion des pucerons grâce à l'interférence ARN médiée par les plantes (ARNi). Ici, nous avons identifié et caractérisé un nouveau gène cible potentiel d'ARNi (*SmDSR33*) qui était un gène codant pour une protéine salivaire putative. Des lignées de blé transgéniques stables exprimant l'ARNdb pour le silençage ciblé de *SmDSR33* chez les pucerons des céréales par l'intermédiaire de l'ARNi à médiation végétale ont été générées. Après s'être nourris de blé transgénique, les niveaux d'expression atténués de *SmDSR33* chez les pucerons ont été observés par rapport aux pucerons se nourrissant de plantes de type sauvage. La diminution des niveaux d'expression de *SmDSR33* a réduit de manière significative la fécondité et la survie des pucerons. Nous avons également observé des comportements alimentaires modifiés des pucerons, tels qu'une durée plus longue de la voie du stylet intercellulaire et une durée plus courte de l'ingestion passive dans les essais d'électropénétrographie. De plus, les pucerons survivants et leur progéniture présentaient des taux de survie et de fécondité réduits, ce qui indique que l'effet de silence pourrait être persistant et transgénérationnel chez les pucerons des céréales. *SmDSR33* peut être sélectionné comme cible ARNi efficace pour le contrôle du puceron du blé. Le silence d'un gène de protéine salivaire essentielle impliqué dans l'ingestion par l'ARNi médié par les plantes était une deuxième cible et pourrait être exploité comme une stratégie efficace

pour le contrôle des pucerons dans le blé.

La technologie CRISPR (CRISPR)/CRISPR-associated protein 9 (Cas9) à développement rapide a été largement appliquée pour les études de génomique fonctionnelle et l'amélioration des cultures. L'azote (N) est appliqué en quantités relativement importantes dans les pratiques agricoles pour favoriser la croissance et le développement des plantes. Pour maximiser le rendement, les variétés de cultures semi-naines de la Révolution verte (GRV) nécessitent un apport élevé en engrais azoté. Avec la croissance de la population mondiale et l'augmentation de la pollution de l'environnement, l'amélioration de la productivité des cultures avec des apports limités en N est un obstacle critique. L'amélioration de l'efficacité de l'utilisation de l'azote (NUE) sera nécessaire pour la durabilité agricole à l'avenir. Le blé (*Triticum aestivum* L.) est une culture vivrière de base consommée par plus de 30% de la population mondiale. Les engrais azotés (N) ont été largement appliqués dans les pratiques agricoles pour améliorer le rendement du blé afin de répondre aux demandes croissantes de production alimentaire. Cependant, l'application excessive d'engrais azotés et la faible efficacité d'utilisation de l'azote (NUE) des variétés de blé modernes aggravent la pollution de l'environnement et la détérioration écologique. Dans des conditions limitant l'azote, le mutant répresseur1 (*are1*) de la réponse anormale de la cytokinine1 (*are1*) du riz (*Oryza sativa* L.) présente une NUE accrue, une sénescence retardée et, par conséquent, une augmentation du rendement en grain. Cependant, la fonction de l'orthologue *ARE1* chez le blé reste inconnue. Ici, nous avons isolé et caractérisé trois homéologues *TaARE1* du cultivar de blé d'hiver chinois d'élite ZhengMai 7698. Nous avons ensuite utilisé la mutagenèse ciblée médiée par CRISPR/Cas9 pour générer une série de lignées mutantes avec des allèles taare1 partiels ou triple nuls. Toutes les lignées mutantes ont montré une tolérance accrue à la privation de N, une sénescence retardée et une augmentation du rendement en grain dans les conditions de terrain. En particulier, les lignées mutantes *AABBdd* et *aabbDD* ont présenté une sénescence retardée et un rendement en grain significativement accru sans défauts de croissance par rapport au témoin de type sauvage. Ensemble, nos résultats ont souligné le potentiel de manipulation des orthologues *ARE1* par l'édition de gènes pour la sélection de blé à haut rendement ainsi que d'autres cultures céréalières avec une NUE améliorée.

En conclusion, nous avons conçu de nouveaux germoplasmes de blé résistants aux pucerons et efficaces pour l'utilisation de l'azote grâce à la technologie ARNi médiée par les plantes et à la technologie d'édition du génome médiée par CRISPR/Cas9.

Mots clés: Puceron des céréales, *Sitobion miscanthi*, interférence ARN (ARNi), protéine salivaire, blé, lutte contre les pucerons, azote, efficacité d'utilisation de l'azote, plante cultivée.

Acknowledgment

I would like to express my sincere appreciation and thanks to all of the members who helped and encouraged me during my PhD study. I would like to thank the Functional & evolutionary Entomology, Gembloux Agro-Bio Tech, University of Liege, Institute of Crop Sciences, and Chinese Academy of Agricultural Sciences. Thanks for the China Scholarship Council project provided me the financial support in Belgium.

I wish to express my deepest gratitude to my supervisors, Prof. Frédéric Francis and Prof. Lanqin Xia. Thanks for the opportunity they offered me to study in Beijing and Gembloux. Their professional guidance and patience helped me to gain more scientific knowledge and develop logical thinking during my study. I would like to thank my thesis committee: Prof. Eric Haubruge (Chairman), Prof. Massart Sébastien, Prof. Vanderschuren Hervé, and Prof. Dumont Benjamin for their insightful comments and encouragement. I would like to thank Prof. Youzhi Ma and Prof. Julian Chen for their professional guidance on wheat yield assessment and aphid-resistance research.

A large part of my work would not have been possible without efficient collaboration with Shaoya Li, Lei Yan, Chen Zhang, Jilin Chen, Jingying Li, Yongwei Sun, Huating Zhang, Mingxin Cao, Xue Zhong, Jiajing Xu, Chengli Tian, and Wenxue Wang during my study at the Institute of Crop Science of CAAS, Rudy Caparros, Emilie Bera, Gregoire Noel, Arnaud Segers, Lallie Glacet, Lisa Iannello, BeiBei Li, Lu Tian, HuiYuan Li, and Xin Jiang during my study at the lab of Functional & evolutionary Entomology.

Most importantly, I wish to appreciate my family and my friends. I would like to express my heartfelt gratitude and best wishes to all of them.

Jiahui Zhang
8/12/2022 in Gembloux, Belgium

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List of Abbreviations

	Chapter I
RNAi	RNA interference
HIGS	Host-induced gene silencing
SIGS	Spray-induced gene silencing
dsRNAs	Double-stranded RNA
N	Nitrogen
NUE	N use efficiency
ARE1	Abnormal cytokinin response1 repressor1
	Chapter II
CHS	Chalcone synthase
ssRNA	Single-stranded RNA
miRNAs	microRNAs
WCR	Western corn rootworm
CathB3	Cysteine protease Cathepsin B3
NTA	Non-tobacco-adapted
CbE E4	Carboxylesterase E4
lmf2-like	Lipase maturation factor 2-like
CHS1	Chitin synthase 1
Gqα	Gq protein alpha subunit
SaZFP	Zinc finger protein gene of *Sitobion avenae*
Tor	Carotene dehydrogenase
Bcat	Branched chain-amino acid transaminase
Hem	Hemocytin
TREH	Trehalase
ATPD	V-type proton ATPase subunit D
ATPE	V-type proton ATPase subunit E
EβF	(E)- β -farnesene
AChE	Acetylcholinesterase
Vg	Vitellogenin
VgR	Vitellogenin receptor
CP19	Cuticle protein 19
AcGNBP1	Gram-negative binding protein gene of *Aphis citricidus*
CdeB	Carotenoid desaturase gene
Ubx	Ultrabithorax
SHP	Sheath protein
siRNA	Short interfering RNA
BAPCs	Branched amphiphilic peptide capsules
	Chapter III
GRV	Green Revolution

NupE	Nitrogen uptake efficiency
NAE	Nitrogen assimilation efficiency
NUtE	Nitrogen utilization efficiency
NRE	Nitrogen remobilization efficiency
NO_3^-	Nitrate
NH_4^+	Ammonium
Rht-1	Reduced height-1
SD1	Semi-dwarf1
GA	Gibberellin
SLR1	SLENDER RICE1
LATS	Low-affinity transport system
HATS	High-affinity transport system
GS	Glutamine synthetase
GOGAT	Glutamine 2-oxoglutarate amidotransferase
Fd-GOGAT	Ferredoxin-dependent glutamate 2-oxoglutarate aminotransferase
NADH-GOGAT	Nicotinamide adenine dinucleotide-dependent glutamate 2-oxoglutarate aminotransferase
rbcS	Rubisco
GRF4	GROWTH-REGULATING FACTOR 4
ZM	ZhengMai 7698
KN199	Kenong 199
DELLAs	DELLA proteins
	Chapter IV
pI	Isoelectric point
MW	Molecular weight
BLAST	Basic Local Alignment Search Tool
DIG	Digoxigenin
qRT-PCR	Quantitative real-time PCR
Rps27	Ribosomal protein S27 A
pd	Potential drops
DAF	Days after feeding
APOP	Preoviposition period
TPOP	Total preoviposition period
R_0	Net reproductive rate
T	Mean generation time
r_m	Intrinsic rates of increase
DT	Doubling times of the population
EPG	Electropenetrography
E2	Phloem ingestion
	Chapter IV
NRTs	Nitrate transporters

AMTs	Ammonium transporters
NR	Nitrate reductase
NiR	Nitrite reductase
Ghd7	Grain number, plant height, and heading date 7
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein 9
AK	AiKang 58
CDC	Cell division control protein
T7E1	T7 Endonuclease I
SPAD	Soil-plant analysis development
SD	Standard deviation
SNP	Single-nucleotide polymorphism
YFP	Yellow fluorescent protein
PCR/RE	Polymerase chain reaction-based restriction enzyme
TRL	Total root length
TRSA	Total root surface area
TRV	Total root volume
RAD	Root average diameter

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Chapter I General introduction

I.1. Context and objectives

I.1.1. Context

Aphids are one of the most destructive agricultural pest group, which reduce the quality and quantity of grain significantly, both directly via feeding and indirectly through the transmission of harmful plant viruses (Tagu et al., 2008; Yu et al., 2016). Chemical treatment could successfully reduce aphid populations, but this practice has accelerated the emergence of insecticide resistance and led to the resurgence of pests. The overuse of chemical pesticides has caused serious environmental issues and threatens human health (Budzinski and Couderchet, 2018). Nitrogen (N) is primary driver of crop production and one of the most predominantly limiting factors for productivity. Considerable amounts of N fertilizers are applied each year in modern agriculture to enhance yield. However, excess N compounds released from agricultural systems have led to several environmental issues, which threatens the quality of air, water, and soil, leading to eutrophication (Tilman et al., 2002; Wang et al., 2012; Gastal et al., 2015). Previous studies reported that increased N inputs could increase insect populations by improving host plant nutritional quality (Cisneros and Godfrey, 2001; Aqueel and Leather, 2011). High amounts of N fertilizer may also increase the abundance of aphids in wheat (Schütz et al., 2008; Kang et al., 2018; Wang et al., 2020).

With the aim of searching for aphid-resistant germplasm, great efforts have been undertaken in conventional breeding programs. However, fewer achievements have been obtained due to the complexity of plant-aphid interactions and the accelerated development of resistant aphid biotypes (Tagu et al., 2008; Yu et al., 2016). As a result, developing effective and environmentally friendly pest management strategies to control aphid damage to cereals is critical to ensuring food safety and security. Considering future challenges of food security and exhaustion of natural resources, reducing the consumption of N fertilizer and increasing nitrogen use efficiency (NUE) are urgently demanding for the development of sustainable and productive agriculture (Tilman et al., 2002; Gastal et al., 2015; Rakotoson et al., 2017).

RNAi has been developed as a novel potential approach for pest control since the discovery that double-stranded RNA (dsRNA) can suppress the transcript abundance of target genes (Pitino et al., 2011; Xu et al., 2014; Zand Karimi and Innes, 2022). Host-induced gene silencing (HIGS) harnesses dsRNA production through the thoughtful and precise engineering of transgenic plants, and spray-induced gene silencing (SIGS) that uses surface applications of a topically applied dsRNA molecule are being exploited in crop protection and production (Koch and Kogel, 2014; Christiaens et al., 2020). Current studies focused on the successful application of RNAi-mediated control in phloem-feeding aphid species by individually targeting essential genes involved in feeding, molting, development, and reproduction.

As sap-sucking insects, aphids secrete both watery and gel saliva (Khan and Naveed, 2022). Gel saliva surrounds the aphid's stylets, which help to stabilize and protect the stylets and may suppress plant defense responses to watery saliva components

(Abdellatef et al., 2015). Watery saliva contains a combination of amino acids, proteins, and digestive enzymes that assist in digesting organic material and reducing plant defense responses (Hogenhout and Bos, 2011; Elzinga et al., 2014). Aphid protein effectors in watery saliva play critical role in the co-evolution of aphids and host plants (Pan et al., 2015). Based on our previous study, we identified a candidate gene *SmDSR33* that had high mortality effects on aphid survival after dsRNA feeding, which is homologous to the salivary protein MYS2 in pea aphid (*Acyrthosiphon pisum*). This gene has one homologue in the salivary gland ESTs database of the aphids, which may have significant effects on nutrition uptake through the mouthpart to the gut (ingestive and digestive system) of the aphids (Wang et al., 2015a). In this thesis (chapter IV), we isolated and characterized a putative salivary protein gene, *SmDSR33*, in the grain aphid. We investigated the relative expression levels of *SmDSR33* in aphids when feeding on transgenic wheat lines. We also evaluated the silencing impact of *SmDSR33* by investigating the fitness parameters and the feeding behavior of the aphids fed on different transgenic lines.

The fast-developing clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR associated (Cas) system opens up a new era of genome editing duo to its specificity, simplicity, versatility, robust and cost-effectiveness, which has revolutionized genome editing (Cong et al., 2013; Feng et al., 2013; Gao and Zhao, 2014; Xie et al., 2014; Ma et al., 2015; Sun et al., 2016; Li and Xia, 2020). The Cas9 endonuclease generates double strand DNA breaks (DSBs) in target genes in a guide RNA dependent manner (Cong et al., 2013). DSBs are repaired either through the error-prone non-homologous end joining (NHEJ) pathway or the homology-directed DNA repair (HDR) pathway. NHEJ is frequently associated with small indels and has been widely employed to generate knock-out mutants (Ma et al., 2015; Zhang et al., 2018a). HDR or homologous recombination-based gene targeting (GT) plays essential roles in targeted allele/gene replacement or precise modifications (Li and Xia, 2020). CRISPR/Cas9-mediated targeted mutagenesis in hexaploid common wheat is still a significant challenge.

are1 was a genetic suppressor of a rice *fd-gogat* mutant defective in nitrogen assimilation. Loss-of-function mutations in ARE1 caused delayed senescence of rice and resulted in 10-20% grain yield increased and enhanced NUE under nitrogen-limiting conditions (Wang et al., 2018a). Grain number, plant height, and heading date7 (Ghd7) encoded a transcription factor which modulates multiple nitrogen related agronomic traits. Ghd7 positively regulated nitrogen utilization through directly binds to two Evening Element-like motifs in ARE1 to repress its expression. The combination of Ghd7 and ARE1 elite alleles can increase NUE and grain yield under low nitrogen conditions (Wang et al., 2021a). However, the *ARE1* ortholog in wheat has not been functionally characterized yet. In this thesis of chapter V, we concentrate on the NUE-related gene-*ARE1* using the CRISPR/Cas system. We cloned and characterized the homologous gene in wheat, obtained *TaARE1* knock-out wheat plants and evaluated NUE of edited wheat lines.

I.1.2. Objectives

Cereal plants are frequently attacked sequentially or simultaneously by different aphid species, significantly reducing grain quality and quantity. To guarantee food safety and security, it is important and imperative to develop effective and environmentally friendly pest management strategies to control aphid damage to cereals. Nitrogen (N) is one of the essential nutrients required for plant growth. it is essential to resilient wheat production by improving NEU, decreasing the nitrogen application and maintaining the yield stability.

Therefore, the general objectives of this study were to engineer novel types of wheat varieties with increased aphid-resistance and nitrogen-use efficiency.

Two specific objectives are as follows:

(1) To engineer novel aphid-resistant wheat plants through plant-mediated RNA interference of aphid *SmDSR33* gene;

(2) To engineer novel nitrogen-use efficient wheat plants via CRISPR/Cas9-mediated genome editing of *TaARE1* gene.

I.2. Research roadmap and outline

I.2.1. Research roadmap

The research roadmap is presented in Figure 1-1.

I.2.2. Outline

Chapter IV develops aphid-resistant wheat plants through RNA interference of a key putative salivary protein encoding gene *SmDSR33*.

Reference: Zhang, J., Li, H., Zhong, X., Tian, J., Segers, A., Xia, L., and Francis, F. (2022). Silencing an aphid-specific gene *SmDSR33* for aphid control though plant-mediated RNAi in wheat. (*Frontiers in Plant Science*-Minor revision)

Chapter V describes using CRISPR/Cas9 mediated genome editing to increasing yield potential through manipulating of an ARE1 ortholog related to nitrogen use efficiency in wheat.

Reference: Zhang, J., Zhang, H., Li, S., Li, J., Yan, L., and Xia, L. (2021). Increasing yield potential through manipulating of an ARE1 ortholog related to nitrogen use efficiency in wheat by CRISPR/Cas9. J. *Integr. Plant Biol*. 63: 1649-1663.

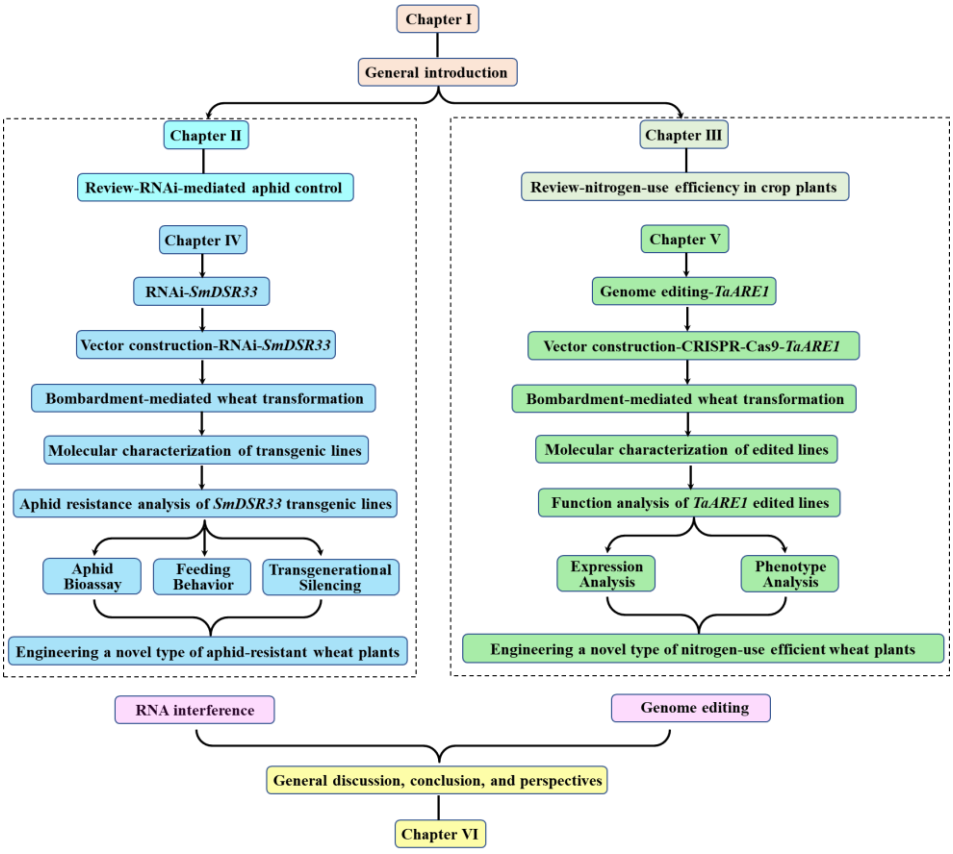

Figure 1-1 The overview contents and research roadmap of thesis

I.3. Reference

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2

Chapter II Literature review on RNA interference-mediated aphid control in crop plants

Abstract

Crop plants suffer severe yield losses due to the significant damages caused by aphids. RNA interference (RNAi) technology is a versatile and environmentally friendly method for pest management in crop protection. Transgenic plants expressing siRNA/dsRNA and non-transformative methods, such as microinjection, feeding, and nanocarrier-delivered mediated RNAi approach, have been successfully applied in agricultural practice for insect pest management. In this review, we summarize the application of host-induced gene silencing (HIGS)-mediated RNAi, spray-induced gene silencing (SIGS)-mediated RNAi, and other delivery method-mediated RNAi to aphid control. We further discuss the challenges to RNAi application, and propose potential solutions to enhance RNAi efficiency.

Key words: RNA interference (RNAi), host-induced gene silencing (HIGS), spray-induced gene silencing (SIGS), aphid control, RNAi efficiency

II.1. Introduction

Cereal plants are frequently attacked sequentially or simultaneously by different aphid species, reducing the quality and quantity of grain significantly. Although chemical control could effectively suppress aphid populations, this method has accelerated insecticide resistance development and resulted in pest resurgence. Overuse of chemical pesticides has led to severe environmental problems and threatens human health (Budzinski and Couderchet, 2018). Therefore, to guarantee food safety and security, it is important and imperative to develop effective and environmentally friendly pest management strategies to control aphid damage to cereals. Extensive research has typically focused on further understanding the relationship between crops and aphids in recent decades, which has greatly contributed to the development of sustainable aphid management strategies (Luo et al., 2021).

RNA interference (RNAi) is a biological process that can be triggered by exogenously applied or endogenously expressed double-stranded RNAs (dsRNA). In this process, sequence-specifically silencing is induced at the transcriptional or post-transcriptional level by directing inhibitory chromatin modifications or by decreasing the stability or translation potential of the targeted mRNA (Dang et al., 2011; Li et al., 2013b; Nicolás and Ruiz-Vázquez, 2013; Bologna and Voinnet, 2014; Guo et al., 2019; Song et al., 2021). RNAi technique has enormous potential applications in agricultural practices, extending to viruses, bacteria, fungi, nematodes, insects, and plants. RNAi-mediated control has been exploited for several species of phloem-feeding aphids by targeting critical genes involved in feeding, molting, development, and fecundity individually (Jain et al., 2021). With applications in crop protection and production, host-induced gene silencing (HIGS) harnesses dsRNA production through the thoughtful and precise engineering of transgenic plants, and spray-induced gene silencing (SIGS) that uses surface applications of a topically applied dsRNA molecule are being exploited. Here, we summarized RNAi-based protection against different aphid species in crop plants, discussed the challenges associated with RNAi application, and proposed potential solutions to improve RNAi efficiency.

II.1.1. RNA interference-based aphid control in crop plants

The first evidence of RNA-induced gene silencing was described more than 30 years ago. When they attempted to overexpress a key gene involved in flavonoid biosynthesis in pigmented petunia petals named chalcone synthase (CHS), but blocked anthocyanin biosynthesis via a post-transcriptional mechanism (Napoli et al., 1990). A subsequent investigation demonstrated that double-stranded RNA (dsRNA) caused a decrease or elimination of target transcript expression in *C. elegans*. This discovery established that dsRNA was more effective than single-stranded RNA (ssRNA), which represented an extraordinary milestone in the RNAi revolution (Fire et al., 1998). Since the discovery that dsRNA induces effective target gene silencing, various methods have been tested or applied to deliver dsRNA in insect species. In laboratory or agricultural practice, exogenous RNAs are applied through surface treatments or invasive methods, such as spraying, soaking, injection, infiltration,

soil/root drench, and petiole absorption (Numata et al., 2014; Dalakouras et al., 2016; San Miguel and Scott, 2016; Ghosh et al., 2017; Dalakouras et al., 2018). Plant-mediated and insect-mediated RNAi has been developed as a potential alternative strategy for pest management (Pitino et al., 2011; Xu et al., 2014; Chung et al., 2018). Application of RNAi through expression of dsRNA in transgenic crop plants or direct application of dsRNA as an insecticide appears promising for agricultural pest control, which can be achieved by host-induced gene silencing (HIGS) and spray-induced gene silencing (SIGS) (Koch and Kogel, 2014; Christiaens et al., 2020) (Figure 2-1).

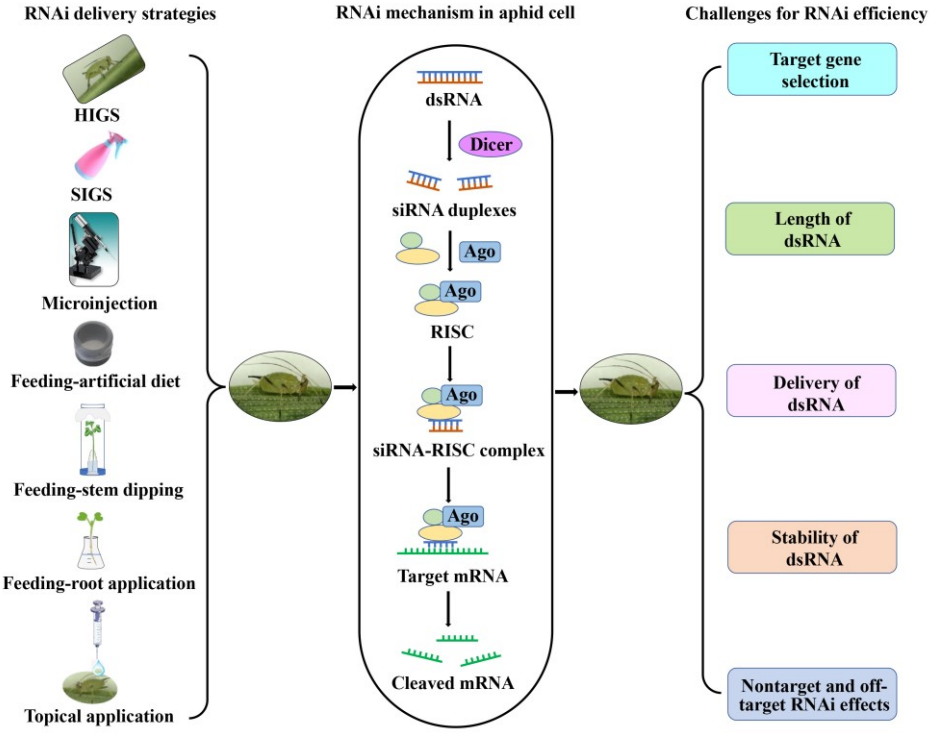

Figure 2-1 Schematic of RNA interference (RNAi) delivery strategies, RNAi mechanism in aphid cells, and challenges affecting RNAi efficiency in aphids.

HIGS: host-induced gene silencing, SIGS: spray-induced gene silencing, dsRNA: double-stranded RNA, Dicer: Dice-like, siRNA: short interfering RNA, Ago: ARGONAUTE, RISC: RNA-induced silencing complex.

II.1.1.1. Host-induced gene silencing

Host-induced gene silencing is a plant-mediated transgenic technique in which plants are genetically engineered to produce pathogen or pest gene-targeting sRNAs or dsRNAs. These RNAs are subsequently transported into the pest or pathogen to silence target genes (Santala and Valkonen, 2018; Sang and Kim, 2020).

The HIGS molecular mechanisms in insects and nematodes may differ from those in fungi and oomycetes. In herbivorous insects, long dsRNAs (including hpRNAs) appear to be taken up directly from the host. Then RNAi machinery within the insect or nematode is used to induce gene silencing. In filamentous pathogens, the available evidence indicates that the pathogen takes up siRNAs and microRNAs (miRNAs) produced within the host plant and then induces gene silencing (Zand Karimi et al., 2022).

Host-induced gene silencing (HIGS) was first reported in *Arabidopsis thaliana* with an expressed hpRNA of a nematode *16D10* gene, transgenic plants exhibited effective resistance against four major root knot nematode species (Huang et al., 2006). The first proof-of-concept research on plant-mediated dsRNA delivery for insect pest management was reported in western corn rootworm (WCR). In a growth chamber assay, transgenic maize plants expressing WCR dsRNAs significantly reduced the damage caused by WCR feeding (Baum et al., 2007). Subsequently, numerous studies have been reported using HIGS in crop plants to confer resistance to various plant pathogens and pests, including fungi (Nowara et al., 2010; Koch et al., 2013), oomycetes (Govindarajulu et al., 2015; Jahan et al., 2015), and insects (Abdellatef et al., 2015; Zhang et al., 2015b).

II.1.1.2. Host-induced gene silencing-based protection of crop plants from aphid

HIGS has great potential to manage insects from the order Hemiptera that feed on plants, especially aphids. The application of HIGS has been exploited in different aphid species, such as the peach aphid (*Myzus persicae*) and grain aphid (*Sitobion avenae*) (Table 2-1A).

Many studies of HIGS focused on *M. persicae* through various transgenic plants, for example, *Arabidopsis thaliana*, *Nicotiana benthamiana*, and *Solanum lycopersicon*. Some salivary effectors have been identified in aphids, such as MpC002, MpPIntO1 (Mp1), MpPIntO2 (Mp2), and Mp55. The knockdown of these genes reduced the reproduction of aphids, which indicated that these effectors could be selected as potential RNAi targets (Pitino et al., 2011; Pitino and Hogenhout, 2013; Elzinga et al., 2014). Rack-1 was a conserved multifunctional scaffold protein which was identified as a luteovirus-binding protein in peach aphid. Knockdown of *Rack-1* reduced the fecundity of peach aphids (Pitino et al., 2011). Based on previous studies of *Rack1*, *MpC002*, and *MpPIntO2*, the persistence and transgenerational effects of plant-mediated RNAi were also investigated through transgenic *Arabidopsis* (Coleman et al., 2015). Transgenic tomato plant-mediated RNAi has been shown to effectively silence the Acetylcholinesterase 1(*Ace1*) gene and reduce the fecundity of peach aphid when fed on transgenic plants (Faisal et al., 2019). A study reported that knockdown of the cysteine protease Cathepsin B3 (*CathB3*) gene improved the performance of a non-tobacco-adapted (NTA) aphid lineage on tobacco. *CathB3* elicited host defenses to suppress phloem sap ingestion by the aphid (Guo et al., 2020). Plastid-mediated RNA interference (RNAi) was successfully employed to silence *MpDhc64C*. Both transgenic and transplastomic tobacco plants exhibited significant

resistance to peach aphids as demonstrated by decreased survival fecundity and survivor weight (Dong et al., 2022).

Most of the studies on *S. avanae* were applied by wheat-mediated HIGS. A Particle bombardment mediated wheat transformation method was used to obtain stable transgenic wheat plants. Feeding on transgenic wheat expressing the carboxylesterase (*CbE E4*) gene could suppress the expression level of *CbE E4* in grain aphids and impair larval tolerance to Phoxim insecticides (Xu et al., 2014). Silencing of the lipase maturation factor 2-like (*lmf2-like*) gene reduced the molting number and decreased the survival and reproduction of aphids (Xu et al., 2017). Similarly, knockdown of the Chitin synthase 1 (*CHS1*) gene reduced the molting and the survival of aphid (Zhao et al., 2018). Silencing of the G protein (*Gqα*) gene could also reduce the reproduction and molting in grain aphids (Hou et al., 2019). Silencing of the zinc finger protein (*SaZFP*) gene led to high mortality and decreased fecundity of grain aphids. The transgenerational silencing effect was investigated in the successive first to fourth generations (Sun et al., 2019).

II.1.1.3. Spray-induced gene silencing

Although transgenes are convenient, they are not required for the ectopic activation of gene silencing in pathogens or pests. According to some research, many eukaryotic pathogens or pests, including aggressive fungi or nematodes, are able to take up RNAs from the environment (Whangbo and Hunter, 2008; Wang et al., 2016b; Qiao et al., 2021). This phenomenon was defined as 'Environmental RNAi', in which the transferred RNAs that have complementary sequences to the genes in the organism can potentially induce highly effective target gene silencing (Whangbo and Hunter, 2008; Ghosh et al., 2018). These studies prompted the development of spray-induced gene silencing (SIGS). In spray-induced gene silencing, dsRNAs or sRNAs that target pathogen or pest genes are sprayed directly onto plant material. These RNAs then move into the pathogen or pest cells and silence target genes through 'Environmental RNAi' (Wang and Jin, 2017; Qiao et al., 2021).

The first evidence of exogenous application of dsRNA against insect pests was in citrus and grapevine trees, in which dsRNA targeting the arginine kinase gene was used to control psyllids and sharpshooter pests (Hunter et al., 2012). *Fusarium graminearum* development in barley leaves was suppressed by spraying dsRNA to target the fungal cytochrome P450, establishing the proof of concept for spray-induced gene silencing (SIGS) (Koch et al., 2016). Also, the potential non-transgenic, spray-based exogenous dsRNA or sRNA (SIGS) application has been widely used on crop plants to decrease disease caused by plant pathogenic fungi, oomycetes, viruses, and insect pests (Cagliari et al., 2019; Dalakouras et al., 2020; Vetukuri et al., 2021; Bilir et al., 2022).

II.1.1.4. Spray-induced gene silencing-based aphid control

The delivery of siRNA and dsRNA via nanoparticle carriers is a novel approach that has been successfully used in a few insect systems (Zhang et al., 2010; Li-Byarlay et al., 2013; Das et al., 2015). The majority of SIGS-based studies employed nanocarrier

delivery systems for aphid control (Table 2-1B).

tor is a carotene dehydrogenase gene which played an important role for pigmentation in *A. pisum*. The branched chain-amino acid transaminase (*bcat*) gene is essential in the metabolism of branched-chain amino acids in aphids. Aerosolizing siRNA-nanoparticle delivery strategy induced modest *tor* gene knockdown in *A. pisum* and *bcat* gene knockdown in *Aphis glycines* as well as associated phenotype. These results indicated that the aerosolized siRNA-nanoparticle method was an effective RNAi delivery system (Thairu et al., 2017). With the use of a nanocarrier and detergent, a novel dsRNA formulation was exploited, which can quickly penetrate through the body wall of *A. glycines* and effectively suppress gene expression. This formulation suggests that transdermal dsRNA delivery could be developed into an effective SIGS-based aphid control strategy. Hemocytin (*Hem*) is an important factor in the hemocyte and fat body of insects, which might regulate aphid population density. When spraying dsRNA-*HEM* nanocarrier/detergent formulation on *A. glycines*, the expression level of *hemocytin* was efficiently silenced, which impaired the survival and fecundity of aphids and suppressed aphid population growth (Zheng et al., 2019).

According to previous studies, Yan et al. (2020a) selected soluble trehalase (*TREH*), V-type proton ATPase subunit D (*ATPD*), V-type proton ATPase subunit E (*ATPE*) and chitin synthase 1 (*CHS1*) genes as RNAi target genes to test the silencing effect in *A. glycines* (Whyard et al., 2009; Chen et al., 2010; Zhang et al., 2010; Upadhyay et al., 2011). This study indicated that *A. glycines* exhibited higher mortality when fed on soybean seedlings sprayed with dsATPD + dsCHS1 nanoparticle formulation. They also demonstrated that a water-soluble cationic dendrimer (nanocarrier) was an efficient gene carrier (Yan et al., 2020a). Another study also investigated the RNAi efficacy of the *ATPD* gene in woolly apple aphid (*Eriosoma lanigerum*) via a nanocarrier-mediated transdermal dsRNA delivery system. Their result suggested that the interference efficiency was greatly increased using nanocarriers and induced high aphid mortality (Guo et al., 2022).

Biedenkopf et al. (2020) reported that application of dsRNA to detached barley leaves resulted in effective SIGS of the sheath protein (*Shp*) gene in grain aphid. Systemic RNAi was also observed in *Hordeum vulgare* after spray treatment in which sprayed dsRNA moved from barley leaves to stems and root tissues. This research contributed significantly to understanding the mechanism of RNA spray technology, especially for SIGS. However, another study in barely suggested that grain aphids fed on barley seedlings sprayed with naked *SaMIF*-dsRNAs did not affect the survival of nymphs, which indicated that aphids were unable to absorb dsRNA from these plants (Liu et al., 2021b). According to a recently published paper, the spray-based application of the nanocarrier-mediated transdermal dsRNA delivery system effectively silenced a putative salivary effector Sg2204 in *Schizaphis graminum* and its homologs from four other aphid species. Aphids silenced with *Sg2204* exhibited a stronger defense response and induced a negative impact on aphid survival, fecundity, and feeding behavior (Zhang et al., 2022a).

II.1.1.5. Other delivery method mediated gene silencing for aphid control

Microinjection was an efficient and widely used research method for delivering dsRNAs. The first evidence of successful dsRNA microinjection was applied to silence *frizzled* and *frizzled 2* genes in *Drosophila melanogaster* embryos by injecting their corresponding dsRNAs (Kennerdell and Carthew, 1998). Since then, microinjection has become a potential method for delivering dsRNA into various insect species. This method was reported to apply in many aphid species, namely *A. gossypii*, *A. pisum*, *M. persicae*, *S. avenae* (Table 2-1C). Injection of siRNA-*C002* into pea aphid decreased the transcription level of *C002* (Mutti et al., 2006). Injected the dsRNAs of different aphid genes which played important roles in aphid sheath formation (*SHP*, Will and Vilcinskas (2015)), cuticular waterproofing (*CYP4G51*, Chen et al. (2016)), (E)-b-farnesene (EβF) reception (*ApisOR5*, *ApisOBP3* and *ApisOBP7*, Zhang et al. (2017b)), chitin biosynthesis (*CHS*, Ye et al. (2019)), molting (*ApCCAP* and *ApCCAPR*, Shi et al. (2022)), flight musculature formation and wing extension (*flightin*, Chang et al. (2022)) induced effective target gene silencing .

Feeding was another basic delivery method for aphids because of its less laborious and easier operation. Aphids fed on a diet containing synthetic dsRNA were more appliable for target gene knockdown. It was first reported that feeding on *E. coli* bacteria expressing dsRNA in *C. elegans* conferred silencing effects on the nematode larvae (Timmons and Fire, 1998). In *Aphis citricidus*, RNAi was performed by feeding dsRNAs of target genes with citrus leaf through stem dipping. Acetylcholinesterase (*AChE*) is an important gene targeted by insecticides based on organophosphates and carbamates. Silencing of two aphid *AChE* genes, *Tcace1* and *Tcace2*, increased susceptibility to malathion and carbaryl insecticides. Furthermore, *Tcace1* silencing resulted in higher mortality in the aphid than *Tcace2* silencing, which indicated that *TcAChE1* was essential for *A. citricidus* postsynaptic neurotransmission (Mou et al., 2017). Knockdown of Vitellogenin (*Vg*) and its receptor (*VgR*) had a negative impact on embryonic and postembryonic development, which led to nymph-adult transition delay, longer pre-reproductive period, and shorter reproductive period (Shang et al., 2018). Cuticle protein is a primary target in insect development and molting. Silencing of the cuticle protein 19 (*CP19*) gene in *A. citricidus* led to aphid mortality (Shang et al., 2020). Similarly, aphids fed with dsRNA of a Gram-negative binding protein gene (*AcGNBP1*) caused target gene silencing and high mortality (Ye et al., 2021). The same delivery strategy was applied in *A. pisum*. Different dsRNAs were fed with the bean leaf through stem dipping. Silencing of the *CP19* gene in pea aphid also led to high mortality (Shang et al., 2020). Parental silencing of the carotenoid desaturase gene (*CdeB*) reduced the intensity of the body color in vivo in the treated aphids and subsequent generations and negatively affected aphid performance (Ding et al., 2020). Silencing of *ApGNBP1* but not *ApGNBP2* in *A. pisum* decreased immune-related phenoloxidase activity (Ye et al., 2021). Feeding on *Brassica* leaf inserted into a solution containing *MpCP19* and *MpGNBP1* dsRNA also induced effective target gene silencing (Shang et al., 2020; Ye et al., 2021). With the aim of decreasing insecticide use and eliminating pesticide-resistant evolved populations, RNAi has also been used to increase the susceptibility of aphids to insecticides. A study reported that *RpAce1* suppression increased susceptibility to pirimicarb and malathion in *Rhopalosiphum padi*. Silencing *SaAce1* also increased *S. avenae* susceptibility to

pirimicarb (Xiao et al., 2015).

It has also been demonstrated that mechanical inoculation can help deliver dsRNA and induce RNAi by spreading dsRNA with soft sterile brushes and gentle rubbing inoculation (Lau et al., 2015; Dubrovina and Kiselev, 2019). The molecules were rapidly absorbed by tomato plants and were taken up by peach aphids (*M. persicae*) when the tomato leaves were gently rubbed with dsRNA solution (Gogoi et al., 2017). The nanocarrier-delivered RNAi method was also used to silence *flightin, vestigial* (*vg*), and *Ultrabithorax* (*Ubx*) genes, which suppressed the wing development in *M. persicae* (Chang et al., 2022; Zhang et al., 2022c). In *Sitobion miscanthi*, a putative salivary effector Sg2204 was effectively silenced via a nanocarrier-mediated transdermal dsRNA delivery system. The fecundity and survival of *S. miscanthi* dramatically decreased after *Sm9723* silencing, and the aphid feeding behavior was also impaired (Zhang et al., 2022b).

II.1.2. Challenges for enhancing RNA interference efficiency

II.1.2.1. Target gene selection

Selection of the target gene is critical to the success of RNAi-based insect control. RNAi efficiency varies considerably among the same transcripts of different insect species (Baum et al., 2007; Ulrich et al., 2015). The efficiency can vary in the same species with different transcripts, genotypes, and tissues, even among the same transcript from different areas (Baum et al., 2007; Zhu et al., 2011; Luo et al., 2013; Zhang et al., 2013b; Camargo et al., 2015; Bona et al., 2016). The ideal RNAi gene target should be essential for insect survival and highly expressed. It should not have functional redundancy, allowing the silencing effect to be easily seen (Lomazzo et al., 2011; Li et al., 2013a). Therefore, potential target genes should be thoroughly vetted, for the ability to suppress specific transcripts and the ability to cause mortality to enhance the efficiency of RNAi-based pest management.

RNAi targets are initially selected based on the knowledge of essential genes in other organisms or by cDNA library screening. Numerous research had indicated that genome-wide screens of high sensitivity target genes were effective in RNAi. More sophisticated, high-throughput approaches continue to be developed. For instance, RNA-seq and digital gene expression tag profile (DGE-tag) were used in the Asian corn borer (ACB; *Ostrinia furnacalis*) to identify potential RNAi targets (Wang et al., 2011). Expression profiling and transcriptome reconstruction of an increasing number of insects have been made possible by second generation sequencing. High-throughput screens such as feeding assays (Whyard et al., 2009), or topical application of dsRNA (Wang et al., 2011; Thairu et al., 2017; Zheng et al., 2019), are also a powerful tool to identify potential RNAi targets. With existing databases growing, tissue-specific and developmental stage-specific expression profiles of insects may narrow down candidate pools for target gene selection. After identifying candidate genes, screening for dsRNA-induced mortality is required to determine the ability of specific dsRNAs to induce the desired phenotype.

Then the candidate dsRNA sequences can be employed in subsequent experiments to test their capacity to cause mortality in multiple life stages. Targeting multiple

genes, dsRNA concatemerization, or using different dsRNA structures can all be used to improve the efficiency of RNAi (Kwon et al., 2016; Sharath et al., 2019; Abbasi et al., 2020; Silver et al., 2021).

II.1.2.2. Length of dsRNA

In some insect species, the uptake and silencing efficiency of RNAi are determined by the length of expressed dsRNA. Different insect species require different minimum lengths of dsRNA to achieve maximal RNAi silencing (Bolognesi et al., 2012). In *Tribolium castaneum*, analysis revealed that dsRNA length had a strong influence on the efficacy of the RNAi response. Longer dsRNA is proving to be more effective at suppressing gene expression. A minimum of 70 nucleotides was required to achieve the desired interference (Miller et al., 2012). The length of dsRNA sequences between 139 bp and 773 bp was used in the majority of the aphid feeding experiments to obtain successful RNAi (Table 2-1).

As we described above, siRNA injection was able to suppress the target gene (*C002*) expression in pea aphid which dramatically reduced aphid survival (Mutti et al., 2006). In grain aphids, RNAi targeting the sheath protein (*SHP*) gene by transgenic barley plants expressing 491 bp length *shp*-dsRNA strongly inhibits feeding and reproductive behavior of grain aphids and negatively impacts their survival (Abdellatef et al., 2015). Gq proteins play important roles in insect cellular signal transduction. Downregulation of *Gqα* gene with a 540 bp fragment of dsRNA resulted in decreased fecundity and molting rate (Hou et al., 2019). A 198 bp fragment of dsRNA complementary to the zinc finger protein (*SaZFP*) gene could induce target gene silencing in grain aphid when feeding on transgenic wheat plants, resulting in decreased reproduction and survival rates (Sun et al., 2019).

Therefore, both short and long dsRNAs induced gene silencing effectively depending on the target pest and target gene. Longer dsRNAs may increase the possibility of off-target effects on beneficial organisms due to the generation of potentially large siRNA pools. Accordingly, RNAi efficiency will be improved by determining the optimal length of target-specific RNAi effectors in conjunction with effective siRNA analysis (Jain et al., 2021).

II.1.2.3. Delivery of dsRNA

Various dsRNA delivery methods, including microinjection, feeding, soaking, HIGS-mediated by transgenic plants, and SIGS-mediated by spraying, have been applied in pest management. As we discussed before, microinjection and feeding are the two basic delivery methods. The soaking delivery method was usually applied in insect cell lines by the direct addition of dsRNA into the cell culture medium (Clemens et al., 2000; Johnson et al., 2010), and some researches have reported the penetration of topically applied dsRNA/siRNA formulation into the insect cuticles to induce lethal effects (Pridgeon et al., 2008; El-Shesheny et al., 2013; Gong et al., 2013; Zhang et al., 2015a; San Miguel and Scott, 2016; Dias et al., 2019). Transgenic plants expressing dsRNA or siRNA have lots of advantages for pest control (Mao et al., 2007). The SIGS-mediated delivery method didn't require plant genetic engineering.

dsRNAs/siRNAs were applied topically to the plant surface via spraying in this silencing type (Qiao et al., 2021).

To improve dsRNA delivery efficiency, various new technologies have been exploited, such as cationic liposome-assisted and nanoparticle-enabled. The combination of nanotechnology and RNAi had the potential to be a more environmentally friendly pest management strategy. In the first investigation of nanoparticle-mediated dsRNA delivery, chitosan was used to silence the chitin synthase genes in *Anopheles gambiae*, and the RNAi effectiveness was found to be enhanced (Zhang et al., 2010). Short interfering RNA (siRNA)-nanoparticle complexes, peptide nano-material-branched amphiphilic peptide capsules (BAPCs), and nanocarrier-based transdermal dsRNA delivery systems were demonstrated to be successful for aphid RNAi, which could efficiently silence gene expression (Thairu et al., 2017; Avila et al., 2018; Zheng et al., 2019; Yan et al., 2020a).

II.1.2.4. The stability of dsRNA

RNAi stability and efficiency vary drastically depending on the concentration and the length of the dsRNA, delivery method and technique, plant organ-specific processes, adverse environmental conditions, insect life stage, and target gene selection (Zhang et al., 2017a; Dias et al., 2019; Marques et al., 2021). Environmental microorganisms can degrade dsRNA before it is consumed by pathogens or pests. Microorganisms in the environment can degrade dsRNA prior to its uptake by pathogens or pests. Nucleases in pest saliva, gut lumen, and haemolymph may also degrade dsRNA rapidly (Kennedy et al., 2004; Allen and Walker III, 2012; Katoch and Thakur, 2012; Luo et al., 2013; Chung et al., 2018; Guan et al., 2018).

The stability of dsRNA in the insect gut is critical for a successful RNAi response, and increased nuclease expression can result in dsRNA degradation and subsequent RNAi failure (Dias et al., 2020). The activity of gut nucleases can be impacted by the high or low pH present in the gut lumens of particular pests, which can decrease dsRNA stability directly or indirectly (Cooper et al., 2019). Some strategies have already been exploited to improve the stability of dsRNA. For example, the nanoparticle-mediated dsRNA delivery system was demonstrated to be efficient in increasing the stability and efficacy of dsRNA, which has been applied to improve the persistence, penetration, and transport of dsRNA into plants or insects (Jain et al., 2021; Laisney et al., 2021; Marques et al., 2021; Pugsley et al., 2021).

II.1.2.5. Nontarget and off-target RNAi effects

Silencing non-target genes in the same organism or in non-target organisms resulted in off-target effects (Xu et al., 2006; Senthil-Kumar and Mysore, 2011; Reddy and Rajam, 2016). To improve RNAi efficiency and minimize off-target effects, species-specific or tissue-specific dsRNA could be selected. A study reported that silencing of *V-ATPase* genes in *T. castaneum*, *M. sexta*, *A. pisum* and *D. melanogaste*r was observed without affecting non-target species using species-specific dsRNA (Whyard et al., 2009).

To design efficient and potent RNAi targets, various web-based computational design approaches have been developed to minimize potential off-target effects via selecting a collection of 21-nt sequences from a given target sequence with a low probability of such off-target effects (Ahmed et al., 2020). Further assessment was applied to selected sequences using software such as ERNAi (https://www.dkfz.de/signaling/e-rnai3/, (Horn and Boutros, 2010)), dsCheck (http://dscheck.rnai.jp/, (Naito et al., 2005)) and by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis against the transcriptomic datasets of human and beneficial insects (Jain et al., 2021).

II.2. Conclusions and perspectives

During the past few years, RNAi has developed as a promising, valuable and effective technique for functional genomic studies. Various RNAi-based approaches have been applied in crop protection for species-specific and eco-friendly pest management. In this review, we summarize the present studies on numerous strategies exploited against different aphid species.

Growing evidence suggested that HIGS-based or SIGS-based crop protection against plant pests is effective. Transgenic plants appeared to be a more beneficial approach to enhancing RNAi effects. Nevertheless, lacking transformation technology in many crop species restricted the widespread application of HIGS. Furthermore, they are still regarded as genetically modified (GM) products in most countries, requiring the plants to undergo a rigorous evaluation before being licensed. The development of transplastomic technology was also restricted by the extensive regulatory process. Global applications of HIGS are limited by public concern over the biosafety of genetically modified organisms (GMOs) (Yan et al., 2020b; Zhao et al., 2021). Using optimized target gene and fragment selection strategies, more effective transformation constructs, and stable transgenic systems, the major challenges for the HIGS strategy will be overcome (Liu et al., 2021a). SIGS does not produce GMOs in comparison to HIGS. However, it has become clear that the instability of naked dsRNA is a significant limitation of SIGS, resulting in a relatively short period of protection. In order to address this issue and improve the insecticidal activity of non-transformative RNAi products, SIGS based dsRNAs affiliated with different types of nanoparticles would be an efficient technique (Mitter et al., 2017a; Mitter et al., 2017b; Huang et al., 2019; Kumar et al., 2019; Li et al., 2019; Fletcher et al., 2020; Cai et al., 2021). These prospective strategies may decrease the cost and improve the dependability of the present delivery techniques. They may also create new opportunities to study the roles of important genes. Another consideration for RNAi application is to exclude potential off-target effects and effects on non-target organisms. To support biosafety claims of RNAi applications, a combination of bioinformatics and ecological bioassays using selected target species is essential.

With the development of new technology, clustered regularly interspaced short palindromic repeat /CRISPR-associated endonuclease Cas9 (CRISPR/Cas9) based genome editing had been reported in *Spodoptera exigua* (Zuo et al., 2017), *Helicoverpa armigera* (Wang et al., 2016a; Ye et al., 2017; Dong et al., 2019), *S. litura*

(Bi et al., 2016; Zhu et al., 2017), and *Nilaparvata lugens* (Lu et al., 2018; Zhao et al., 2019). However, many of them focus on insect genomic function. Further study is needed to exploit genome editing as a viable strategy to create resistant varieties against numerous insect pests and enhance pest resistance in crops (Seni, 2021).

Overall, with greater understanding of the RNAi machinery and dsRNA delivery strategies, RNAi will be more effectively used in aphid control for crop protection.

II.3. References

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Table 2-1 Summary of RNAi application for aphid control.

A. Host-induced gene silencing-based protection of plants from aphids

Types of gene silencing	Aphid species	Plant species	Delivery strategy	Target genes	Molecule	Size	Main effects	Reference
HIGS	*Myzus persicae*	*N. benthamiana and A. thaliana*	Transgenic *N. benthamiana* and *A. thaliana*	*MpC002, Rack1*	dsRNA	710 bp, 309 bp	Knock down of target genes.	(Pitino et al., 2011)
	Myzus persicae	*N. benthamiana and A. thaliana*	Transgenic *N. benthamiana* and *A. thaliana*	*MpC002, MpPIntO1 (Mp1), MpPIntO2 (Mp2)*	dsRNA	710 bp, 263 bp, 254 bp	Silencing of *MpC002* and *MpPIntO2* reduced nymph production.	(Pitino and Hogenhout, 2013)
	Myzus persicae	*N. tabacum, A. thaliana, and N. benthamiana*	Transgenic *N. tabacum, A. thaliana*, and *N. benthamiana*	*Mp55*	dsRNA	>900 bp	Reduced aphid reproduction.	(Elzinga et al., 2014)
	Myzus persicae	*A. thaliana*	Transgenic *A. thaliana*	*Rack1*, *MpC002, MpPIntO2 (Mp2)*	dsRNA	309 bp, 710 bp, 254 bp	Reduced aphid reproduction.	(Coleman et al., 2015)
	Myzus persicae	*A. thaliana*	Transgenic *A. thaliana*	Cuticular protein *MyCP*	dsRNA	327 bp	Attenuation of fecundity in aphids.	(Bhatia and Bhattacharya, 2018)
	Myzus persicae	tomato	Agrobacterium-mediated transformation, transgenic tomato	Acetylcholinesterase 1 (*Ace 1*)	dsRNA	571 bp	Silenced the target gene (*Ace 1*) and inhibited of fecundity.	(Faisal et al., 2019)

HIGS	*Myzus persicae*	tobacco	Injection and feeding on transgenic tobacco	Cysteine protease Cathepsin B3 (*CathB3*)	dsRNA	230 bp	Improved the performance of non-tobacco adapted lineages on tobacco.	(Guo et al., 2020)
	Myzus persicae	tobacco	Plastid-mediated RNA interference, transgenic tobacco	MpDhc64C	dsRNA	269 bp	Reduced insect survival, impaired fecundity, and decreased weight of survivors.	(Dong et al., 2022)
	Sitobion avenae	wheat	Particle bombardment, transgenic wheat	Carboxylesterase (*CbE E4*)	dsRNA	350 bp	Suppressed *CbE E4* expression impaired *S. avenae* larval tolerance of Phoxim insecticides.	(Xu et al., 2014)
	Sitobion avenae	wheat	Particle bombardment, transgenic wheat	Lipase maturation factor 2-like gene, *lmf2-like*	dsRNA	543 bp	Reductions in molting number, survival and reproduction.	(Xu et al., 2017)
	Sitobion avanae	wheat	Particle bombardment, transgenic wheat	Chitin synthase 1 (*CHS1*)	dsRNA	550 bp	Decreased *CHS1* expression level and reduced total and molting aphid numbers.	(Zhao et al., 2018)
	Sitobion avenae	wheat	Particle bombardment, transgenic wheat	G protein (*Gqα*)	dsRNA	517 bp	Reduced reproduction and molting in aphid.	(Hou et al., 2019)
	Sitobion avenae	wheat	Particle bombardment, transgenic wheat	Zinc finger protein (*SaZFP*)	dsRNA	198 bp	High mortality and decreased fecundity.	(Sun et al., 2019)

B. Spray-induced gene silencing-based aphid control

Type of gene silencing	Aphid species	Plant species	Delivery strategy	Target genes	Molecule	Size	Main effects	Reference
SIGS	*Aphis glycines*		Aerosolized siRNA-nanoparticle delivery method	Carotene dehydrogenase (*tor*), branched chain-amino acid transaminase (*bcat*)	siRNA	25 nt	Knockdown of target genes.	(Thairu et al., 2017)
	Aphis glycines		Nanocarrier-based transdermal dsRNA delivery system	*TREH*, *ATPD*, *ATPE* and *CHS1*	dsRNA	431 bp, 504 bp, 536 bp, 429 bp	Silenced the target gene expression, led to high mortality.	(Yan et al., 2020a)
	Acyrthosiphon pisum		Aerosolized siRNA-nanoparticle delivery method	*Tor* and *bcat*	siRNA	25 nt	Knockdown of target genes.	(Thairu et al., 2017)
	Sitobion avenae	Barley	Spraying	Structural sheath protein (*SHP*)	dsRNA	491 bp	Reduced *shp* expression level.	(Biedenkopf et al., 2020)
	Sitobion avenae	Barley	Spraying and feeding	Macrophage migration inhibitory factors, *SaMIF1*, *SaMIF2*, and *SaMIF3*	dsRNA	223 bp, 323 bp, 212 bp	Fed on artificial diet caused high mortality rates, sprayed with naked *SaMIF*-dsRNAs didn't alter nymphs' survival.	(Liu et al., 2021b)

	Schizaphis graminum		Aerosolized siRNA-nanoparticle delivery method	*Tor* and *bcat*	siRNA	25 nt	Knockdown of target genes.	(Thairu et al., 2017)
SIGS	*Schizaphis graminum*	Wheat	Nanocarrier-mediated transdermal dsRNA delivery system	*Sg2204*	dsRNA	/	Induced a stronger wheat defense response and resulted in negative impacts on aphid feeding behavior, survival, and fecundity.	(Zhang et al., 2022a)

C. Other delivery methods mediated gene silencing for aphid control

Type of gene silencing	Aphid species	Delivery strategy	Target genes	Molecule	Size	Main effects	Reference
Other delivery method	*Aphis citricidus*	Feeding, citrus stem dipping	Insulin receptor genes *AcInR1* and *AcInR2*	dsRNA	511 bp, 609 bp	Developmental defects, co-silencing of *AcInR1* and *AcInR2* resulted in high mortality.	(Ding et al., 2017)
	Aphis citricidus	Feeding, citrus stem dipping	Acetylcholinest erase, *TcAChE1* and *TcAChE2*	dsRNA	435 bp, 421 bp	High mortality, increased the susceptibility of *A. citricidus* to malathion and carbaryl.	(Mou et al., 2017)
	Aphis citricidus	Feeding, citrus stem dipping	Vitellogenin (*AcVg*), Vitellogenin receptor (*AcVgR*)	dsRNA	557 bp, 577 bp	A delay in the nymph-adult transition, a prolonged pre-reproductive period, and a shortened reproductive period, which in turn resulted in slower embryonic development and fewer newborn nymphs.	(Shang et al., 2018)
	Aphis citricidus	Feeding, citrus stem dipping	*AcCP19*	dsRNA	183 bp	Induced target genes silencing, high mortality.	(Shang et al., 2020)
	Aphis citricidus	Feeding, citrus stem dipping	*AcGNBP1*	dsRNA	431 bp	Decreased the activity of immune-related phenoloxidase.	(Ye et al., 2021)

	Aphis glycines	Topical application, nanocarrier and detergent mediated transdermal delivery system	Hemocytin, *Hem*	dsRNA	555 bp	Reduced the target gene expression and aphid population density.	(Zheng et al., 2019)
	Aphis gossypii	Feeding	Carboxylesterase, *CarE*	dsRNA	686 bp	Decreased resistance to organophosphorus insecticides.	(Gong et al., 2014)
Other delivery method	*Aphis gossypii*	Feeding	Cytochrome P450 monooxygenase gene *CYP6A2*	dsRNA	773 bp	Increased sensitivity to spirotetramat and alpha-cypermethrin.	(Peng et al., 2016)
	Aphis gossypii	Feeding	Odorant-binding proteins *AgOBP2*	dsRNA	434 bp	Interfered the odorant perception of aphid.	(Rebijith et al., 2016)
	Aphis gossypii	Feeding	*CYP6CY14*	dsRNA	459 bp	Increased the sensitivity of the resistant aphid to thiamethoxam.	(Wu et al., 2018)
	Aphis gossypii	Feeding	*CYP380C6*	dsRNA	436 bp	Increased the sensitivity of the resistant adults and nymphs to spirotetramat.	(Pan et al., 2018)
	Aphis gossypii	Feeding	*dsCYP6DC1*, *dsCYP6CY14*, and *dsCYP6CZ1*	dsRNA	494 bp, 499 bp, 499 bp	Increased the sensitivity of *Ace-R* strain to acetamiprid.	(Ullah et al., 2020a)

	Aphis gossypii	Feeding	Ecdysone receptor (*EcR*)	dsRNA	486 bp	High mortality rates and the lowered longevity and fecundity.	(Ullah et al., 2022a)
	Aphis gossypii	Injection	crustacean cardioactive peptide (*ApCCAP*), crustacean cardioactive peptide receptor (*ApCCAPR*)	dsRNA	339 bp, 519 bp	Developmental failure during nymph-adult ecdysis.	(Shi et al., 2022)
	Acyrthosiphon Pisum	Injection	*C002*	siRNA	21-23 nt	Knock down the transcript level of *C002.*	(Mutti et al., 2006)
Other delivery method	*Acyrthosiphon Pisum*	injection	Calreticulin, *cathepsin-L*	dsRNA	434 bp, 353 bp	Induced target genes silencing.	(Jaubert-Possamai et al., 2007)
	Acyrthosiphon Pisum	Feeding	*ApAQP1*	dsRNA	451 bp	Knockdown the *ApAQP1* expression level, resulted in elevated osmotic pressure of the haemolymph.	(Shakesby et al., 2009)
	Acyrthosiphon Pisum	injection	*vATPase*	dsRNA	185 bp	Induce high levels of mortality	(Whyard et al., 2009)
	Acyrthosiphon Pisum	Feeding	*Hunchback*	dsRNA	524 bp, 497 bp	Reduction of *Aphb* transcripts and rise of insect lethality.	(Mao and Zeng, 2012)
	Acyrthosiphon pisum	Injection and feeding	Enzyme *Cathepsin-L*	dsRNA	357 bp	Induced lethal effects.	(Sapountzis et al., 2014)
	Acyrthosiphon pisum	Injection	*ACYPI39568*	dsRNA	246 bp	Reduced *ACYPI39568* expression level but not affected the survival rate.	(Guo et al., 2014)

	Acyrthosiphon Pisum	Injection	Angiotensin-converting enzymes *ACE1*, *ACE2*	dsRNA	313 bp, 468 bp	Knockdown of *ACE1* and *ACE2* resulted in a higher mortality rate.	(Wang et al., 2015c)
	Acyrthosiphon Pisum	Injection	*ApMIF1*	dsRNA	213 bp	Disturbs their ability to feed from phloem sap.	(Naessens et al., 2015)
	Acyrthosiphon Pisum	Injection	*Armet*	dsRNA	286 bp	Disturbed feeding behavior and led to a shortened life span.	(Wang et al., 2015b)
Other delivery method	*Acyrthosiphon Pisum*	Injection	Structural sheath protein (*SHP*)	dsRNA	491 bp	Disrupted sheath formation prevented efficient long-term feeding from sieve tubes and a silencing effect on reproduction but not survival.	(Will and Vilcinskas, 2015)
	Acyrthosiphon Pisum	Injection	Peroxiredoxins, *ApPrx1*	dsRNA	206 bp	Decreased survival rate.	(Zhang and Lu, 2015)
	Acyrthosiphon Pisum	Injection and ingestion	Cytochrome P450 gene, *CYP4G51*	dsRNA	310 bp, 325 bp	Reduced *CYP4G51* expression, caused reductions in internal and external long-chain hydrocarbons (HCs) and increased mortality.	(Chen et al., 2016)
	Acyrthosiphon Pisum	Injection	Odorant receptors, *ApisOR5*, odorant-binding proteins, *ApisOBP3*, and *ApisOBP7*	dsRNA	/	The repellent behavior of *A. pisum* to EBF disappears.	(Zhang et al., 2017b)

	Acyrthosiphon Pisum	Feeding	Cuticular protein, *Stylin-01*, *Stylin-02*	siRNA	19 bp	Silencing the *stylin-01* decreased the efficiency of Cauliflower mosaic virus transmission by *M. persicae.*	(Webster et al., 2018)
	Acyrthosiphon Pisum	Injection	Neuropeptide F (*NPF*), NPF receptor (*NPFR*)	dsRNA	232 bp, 354 bp	Reduced aphid food intake, indicated a lower appetite for food after *NPF* knockdown.	(Li et al., 2018c)
	Acyrthosiphon Pisum	Feeding	*amiD*, *ldcA1*	dsRNA	311 bp, 353 bp	Reduction in *Buchnera* abundance and activity, accompanied by depressed aphid growth rates.	(Chung et al., 2018)
	Acyrthosiphon Pisum	Injection	Gap gene *Hunchback*	dsRNA	448 bp	Knock down of target gene.	(Ye et al., 2018)
Other delivery method	*Acyrthosiphon Pisum*	Injection and ingestion	Chitin synthase, *CHS*	dsRNA	364 bp	Induced mortality and development deformity.	(Ye et al., 2019)
	Acyrthosiphon Pisum	Injection	*ApHRC*	dsRNA	263 bp	Serratia-infected aphids displayed shorter phloem-feeding duration and caused Ca^{2+} elevation and ROS accumulation in plants.	(Wang et al., 2020)
	Acyrthosiphon pisum	Feeding, bean stem dipping	Cuticle protein gene, *ApCP19*	dsRNA	216 bp	Induced target genes silencing, high mortality.	(Shang et al., 2020)
	Acyrthosiphon pisum	Feeding, bean stem dipping	Carotenoid desaturase, *CdeB*	dsRNA	431 bp	Reduced aphid performance as reflected by a delay in nymphal developmental duration, lower weight, smaller number, and altered the population age structure.	(Ding et al., 2020)

Other delivery method	*Acyrthosiphon pisum*	Feeding, bean stem dipping	Gram-negative binding proteins, *ApGNBP1*, *ApGNBP2*	dsRNA	550 bp, 518 bp	Decreased the activity of immune-related phenoloxidase.	(Ye et al., 2021)
	Acyrthosiphon Pisum	Injection	CCHamide-2 receptor (*CCHa2-R*)	dsRNA	478 bp	Reduced *CCHa2-R* expression, food intake in adult aphids, and reproduction but not survival.	(Shahid et al., 2021)
	Acyrthosiphon Pisum	Injection	Fatty acid synthase 1 (*FASN1*) and diacylglycerol o-acyltransferase 2 (*DGAT2*)	dsRNA	609 bp, 388 bp	Prolonged the nymphal growth period and decreased the aphid body weight.	(Zhou et al., 2021)
	Acyrthosiphon pisum	Injection, nanocarrier-delivered RNAi method	*flightin*	dsRNA	374 bp	Malformed wings, the shape of dorsal longitudinal muscle (*DLM*) was deformed, the dorsoventral flight muscle (*DVM*) became wider and looser in aphids.	(Chang et al., 2022)
	Eriosoma lanigerum Hausmann	Topical application, nanocarrier-mediated transdermal dsRNA delivery system	V-ATPase subunit D (*ATPD*)	dsRNA	/	Induced target gene silencing, led to high mortality.	(Guo et al., 2022)

	Myzus nicotianae	Feeding	*TRV-ALY*, *TRV-Eph*	dsRNA	182 bp, 249 bp	Inhibition of target genes.	(Mulot et al., 2016)
	Myzus persicae	Injection	*MpMIF1*	dsRNA	205 bp	Disturbs their ability to feed from phloem sap.	(Naessens et al., 2015)
	Myzus persicae	Foliar application	*ZYMV HC-Pro*	dsRNA	588 bp	Insect successfully uptake dsRNA; the dsRNA was processed into siRNA by the insect RNAi machinery.	(Gogoi et al., 2017)
	Myzus persicae	Feeding	*Stylin-01*, *Stylin-02*	siRNA	19 bp	Silencing the *stylin-01* decreased the efficiency of Cauliflower mosaic virus transmission by *M. persicae.*	(Webster et al., 2018)
Other delivery method	*Myzus persicae*	Feeding	Voltage-gated sodium channels *MpNav*	dsRNA	289 bp	Induced high mortality and lower fecundity and longevity.	(Tariq et al., 2019)
	Myzus persicae	Feeding, Brassica leaf	*MpCP19*	dsRNA	139 bp	Induced target genes silencing, high mortality.	(Shang et al., 2020)
	Myzus persicae	Feeding, Brassica leaf	*MpGNBP1*	dsRNA	450 bp	Decreased the activity of immune-related phenoloxidase.	(Ye et al., 2021)
	Myzus persicae	Feeding	*Mp58*, *OBP2*	dsRNA	423 bp, 428 bp	Induced high mortality.	(Mahmood et al., 2021)
	Myzus persicae	Topical and root applications, nanocarrier-mediated delivery system	vestigial (*vg*), Ultrabithorax (*Ubx*)	dsRNA	489 bp, 359 bp	Downregulated target genes, caused wing aberration.	(Zhang et al., 2022c)

Other delivery method	*Myzus persicae*	Injection	ATP -binding cassette transporter gene (*ABCG4*), DnaJ homolog subfamily C member 1 (*DnaJC1*)	dsRNA	~400 bp	Increased mortality rate.	(Ding et al., 2022)
	Megoura viciae	Injection	Tyrosine hydroxylase *MV-TH*	dsRNA	400 bp	Reduced the *L-DOPA* level in aphid and a slight decrease in exuvia tanning.	(Wang et al., 2019)
	Rhopalosiphum padi	Injection	Acetylcholinest erase gene *RpAce1*,	dsRNA	383 bp	Increased susceptibilities to pirimicarb and malathion in *R. padi*, and reduced fecundity.	(Xiao et al., 2015)
	Sitobion avenae	Feeding	Catalase *CAT*	dsRNA	471 bp	Reduced survival rate and ecdysis index	(Deng and Zhao, 2014)
	Sitobion avenae	Feeding	Unigenes *DSR8*, *DSR32*, *DSR33*, *DSR48*	dsRNA	162 bp, 411 bp, 439 bp, 397 bp	Down-regulation of target genes and aphid mortality.	(Wang et al., 2015a)
	Sitobion avenae	Injection	Acetylcholinest erase gene *SaAce1*	dsRNA	400 bp	Increased susceptibility to pirimicarb in *S. avenae*, and reduced fecundity.	(Xiao et al., 2015)
	Sitobion avenae	Feeding	Ecdysone receptor (*SaEcR*), ultraspiracle protein (*SaUSP*)	dsRNA	469 bp, 411 bp	Significantly decreased the survival of aphids.	(Yan et al., 2016)

	Sitobion avenae	Feeding	*Laccase 1*, *SaLac 1*	dsRNA	613 bp	Inhibited the transcript levels of *SaLac 1*, decreased the survival rate.	(Zhang et al., 2018b)
	Sitobion avenae	Feeding	odorant binding protein (*SaveOBP9*)	dsRNA	501 bp	Reduced the expression level of *SaveOBP9* and induced the non-significant response of *S. avenae* to the tetradecane, octanal, decanal, and hexadecane.	(Ullah et al., 2020b)
	Sitobion avenae	Feeding	odorant binding protein (*SaveOBP10*)	dsRNA	432 bp	Aphid showed nonattraction towards β-caryophyllene and nonsignificant behavioural response to pentadecane, butylated hydroxytoluene and tetradecane.	(Ullah et al., 2022b)
Other delivery method	*Schizaphis graminum*	Feeding	*SgC002*	siRNA	476 bp	Fed on artificial diet for 3 days then transferred on aphid-susceptible wheat suppressed *SgC002* expression and led to lethality.	(Zhang et al., 2015c)
	Schizaphis graminum	Feeding	*MRA*, *GAT*, *TLP*	dsRNA	376 bp, 433 bp, 422 bp	Increased susceptibility to imidacloprid.	(Zhang et al., 2019)
	Sitobion miscanthi	topical application, nanocarrier-mediated transdermal dsRNA delivery system	*Sm9723*	dsRNA	/	Decreased fecundity and survival and negatively affected the feeding behavior.	(Zhang et al., 2022b)

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3

Chapter III Literature review on current status of nitrogen use efficiency in crop plants

Abstract

Nitrogen (N) is applied in relatively large amounts in agricultural practice to promote plant growth and development. To maximize yield, semi-dwarf crop varieties of the Green Revolution (GRV) require a high nitrogen fertilizer input. With the world's population growing and environmental pollution increasing, enhanced crop productivity with limited N supplies is a critical barrier. Improved nitrogen use efficiency (NUE) will be necessary for agricultural sustainability in the future. Here, we summarize the present studies on nitrogen metabolism genes involved in nitrogen uptake, transport, assimilation, and redistribution in crop plants, especially in rice and wheat. Pyramiding various genes combine with precise gene editing strategies will enhance the NUE of crop plants and increase grain yield for sustainable agriculture.

Keywords: Nitrogen (N), Green Revolution (GRV), nitrogen use efficiency (NUE), crop plant, grain yield

III.1. Introduction

Nitrogen (N) is a primary constituent of the nucleotides and proteins that are essential elements for all living organisms. The availability of N is a significant limiting factor for plant growth and development. For humans and animals, the nitrogen nutrients derived from plants are primary sources (Kraiser et al., 2011). Since nitrogen is the primary driver of crop production and one of the most predominantly limiting factors for productivity, considerable amounts of N fertilizers are applied each year in modern agriculture to maximize yield. N fertilization is currently one of the most expensive inputs in agricultural practice, and as resources grow more limited, this cost will increase. However, only 30%-50% of applied N fertilizer is absorbed by crop plants. Numerous environmental pollution issues are caused by excess N compounds released from agricultural systems (Tilman et al., 2002; Gastal et al., 2015). It threatens the quality of soil, water, and air. The eutrophication of rivers and acidification of soils are accelerated by the tropospheric production of atmospheric nitrous oxide and reactive N gases (Wang et al., 2012). Therefore, increasing N use efficiency (NUE) and decreasing N fertilizer consumption are critically needed to create sustainable and productive agriculture in light of the issues of food security and resource depletion in the future (Tilman et al., 2002; Gastal et al., 2015; Rakotoson et al., 2017).

With the development of the Green Revolution (GRV), the selection and widespread adaptation of a few semi-dwarf, early-maturing, high-yielding, disease-resistant cultivars that require high input conditions has substituted the conventional varieties (Khush, 1999). Nevertheless, the utilization of nitrogen fertilizer is relatively inefficient in Green Revolution varieties (GRVs). To reduce the negative effects of increased output of fertilizer on the environment and natural resources, it is essential to increase the resource use efficiency of cereal crops. Enhancing nitrogen use efficiency (NUE) in crop plants must be a priority in breeding programs in order to manage sustainable feeding for the expanding global population and limit the impact of increased fertilizer use on climate change (Sandhu et al., 2021).

NUE is defined as a ratio of output (total plant N, grain N, biomass yield, grain yield) and input (total N, soil N, or N-fertilizer applied). It is the optimal balance between nitrogen uptake efficiency (NupE), nitrogen assimilation efficiency (NAE), nitrogen utilization efficiency (NUtE) and nitrogen remobilization efficiency (NRE) (Masclaux-Daubresse et al., 2010). In crop plants, NUE is complex and primarily determined by N uptake, translocation, assimilation, and remobilization (Zeng et al., 2017). NUE mainly depends on soil nitrogen availability for the majority of non-legume plants, as well as how they take up inorganic nitrogen from the soil, assimilate nitrate (NO_3^-) and ammonium ($NH_4^{+)}$, and recycle organic nitrogen (Masclaux-Daubresse et al., 2010). In the past few years, numerous transportation and metabolism genes have been found to enable improved access to the biological processes of N uptake, transport, assimilation, and redistribution in crop plants (Li et al., 2017; Wang et al., 2018c; Han et al., 2020; Jia and von Wirén, 2020). Recently, studies in rice reported that some genes could enhance rice NUE in GRVs, whose products have been revealed to be involved not only in regulating N assimilation but also in promoting plant growth and C and N metabolism coordination (Li et al., 2018a;

Han et al., 2020; Wu et al., 2020). In this review, we summarize the recent advances in the understanding of NUE enhancements and co-regulation of C-N balance in crop plants, especially in rice and wheat.

III.1.1. Nitrogen-cycling network and microorganisms

The network of nitrogen-transforming microorganisms and its impact on global biogeochemical nitrogen cycling interactions are complex. Six distinct nitrogen transforming processes exist in biogeochemical nitrogen cycling, including assimilation, ammonification, nitrification, denitrification, anaerobic ammonium oxidation (anammox), and nitrogen fixation (Kuypers et al., 2018). The majority of studies have reported the reactions of nitrogen-transformation. For example, (1) Nitrogen fixation. Microorganisms carry the nitrogenase metalloenzyme can fix dinitrogen into ammonia. Nitrogenase is found in many bacteria and archaea (Hoffman et al., 2014). (2) Ammonia oxidation to hydroxylamine. Ammonia monooxygenase (AMO) is used by all known aerobic ammonia-oxidizing bacteria and archaea to oxidize ammonia to hydroxylamine (Hooper et al., 1997). (3) Hydroxylamine oxidation to nitric oxide and then to nitrite. AMO and hydroxylamine oxidoreductase (HAO) are present in all ammonia-oxidizing bacteria, which can oxidize ammonia all the way to nitrate (Daims et al., 2015; Van Kessel et al., 2015). (4) Nitrite oxidation to nitrate. The primary metabolic pathway that produces nitrate is nitrite oxidation, which is catalyzed by nitrite oxidoreductase (NXR). NXR is encoded by aerobic nitrite-oxidizing bacteria, anoxygenic phototrophs, and anaerobic ammonium-oxidizing bacteria (Strous et al., 2006; Griffin et al., 2007; Schott et al., 2010; Daims et al., 2016). (5) Nitrate reduction to nitrite. Microorganisms can conduct dissimilatory nitrate reduction to nitrite in all anoxic environments where nitrate is present, including soils (Philippot et al., 2007), oxygen minimum zones (Lam and Kuypers, 2011), marine sediments (Kraft et al., 2014), and the human gastrointestinal tract (Lundberg et al., 2008). (6) Nitrite reduction to ammonium. Nitrite reduction to ammonium is involved in both dissimilatory and assimilatory processes. The majority of bacterial lineages, including the thermophilic *Pyrolobus fumarii* (Blochl, 1997), methane-oxidizing archaea (Ettwig et al., 2016), diatoms (Kamp et al., 2011), and fungi (Zhou et al., 2002), all dissimulate nitrite reduction to ammonium. (7) Nitrite reduction to nitric oxide. Numerous microorganisms, including Proteobacteria, anaerobic ammonium-oxidizing bacteria, and Bacteroidetes (Maia and Moura, 2014), have the capacity to conduct nitrite to nitric oxide. These bacteria can be found in a variety of environments, including soils (Philippot et al., 2007), oxygen minimum zones (Lam and Kuypers, 2011), and marine sediments (Kraft et al., 2014), where nitrate is available and oxygen levels are low. (8) Nitric oxide reduction to nitrous oxide or dinitrogen gas. Various environments, such as wastewater treatment facilities (Kartal et al., 2013), agricultural soils (Philippot et al., 2007; Hallin et al., 2018), marine sediments (Kamp et al., 2011), and marine oxygen minimum zones (Lam and Kuypers, 2011), are habitat to microorganisms that could reduce nitric oxide. (9) Nitrous oxide reduction to nitrogen gas. Nitrous oxide reductase (NOS) is the only enzyme that is known to catalyze microbial nitrous oxide reduction to nitrogen gas

(Zumft and Kroneck, 2006). NOS is used by a variety of bacteria, including those from the Proteobacteria, Bacteroidetes, and Chlorobi phylas as well as archaea from the Crenarchaeota and Halobacteria (Cabello et al., 2004). It would be necessary to have a better understanding of the physiology of the involved microorganisms as well as their ecology and evolution in order to identify the factors that shape nitrogen-transforming networks.

III.1.2. Green revolution and nitrogen use efficiency

In the past 60 years, the widespread application of "green revolution" technology has achieved great success in enhancing food-grain production. The increased cultivation of GRVs, together with improvements in agricultural mechanization, irrigation, and fertilizer use, has greatly increased global crop production (Khush, 1999). Mutant alleles in wheat Reduced height-1 (*Rht-1*) and rice Semi-dwarf1 (*sd1*) loci confer beneficial semi-dwarfism, respectively. In subsequent studies, these mutations are discovered to be involved in GA metabolism and signaling pathways (Peng et al., 1999; Boss and Thomas, 2002; Sasaki et al., 2002). The *sd1* allele decreases bioactive gibberellin (GA) abundance while increasing accumulation of the rice DELLA protein SLENDER RICE1 (SLR1) (Li et al., 2018a). N-terminally truncated Rht-B1b and Rht-D1b proteins are resistant to GA-stimulated destruction (Peng et al., 1999; Van De Velde et al., 2021). The semi-dwarf varieties of GRV have an increased harvest index but exhibited a relatively low nitrogen use efficiency (NUE). To achieve high yields, semi-dwarf GRV varieties require high N fertilizer applications. Consequently, a significant issue for sustainable agriculture is whether increasing NUE by a reduction of N fertilizer can be done without a corresponding yield cost (Wu et al., 2021).

III.1.3. Nitrogen metabolism related genes in crop plants

Nitrate is the main type of inorganic N in aerobic soils, while ammonium predominates in acidic or flooded wetland soils (Xu et al., 2012). Nitrate uptake is at the root level. Two nitrate transport systems coexist and act coordinately to absorb nitrate from the soil. According to the studies, the NRT1 family mediates low-affinity transport system (LATS) (except *NRT1.1*) and the NRT2 family mediates high-affinity transport system (HATS) (Williams and Miller, 2001b; Miller et al., 2007). Ammonium uptake is carried out by plasma membrane (PM)-located AMT/MEP/RH transporters (Khademi et al., 2004). The primary steps of the assimilatory pathway are coupled reactions related to the reduction of nitrate to nitrite catalyzed by nitrate reductase and nitrite reductase via the glutamine synthetase (GS)/2-oxoglutarate amidotransferase (GOGAT) cycle. Glutamine synthetase (GS) catalyzes the conversion of glutamate into glutamine by incorporating a molecule of ammonia. Subsequently, an amide group from glutamine to 2-oxoglutarate is transferred to produce two molecules of glutamate catalyzed by glutamate synthase (GOGAT). Based on the subcellular location, GS is classified into two isoforms, including cytosolic (GS1) and chloroplastic (GS2). GS1 is usually encoded by a multigene family, while GS2 is often encoded by a single gene (Miflin and Habash, 2002;

Chardon et al., 2012). There are two different forms of GOGAT. Fd-GOGAT and NADH-GOGAT use ferredoxin and NADH as the electron donors, respectively. Fd-GOGAT is predominantly localized in leaf chloroplasts, whereas NADH-GOGAT is primarily located in plastids of non-photosynthetic tissues (Temple et al., 1998; Forde and Lea, 2007).

III.1.4. Improving nitrogen use efficiency by modulate nitrogen metabolism genes in crop plants

Several metabolic processes, including uptake, translocation, assimilation, and remobilization, in coordination affect the nitrogen use efficiency of higher plants. Greater yields with fewer inputs seem to be an ideal trait for NUE improvement. Many critical candidate genes have been manipulated by genetic engineering in order to test their effects on biomass and plant nitrogen status.

Numerous transmembrane transporter genes involved in NO_3^- absorption and translocation have been reported. In rice, higher expression of *OsNRT1.1B* results in increases in N absorption, biomass production, and grain yield, whereas the *osnrt1.1b* mutant exhibits lower NO_3^- uptake and root-to-shoot translocation (Hu et al., 2015a). Overexpression of *OsNRT2.3b* increases the capacity of rice plants to buffer pH in response to varying N supplies, which has a significant impact on NUE and grain production in rice (Fan et al., 2016). *OsNPF6.1* allele-specific expression is much more likely to be transactivated by *OsNAC42*, conferring increased NUE and rice yield (Tang et al., 2019). *OsNRT2.1* or *OsNAR2.1* overexpression increases NO_3^- absorption and grain yield.

There is increasing evidence that both *GS1* and *GS2* are critical for effective N usage and high yield potential in major varieties of wheat, rice, and maize (Hirel et al., 2011; Chardon et al., 2012; Thomsen et al., 2014). *Osgs1.1* mutations in rice substantially reduce grain filling and growth rate. Due to low glutamine levels, the total free amino acid content is decreased in the mutant's leaf blades. The results may indicate that the products of *OsGS1.1* are most likely to be responsible for synthesizing glutamine for remobilization through the phloem (Obara et al., 2001; Obara et al., 2004). Overexpression of the *Phaseolus vulgaris GS1* gene in wheat under the control of the *Rubisco (rbcS)* promoter leads to significantly increased root and grain yield with higher N content in grain (Habash et al., 2001). Increased biomass and leaf protein are observed when the *Pisum sativum GS1* gene was overexpressed in *N. tabaccum* under the control of the *CaMV 35S* promoter (Oliveira et al., 2002). In wheat, the amount of N remobilized from the shoot to the grain is correlated with GS activity utilizing cultivars with contrasting NUE via a quantitative genetic method (Habash et al., 2007; Kichey et al., 2007). The N harvest index and spikelet number are raised by overexpressing the *GS1* gene *Gln1* with a *ubiquitin* promoter in rice (Brauer et al., 2011). Transgenic expression of *TaGS2-2Ab* in wheat using its endogenous promoter enhances grain yield and NUE since it increased N uptake, remobilization, and N harvest index (Hu et al., 2018).

Fd-GOGAT and NADH-GOGAT have been shown to play essential roles in plant growth and seed development in rice, barley, and maize (Kendall et al., 1986; Tamura

et al., 2010; Yang et al., 2016; Zeng et al., 2017; Cañas et al., 2020). In *NADH-GOGAT* over-expression rice plants, the grain weight is increased by up to 80% (Yamaya et al., 2002). Studies in rice with both *Fd-GOGAT* and *NADH-GOGAT* suppression show that tiller number, total shoot dry weight, and yield are significantly reduced when compared to control plants (Lu et al., 2011). Ammonium from photorespiration is assimilated by NADH-GOGAT. As a result of photorespiration, rice plants lose a significant amount of volatile NH3 from their leaves. Therefore, increasing the reassimilation of photorespiratory ammonia at the grain-filling stage is a promising method for enhancing NUE (Kumagai et al., 2011).

GROWTH-REGULATING FACTOR 4 (GRF4) transcription factor can promote rice GRV ammonium uptake and integrates nitrogen assimilation, carbon fixation and plant growth (Li et al., 2018a). *ARE1* is a genetic suppressor of a rice *fd-gogat* mutant defective in nitrogen assimilation. Loss-of-function mutations in ARE1 delay rice senescence and increase grain production by 10-20%, which improves NUE in nitrogen-limiting situations (Wang et al., 2018a). *TaARE1* homeologs have been isolated and characterized in wheat *cv* Zhengmai 7698 (ZM7698) and *cv* Kenong 199 (KN199). Mutant lines either with partial or triple null *taare1* alleles via CRISPR/Cas9-mediated target mutagenesis of *TaARE1.* The results indicate that mutant lines exhibited significantly enhanced NUE and increased grain yield in both ZM7698 and KN199 varieties (Guo et al., 2021; Zhang et al., 2021a).

III.1.5. Modulation of growth-metabolism coordination for enhanced nitrogen use efficiency in crop plants

To enhance plant development and grain yield, interaction between C and N metabolisms in plants is crucial for the effective absorption of these two essential nutrients (Zheng, 2009). Plants have evolved sophisticated and coordinated processes to control plant growth, C fixation, and N assimilation in order to efficiently absorb N from soil. Based on previous studies, overexpression of the *Rubisco* (*rbcS*) gene increased N storage in rice leaves (Suzuki et al., 2007), while decrease Rubisco activity lead to an inhibition of N metabolism (Matt et al., 2002). Additionally, it has been demonstrated that the proteins of the plant-specific *Dof1* family regulate the development of the C skeleton. Under low N circumstances, overexpression of either rice *OsDof2* or maize *ZmDof1* could increase grain production and NUE, which indicates that the synergistic enhancement of C/N metabolism (Yanagisawa et al., 2004; Kurai et al., 2011; Iwamoto and Tagiri, 2016). The dep1-1 allele mutation decreased the expression of *OsCKX2* and improved CK biosynthesis, increasing the number of grains and rice yield. More significantly, rice plants with the dominant *dep1-1* allele displayed an N-insensitive phenotype together with enhanced N absorption and assimilation, leading to an increase in yield at moderate N supply levels (Sun et al., 2014). Li et al. (2018a) reported that the reduced NUE in high-yielding GRVs is primarily due to DELLA proteins (DELLAs) accumulation. Their study also demonstrated that an allelic mutation at the *GRF4* locus increases C and N assimilation and reduces the inhibitory effects of rice DELLA protein SLR1. Increasing *GRF4* abundance significantly boosts grain production of elite rice and

wheat GRVs without reducing the yield-enhancing semi-dwarfism conferred by DELLA. Another study demonstrated that higher grain yield and NUE are achieved at low N fertilization levels owing to pyramiding of elite *GRF4* and *NITROGEN-MEDIATED TILLER GROWTH RESPONSE 5* (*NGR5)* alleles that improve tillering and N assimilation (Wu et al., 2020). Therefore, the GRF4-DELLA-NGR5 manipulating module provides a simple and efficient way of enhancing NUE and grain yield in GRV crop plants (Liu et al., 2022).

III.2. Conclusions and perspectives

In both agricultural and natural ecosystems, the macronutrient N frequently serves as a limiting factor for plant productivity. One of the key strategies for increasing crop yields is the application of inorganic N fertilizer. However, the semidwarf GRVs have very poor N uptake and assimilation efficiency. As a result, a lot of inorganic fertilizer is needed to achieve the current global cereal crop yields, raising worries about the significant economic and environmental consequences. To reduce fertilizer consumption in high-yield crops and maintain agricultural sustainability, innovative breeding techniques are required. On the other hand, as a complex polygenic trait, NUE is regulated by a variety of metabolic, developmental, and environmental response network connections that are integrated over the whole life cycle of the plants. Crop NUE and grain yield have been enhanced by targeting genes directly involved in N uptake, assimilation, translocation, and remobilization (Han et al., 2020). As more new genes that control plant growth and nitrogen metabolism are discovered, utilizing precise gene editing will improve crop breeding approaches. A new era of the Green Revolution will proceed.

III.3. References

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4

Chapter IV Silencing an aphid-specific gene *SmDSR33* for aphid control through plant-mediated RNAi in wheat

Abstract

Grain aphid (*Sitobion miscanthi*) is one of the most dominant and devastating insect pests in wheat, which causes substantial losses to wheat production each year. Engineering transgenic plants expressing double strand RNA (dsRNA) targeting an insect-specific gene has been demonstrated to provide an alternative environmentally friendly strategy for aphid management through plant-mediated RNA interference (RNAi). Here we identified and characterized a novel potential RNAi target gene (*SmDSR33*) which was a gene encoding a putative salivary protein. We then generated stable transgenic wheat lines expressing dsRNA for targeted silencing of *SmDSR33* in grain aphids through plant-mediated RNAi. After feeding on transgenic wheat plants expressing *SmDSR33*-dsRNA, the attenuated expression levels of *SmDSR33* in aphids were observed when compared to aphids feeding on wild-type plants. The decreased *SmDSR33* expression levels were thus resulted in significantly reduced fecundity and survival, and decreased reproduction of aphids. We also observed altered aphid feeding behaviors such as longer duration of intercellular stylet pathway and shorter duration of passive ingestion in electropenetrography assays. Furthermore, both the surviving aphids and their offspring exhibited decreased survival rates and fecundity, indicating that the silencing effect could be persistent and transgenerational in grain aphids. The results demonstrated that *SmDSR33* can be selected as an effective RNAi target for wheat aphid control. Silencing of an essential salivary protein gene involved in ingestion through plant-mediated RNAi could be exploited as an effective strategy for aphid control in wheat.

Key words: wheat (*Triticum aestivum* L), grain aphid (*Sitobion miscanthi*), RNA interference (RNAi), salivary protein, aphid control

IV.1. Introduction

Aphids are the most destructive agricultural insect pests, which cause potential yield losses of common wheat (*Triticum aestivum* L) by sap-sucking and virus transmission (Xia et al., 2012; Sun et al., 2019). The grain aphid (*Sitobion miscanthi*) is one of the most devastating wheat aphids that causes substantial damage to wheat, which was previously misidentified as *Sitobion avenae* (Zhang et al., 2013; Yu et al., 2014; Zhang et al., 2022a). Currently, neurotoxic insecticides are still the predominant measure for aphid management. However, intensive use of pesticides can cause aphid resistance and harmfulness to non-target organisms, which leads to environmental issues (Sanahuja et al., 2011). Limited aphid resistance germplasm has significantly hampered the process of conventional breeding projects (Crespo-Herrera et al., 2019). Therefore, it is imperative to search for effective and practical strategies for aphid management in wheat.

RNA interference (RNAi) has been recognized as one of the most potential technologies for pest control. Transgenic plant-mediated RNA interference (RNAi), which provides a protective and environmentally friendly strategy for aphid management, has been proven to be a practicable method in recent years (Price and Gatehouse, 2008). For example, interference of structural sheath protein (SHP) encoding gene in grain aphids by feeding on transgenic barely plants effectively reduce their survival and reproduction rates. Knock-down of *shp* strongly affect feeding behavior and the transgenerational effect can last for the next seven generations (Abdellatef et al., 2015). The dsRNA-transgenic *Arabidopsis* plants of the cuticular protein gene impaired the fecundity of *Myzus persicae* (Bhatia and Bhattacharya, 2018). Transgenic wheat plants expressing *SaZFP*-dsRNA decreased the survival and fecundity significantly in *S. avenae* with effects also observed on offspring (Sun et al., 2019). Plastid-expressed dsRNAs can be efficiently applied for sap-sucking pest control. Aphids feeding on transplastomic plants exhibited significant mortality, decreased aphid fecundity, and reduced weight of survivors (Dong et al., 2022).

As sap-sucking insects, aphids secrete gel saliva during stylet penetration and watery saliva during sap sucking (Khan and Naveed, 2022). Aphid salivary protein plays a pivotal role in the interaction between pest and host plants (Pan et al., 2015). ApC002 was first discovered in *Acyrthosiphon pisum* and has been proven to play a critical role in the foraging and feeding process of pea aphid (Mutti et al., 2008). Transient expression of salivary proteins in *Nicotiana benthamiana*, such as Mp10, Mp42, Mp56, Mp57, and Mp58, caused reduced virulence and fecundity of green peach aphids (Bos et al., 2010; Elzinga et al., 2014; Rodriguez et al., 2014). *M. persicae* salivary proteins Mp1, Mp2, Mp55, and MpMIIF1 were verified to inhibit host plant defense responses and facilitate green peach aphid performance on host plants (Pitino and Hogenhout, 2013; Elzinga et al., 2014; Naessens et al., 2015). Overexpression of salivary proteins Me10 and Me23 enhanced potato aphid infestation and fecundity (Atamian et al., 2013). Knockdown of the transcript of effector protein Armet by RNA interference impeded the feeding behavior of pea aphids. Overexpression of *Armet* in *N. benthamiana* was shown to activate plant-

pathogen interactions and induce salicylic acid-mediated defense in plants, but had no detectable effects on aphid performance (Wang et al., 2015b; Cui et al., 2019). Expression of bird cherry-oat aphid candidate effector Rp1 in transgenic barley plants significantly promoted aphid fecundity and suppressed plant defense responses (Escudero-Martinez et al., 2020). Besides, transient overexpression of salivary effectors Sm9723 and Sg2204 in tobacco inhibited cell death and suppressed plant defense responses. Silencing *Sm9723* through a nanocarrier-mediated dsRNA delivery system significantly decreased the survival rates and fecundity of aphids and affected feeding behavior. Similarly, *Sg2204*-silenced aphids exhibited a strong wheat defense response and negatively impacted aphid survival rate, fecundity, and feeding behavior. The aphid performance on host plants was significantly reduced when silencing the homologs of *Sg2204* from four other aphid species (Zhang et al., 2022a; Zhang et al., 2022b). These results implied that the genes encoding salivary proteins in aphids are the potential candidate for aphid control in plants though plant-mediated RNAi.

Here, we isolated a novel putative salivary protein encoding gene, *SmDSR33*, in grain aphid based on our previous transcriptomic profiling. We found that feeding on transgenic wheat plants expressing *SmDSR33*-dsRNA decreased the survival rate and the fecundity significantly in grain aphids. The surviving aphids exhibited a silencing effect and induced a transgenerational effect on their offspring.

IV.2. Materials and methods

IV.2.1. Plants and Insects

Plants: the hexaploid wheat variety *Triticum aestivum* L. *cv* Zhengmai 7698 (ZM7698) was used in this study. 30-35 wild-type and transgenic wheat plant seeds were sown in pots and were cultured in a climate chamber at 22℃ under a 16-h photoperiod, and with a relative humidity of 40%-60%.

Insects: grain aphids, *S. miscanthi* were reared on two-leaf stage aphid susceptible wheat seedlings in a controlled chamber with similar conditions than for plant growing. Apterous adult grain aphids from a single clonal lineage were reared on wheat seedlings in a continuous culture for 24 hours to produce synchronized nymphs. After that, the adults were removed away, and the offspring were used in subsequent experiments. All experiments were carried out in a climate chamber under the above-mentioned conditions.

IV.2.2. Isolation and Characterization of SmDSR33

Total RNA of pooled adults was extracted by using TransZol Up (TransGen Biotech, Beijing, China). cDNA was synthesized by using FastKing RT Kit (Tiangen, Beijing, China). The full length of the *SmDSR33* gene was obtained using TransStart® FastPfu DNA Polymerase (TransGen Biotech, Beijing, China) following the instructions. The DNA amplification products were sequenced by the Institute of Crop Sciences (Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China). The theoretical isoelectric point (pI) and molecular weight (MW) of SmDSR33 were calculated through ExPASy (https://web.expasy.org/compute_pi/).

Transmembrane region and putative signal peptide were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/), respectively. The counterparts of *SmDSR33* of other aphid species were obtained by the Basic Local Alignment Search Tool (BLAST) against the aphidbase database (http://bipaa.genouest.org/is/aphidbase/). Phylogenetic trees of *SmDSR33* in twelve aphid species were constructed using the nucleotide acid sequences as a matrix via MEGA X software (www.megasoftware.net). The branch strength was analyzed by using the maximum likelihood method and performing 100 bootstrap replications.

IV.2.3. Vector construction and wheat transformation

To amplify the 439 bp *SmDSR33* target sequence, specific primers were designed. A 320 bp fragment of GFP were selected as a control in the aphid bioassay experiment. The amplified PCR products were recovered and inserted at inverted repetitions into the *Spe*I/*Eco*RV and *Sac*I/*Hpa*I sites of the pEasy-Blun-Zero-AdhI vector to construct the hairpin RNAi, Bzero-DSR33-adhI-DSR33. The vector of Bzero-DSR33-adhI-DSR33 was digested by *Ssp* I and *BsrG* I to obtain the expression cassette. The latter was recovered for bombardment. The RNAi fragment was driven by the maize Ubi promoter. Bombardment-mediated transformation was applied to immature embryos isolated from ZM7698. Somatic embryos were induced in tissue culture on medium, and whole plants were then regenerated and selected. Healthy seedlings were transplanted to soil to grow until maturity.

IV.2.4. Southern blot analysis

The CTAB method was used to extract genomic DNA from young T_3 plant leaf tissues as described by Sambrook et al. (1989). The restriction enzyme was used to digest 35 μg of genomic DNA overnight. The products were fractionated for 12-16 h at 60 V on a 0.8% agarose gel in 1×TBE buffer. The Hybond-N^+ membranes were used for blotting (Amersham, UK). The digoxigenin (DIG) High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany) was used for prehybridization, hybridization, washing, and detection of the membranes. The primer sets SmDSR33S-F/R were used to synthesized DNA probes (Supplementary Table 4-S1).

IV.2.5. Quantitative real-time PCR

For the expression level of *SmDSR33* at different development stages, total RNAs of grain aphids were isolated from the four nymphs and adults reared on susceptible wheat. For the expression level of *SmDSR33 in* aphids fed with different transgenic wheat and wild-type plants, the adult aphids were collected and used for total RNA extraction and further experiments.

The cDNA was synthesized following conventional procedures. A quantitative real-time PCR (qRT-PCR) assay was carried out using the SYBRH Green Real-time PCR Master Mix (Tiangen, Beijing, China) in an ABI 7300 Real Time PCR system. The aphid *Actin* gene and ribosomal protein S27 A (*Rps27*) gene were selected as internal controls, and *SmDSR33* specific primers were designed for normalization

(Supplementary Table 4-S1). All qRT-PCR experiments were performed in triplicate. The relative gene expression of each target gene was calculated by using the mean value of the reference genes through the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

IV.2.6. Aphid bioassays

A single clonal lineage of apterous adult grain aphids was reared on wheat seedlings in cages for 24 hours to produce nymphs. The newborn nymphs produced during the period of 24 hours were transferred to fresh transgenic wheat plants.

T_3 homozygous wheat plants with *SmDSR33*-dsRNA expression were selected to evaluate the effects on aphid survival and fecundity. At the 3-4 leaf stage, 20 neonatal first instar nymphs of *S. miscanthi* were placed on the leaf of each plant. The mortality of aphids was recorded every day. Ten plants from each line were used in every experiment. The experiment was repeated three times.

Life cycle parameters were calculated as follows: the net reproductive rate, $R_0 = \sum l_x \cdot \mathrm{m}_x$, the mean generation time, $T = \sum x l_x m_x / \sum L_x m_x$, the intrinsic rate of increase, $r_m = (\ln R_0)/T$, and the finite rate of increase, $\lambda = e^{r_m}$. In the equations, l_x is the surviving rate to a specific age x, and m_x is the number of new-born nymphs produced by per live adult for a specific age x (Biondi et al., 2013).

IV.2.7. Electrical penetration graph technique analysis

The Giga-8 DC EPG amplifier (EPG-Systems, Wageningen, Netherlands) and a Faraday cage was used to record the probing and feeding behaviors of apterous adult aphids on wheat. Firstly, synchronous adult aphids were inoculated on transgenic line 33-592 and control plants for two days, respectively. Then, the aphid was starved for 2 h. After that, water-soluble silver conductive paint was used to attach each aphid to a flexible gold wire (18 μm diameter×2 cm length) through the dorsal thorax individually. The aphids were placed onto the adaxial side of a leaf from transgenic and wild-type wheat plants at the three-leaf stage, and the opposite ends of the gold wires (2 mm in diameter×3 cm length) were connected to copper wire with conducting silver glue, which was connected to a DC amplifier. The plant electrode was inserted into the soil. Under light conditions, the EPG signal of each individual was continuously monitored for 8 h. We monitored 12 behavioral recordings for each treatment. The software Stylet+a (EPG-Systems) was used to analyze EPG signals. According to the method described by Tjallingii (Tjallingii, 1985; Tjallingii, 1994), the different waveforms were correlated with feeding behavior. Non-probing (np) waveform, which reflects stylet external to wheat leaf tissue. Pathway phase contains two waveforms, waveform C, which reflects the intercellular stylet pathway, potential drops (pd), which reflects intracellular punctures during intercellular pathway. Waveform G (xylem phase) is the only waveform that reflects active sap ingestion from xylem elements. Phloem phase can be divided into two phases: E1 always occurs at the start of the phloem phase and reflects saliva secretion into the sieve element, E2 reflects passive phloem sap ingestion. EPG data was analyzed using the EPG-Excel data workbook provided by Sarria et al. (2009).

IV.2.8. Statistical analysis

The two-tailed Student's *t*-test was used to evaluate the differences between wild-type and transgenic wheat lines. For all comparisons, significance (*P* value) was calculated at the 1% or 5% level. The standard error of the mean (SEM) for each treatment was calculated using three biological replicates. For the EPG experiments, means and standard errors of variables were calculated from recordings per individual aphid, and differences were analyzed by Student's *t*-test. All data represents means ± SEM.

IV.3. Results

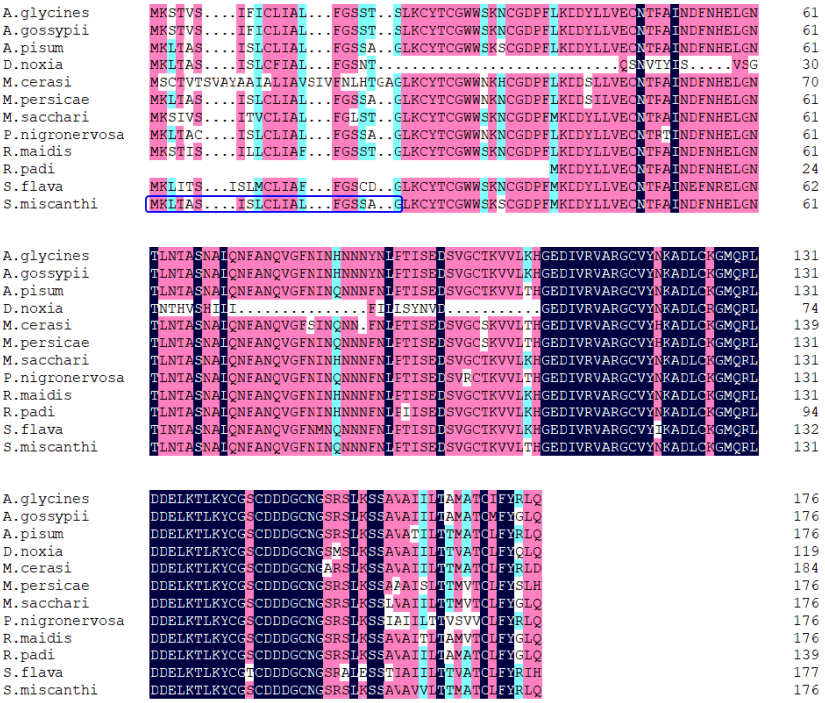
IV.3.1. Characterization of SmDSR33 gene in grain aphids

We identified a candidate gene *SmDSR33*, which encoding a putative salivary protein in grain aphid, based on transcriptomic profiling and dsRNA feeding assay (Wang et al., 2015a). The full-length cDNA sequence of *SmDSR33* was 534 bp in length, encoding a 177 amino acid putative salivary protein. The SmDSR33 protein was predicted to have an Mw of 19.376KDa and a pI of 6.26, possess a signal secretion peptide with a predicted cleavage site between amino acid residues 20 and 21 and have one predicted transmembrane helix, suggesting that SmDSR33 was a secreted protein (Figure 4-1A, Supplementary Figure 4-S1).

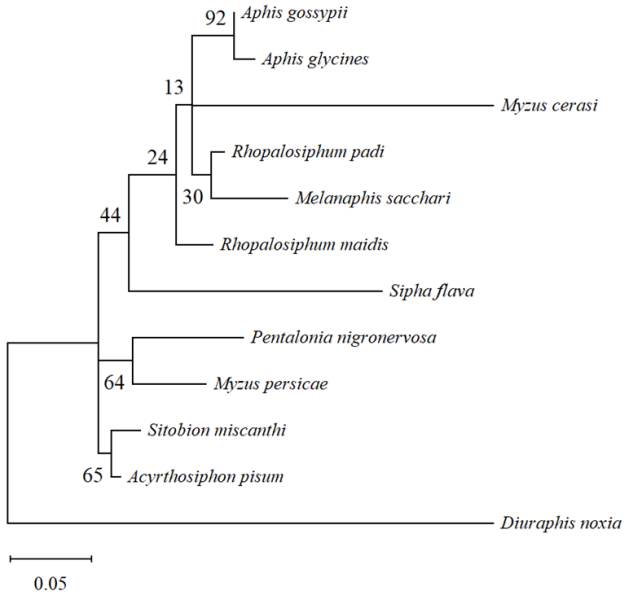
To clarify the evolutionary relationships of this gene in different insect species, sequences of *SmDSR33* counterparts in pea aphid (*A. pisum*), soybean aphid (*Aphis glycines*), cotton aphid (*A. gossypii*), Russian wheat aphid (*Diuraphis noxia*), sugarcane aphid (*Melanaphis sacchari*), black cherry aphid (*Myzus cerasi*), peach aphid (*M. persicae*), banana aphid (*Pentalonia nigronervosa*), corn aphid (*Rhopalosiphum maidis*), bird cherry-oat aphid (*Rhopalosiphum padi*), and yellow sugarcane aphid (*Sipha flava*) were obtained by BLAST against aphidbase database and NCBI. The phylogenetic tree of *SmDSR33* was constructed via MEGA X software. Phylogenetic analysis demonstrated *SmDSR33* was more closely related to its orthologs in the pea aphid (*A. pisum*) (Figure 4-1B).

We the used qRT-PCR to investigate the *SmDSR33* expression level in grain aphids at different developmental stages. Results revealed that *SmDSR33* transcription was accumulated throughout the developmental phases at different levels (Figure 4-1C). The *SmDSR33* expression pattern peaked in the adult aphid and was about 1.6-fold higher compared to first instar nymphs.

(A)


A.glycines
A.gossypii
A.pisum
D.noxia
M.cerasi
M.persicae
M.sacchari
P.nigronervosa
R.maidis
R.padi
S.flava
S.miscanthi

(B)


92
Aphis gossypii
Aphis glycines
13
Myzus cerasi
24
Rhopalosiphum padi
30
Melanaphis sacchari
44
Rhopalosiphum maidis
Sipha flava
Pentalonia nigronervosa
64
Myzus persicae
Sitobion miscanthi
65
Acyrthosiphon pisum
Diuraphis noxia
0.05

(C)

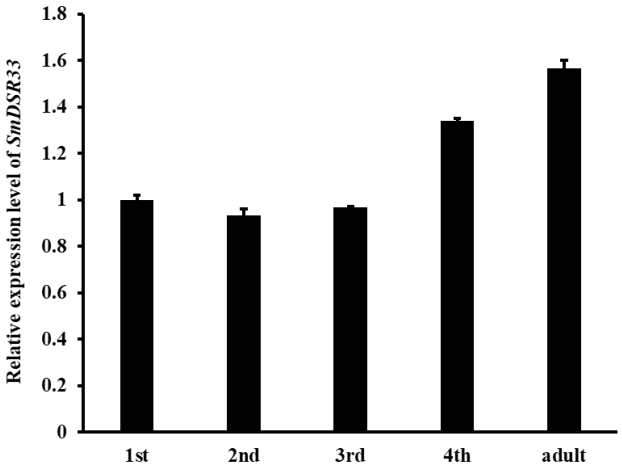


Figure 4-1 Characterization of SmDSR33

(A) Multiple sequence alignment of SmDSR33 protein and orthologs from other aphid species. The deduced amino acid sequences from eleven aphid species include *Acyrthosiphon pisum* (NM_001163178.1), *Aphis glycines* (AG000929-RA), *Aphis gossypii* (XM_027996540.1), *Diuraphis noxia* (XM_015509488.1), *Melanaphis sacchari* (XM_025349621.1), *Myzus cerasi* (Mca00769.t1), *Myzus persicae* (XM_022311485.1), *Pentalonia nigronervosa* (g3912.t1), *Rhopalosiphum maidis* (XM_026949227.1). *Rhopalosiphum padi* (Rpa07522.t1), and *Sipha flava* (XM_025560312.1). Black shades indicate identical amino acids. Pink shades indicate similar amino acid, and blue shades include the sequences with identical and similar residues. Signal peptide of SmDSR33 is highlighted with blue box.
(B) Phylogenetic tree of SmDSR33 and its homologs from other aphid species constructed with the maximum likelihood method. Bootstrap supporting values (1000 replicates) are shown at the branch nodes.
(C) The expression profile of *SmDSR33* in grain aphid at different development stages. The expression profiles of *SmDSR33* at different developmental stages of grain aphids fed on wheat. Values and error bars represent the mean and SEM of three independent biological replicates, each with a pool of 15 individual aphids.

IV.3.2. Wheat plants expressing SmDSR33-dsRNA induce SmDSR33 silencing in aphids upon feeding

To investigate the function of *SmDSR33*, a 439 bp fragment of *SmDSR33* gene was selected as a template for RNAi target (Figure 4-2A). We used BLAST against the NCBI database to evaluate the specificity of the *SmDSR33* fragment. At the nucleotide acid level, no continuous three 21-nt matches were detected between the selected 439 bp fragment and aphid natural enemies or humans (data not shown), which implied

that the selected *dsSmDSR33* fragment would not pose potential risks to non-target organisms (Bachman et al., 2013). Then, the RNAi vector harboring *SmDSR33*-hairpin DNA was constructed (Figure 4-2B). After transformed into wheat immature embryos, we obtained 8 independent transgenic wheat lines, among which, we randomly selected 3 of them for further analysis. Southern blot analysis indicated that the expression cassette of *SmDSR33*-dsRNA had been successfully integrated into the wheat genome with two to twelve copies (Figure 4-2C).

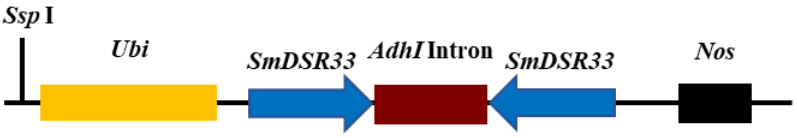
To further investigate whether the expression of the target *SmDSR33* gene in aphids was inhibited when feeding on transgenic wheat plants. The individual synchronous one-day-old nymphs were transferred to wild-type and transgenic wheat plants, respectively. The relative expression levels of *SmDSR33* were detected in adult aphids. The relative expression levels of *SmDSR33* in grain aphids decreased significantly upon feeding on three transgenic wheat lines ($P < 0.01$, Figure 4-2D).

(A)

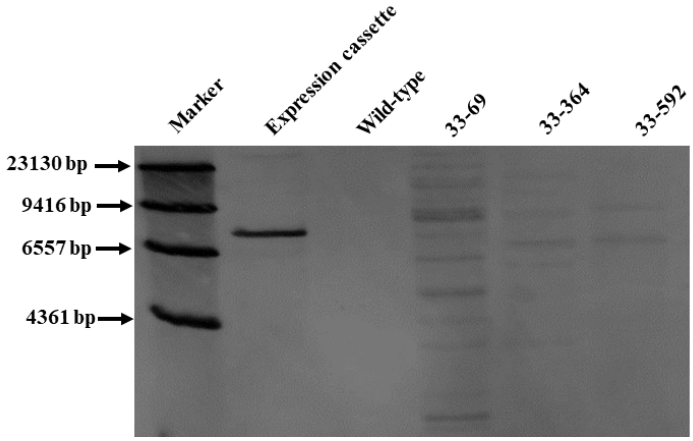
```
1    ATG AAG TTA ACT GCA TCA ATA TCG TTA TGT TTA ATC GCC CTG TTT GGA TCG AGT GCT GGT
1     M   K   L   T   A   S   I   S   L   C   L   I   A   L   F   G   S   S   A   G
61   CTA AAA TGC TAC ACT TGT GGC TGG TGG AGC AAA AGT TGT GGT GAT CCA TTC ATG AAA GAT
21    L   K   C   Y   T   C   G   W   W   S   K   S   C   G   D   P   F   M   K   D

121  GAT TAT CTT TTA GTT GAG TGT AAT ACT AGA GCG ATT AAT GAT TTC AAC CAC GAA CTA GGA
41    D   Y   L   L   V   E   C   N   T   R   A   I   N   D   F   N   H   E   L   G
181  AAC ACC CTA AAT ACG GCC AGT AAT GCT TTA CAA AAT TTC GCA AAT CAG GTG GGT TTC AAC
61    N   T   L   N   T   A   S   N   A   L   Q   N   F   A   N   Q   V   G   F   N
241  ATC AAT CAA AAC AAC AAT TTC AAT TTG CCT ACC ATT AGT GAA GAT TCA GTT GGA TGC ACT
81    I   N   Q   N   N   N   F   N   L   P   T   I   S   E   D   S   V   G   C   T
301  AAA GTA GTA CTT ACA CAC GGA GAA GAT ATT GTA CGA GTG GCT CGA GGA TGC GTT TAT AAC
101   K   V   V   L   T   H   G   E   D   I   V   R   V   A   R   G   C   V   Y   N
361  AAA GCA GAC TTG TGC AAG GGA ATG CAA AGA CTT GAC GAC GAG CTG AAA ACC CTC AAA TAC
121   K   A   D   L   C   K   G   M   Q   R   L   D   D   E   L   K   T   L   K   Y
421  TGT GGT TCG TGC GAC GAC GAC GGA TGT AAC GGT TCT AGG TCG TTG AAG TCT TCG GCG GTC
141   C   G   S   C   D   D   D   G   C   N   G   S   R   S   L   K   S   S   A   V
481  GCA GTA GTT CTG ACC ACG ATG GCT ACG TGC CTG TTC TAC AGG CTA CAG CAC TAA
161   A   V   V   L   T   T   M   A   T   C   L   F   Y   R   L   Q   H   *
```

(B)



(C)



(D)

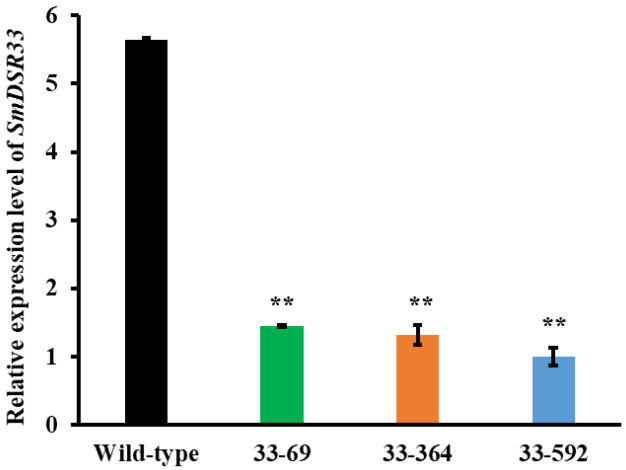


Figure 4-2 RNAi induced silencing of *SmDSR33* gene in wheat

(A) The encoding sequence of SmDSR33 and its deduced amino acid sequence. The sequences selected for construction of the RNAi vector are highlighted in yellow. (B) A schematic show of the *SmDSR33* expression cassette and position of *Ssp I* restriction enzyme. (C) Southern blot analysis of the transgenic wheat lines. Genomic DNA was digested with *Ssp*I and hybridized with a *SmDSR33* gene fragment with the expression cassette digested with *Ssp* I as a positive control. (D) Relative expression levels of *SmDSR33* of grain aphid fed on wild-type and transgenic wheat lines. The

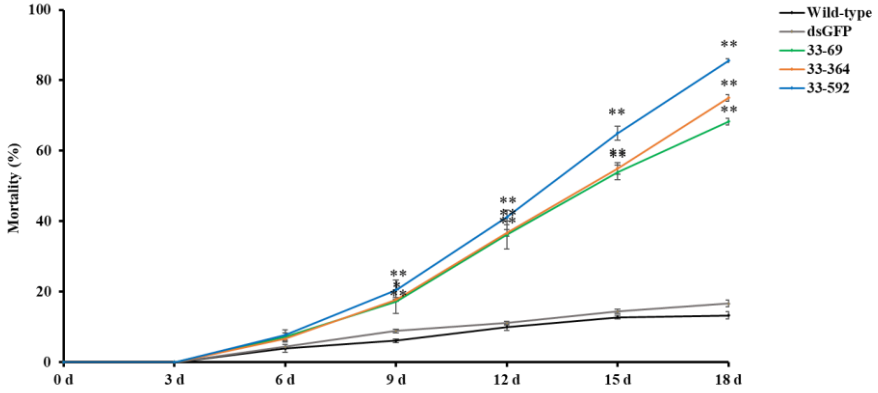
expression level of *SmDSR33* in the adult aphids fed on wild-type and different transgenic wheat lines after inoculation of one-day-old newborn nymphs, respectively. Values and error bars represent the mean and SEM of three independent biological replicates, each with a pool of 15 surviving individual aphids (Student's *t*-test, ** $P<0.01$)

IV.3.3. Fitness of the aphids fed on SmDSR33-dsRNA expressing transgenic wheat lines

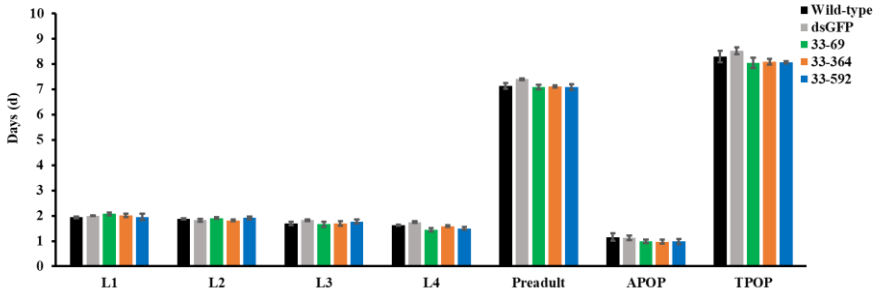
Fitness parameters including life cycle and mortality of aphids upon feeding on different transgenic lines were further investigated to evaluate the silencing impact of *SmDSR33*. The mortality rates of aphids fed on transgenic wheat lines significantly increased when compared to that of aphis fed on wild-type plants at 9 days after feeding (DAF), reaching more than 60% at 18 DAF (Figure 4-3A). We also monitored the development duration of aphids from the nymphal to imago stage. The adult preoviposition period (APOP) and the total preoviposition period (TPOP) of aphids showed no significant difference between host plant lines (Figure 4-3B). The aphid longevity fed on transgenic wheat lines was significantly shorter than on wild-type plants. Similarly, the adult longevity and reproductive period of aphids significantly decreased than wild-type ($P<0.01$) (Figure 4-3C). Consequently, in comparison with the wild-type plants, the aphid total production significantly decreased when fed on all three transgenic wheat lines ($P<0.01$) (Figure 4-3D), and the daily fecundity of aphids fed on 33-592 transgenic lines decreased at a significant level ($P<0.01$) (Figure 4-3D).

All of the population parameters, including the net reproductive rate (R_0), mean generation time (T), the intrinsic rates of increase (r_m) and doubling times of the population (DT), showed differences between grain aphids fed on transgenic lines and those on wild-type plants (Table 4-1). For example, the net reproductive rates (R_0) of aphids were significantly lower when fed on transgenic wheat lines. The mean generation time (T) of aphids fed on 33-592 line was significantly decreased than wild-type ($P<0.01$) (Table 4-1).

(A)



(B)



(C) (D)

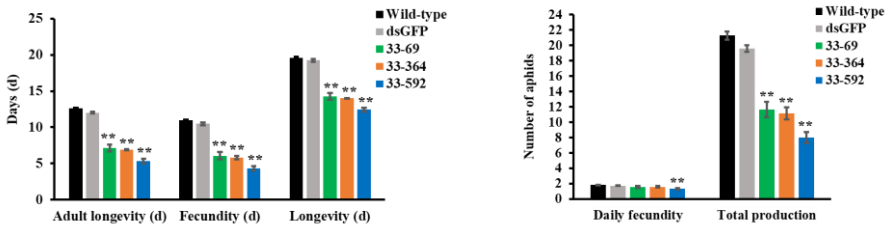


Figure 4-3 Fitness analysis of aphids fed on transgenic plants

(A) Mortality of aphids fed on wild-type and transgenic wheat lines. The mortality of aphids fed on wild-type and *dsSmDSR33* expression transgenic wheat lines. Twenty synchronous one-day-old nymphs were put into clip cages individually on transgenic

and wild-type wheat plants. All experiments were repeated three times. Values and bars represent the mean ± SEM (Student's *t*-test, * $P<0.05$, ** $P<0.01$). (B) The longevity of different stages, adult preoviposition period (APOP) and total preoviposition period (TPOP) of aphids fed on transgenic lines and wild-type control. (C) The adult longevity, fecundity and the total longevity of aphids fed on transgenic wheat lines and wild-type control. (D) The reproduction of aphids fed on transgenic wheat lines and the wild-type control. All experiments were repeated three times, each with 20 synchronous one-day-old nymphs. Values and bars represent the mean ± SEM (Student's *t*-test, * $P<0.05$, ** $P<0.01$).

Table 4-1 Life table parameters of grain aphids fed on wild-type and different transgenic wheat lines

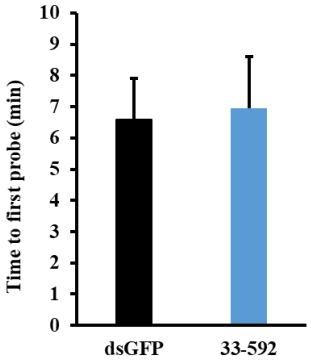
Parameters	Wild-type	33-69	33-364	33-592
R_0	21.27±0.52	11.32±1.08**	10.86±0.73**	7.94±0.77**
T	14.49±0.30	13.40±0.19*	13.38±0.16*	12.57±0.26**
r_m	0.21±0.01	0.18±0.01*	0.18±0.01*	0.16±0.01*
λ	1.24±0.01	1.20±0.01*	1.20±0.01*	1.18±0.01*
DT	3.29±0.08	3.86±0.16*	3.91±0.15*	4.25±0.23*

All data are expressed as means ± SEM based on 3 repeated experiments. R_0, net reproductive rate; r_m, the intrinsic rate of increase; λ, the finite rate of increase; T, the mean generation time; DT, Doubling time (day). Student's *t*-test, n=3, $^{*}P<0.05$, $^{**}P<0.01$.

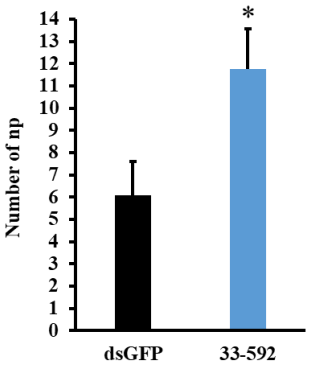
IV.3.4. Feeding behavior of aphids feeding on transgenic wheat plants

To investigate the feeding behavior of aphids, transgenic wheat line 33-592 was selected to perform electropenetrography (EPG) assays. As shown in Figure 4-4A-F, there was no difference between the aphids fed on *SmDSR33* and *dsGFP* wheat plants at time point of first probe activity. The number of non-probing (np) waveforms of *SmDSR33*-silenced aphids fed on 33-592 lines was significantly higher than on wild-type plants. Furthermore, the total duration of np waveforms and C phases of *SmDSR33*-silenced aphids was significantly increased compared to control. Finally, there was no difference in the duration of E1 waveforms, but did of phloem ingestion (E2) with a significant reduction for aphids on control plants. These results indicated that the feeding behavior of grain aphids was affected after *SmDSR33* silencing.

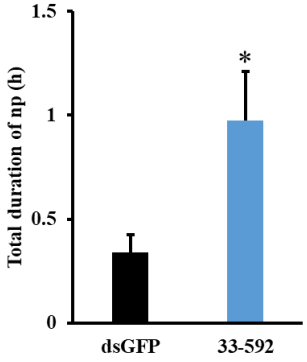
(A)


Time to first probe (min)
10
9
8
7
6
5
4
3
2
1
0
dsGFP
33-592

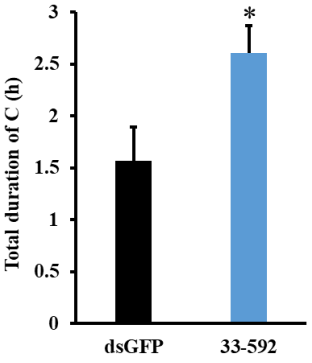
(B)


Number of np
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
*
dsGFP
33-592

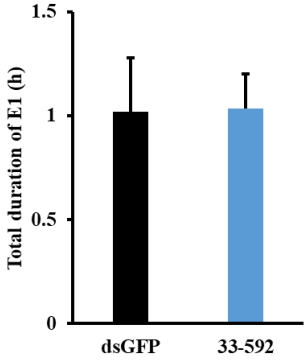
(C)


Total duration of np (h)
1.5
1
0.5
0
*
dsGFP
33-592

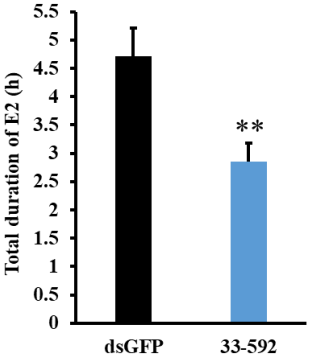
(D)


Total duration of C (h)
3
2.5
2
1.5
1
0.5
0
*
dsGFP
33-592

(E)


Total duration of E1 (h)
1.5
1
0.5
0
dsGFP
33-592

(F)


Total duration of E2 (h)
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
dsGFP
33-592

(G)

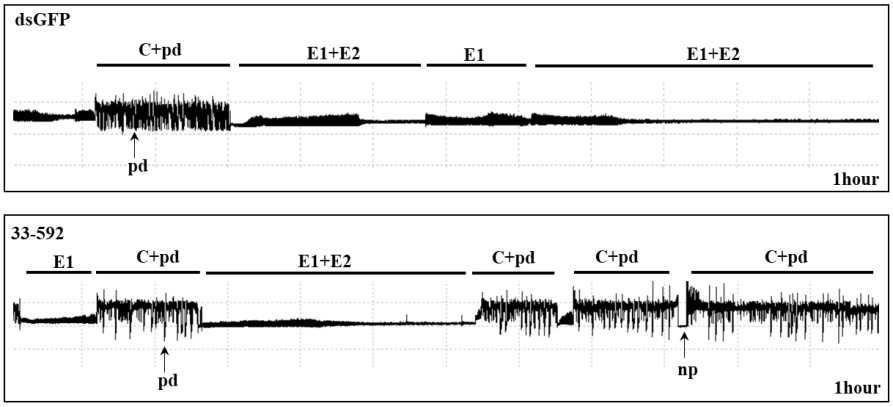


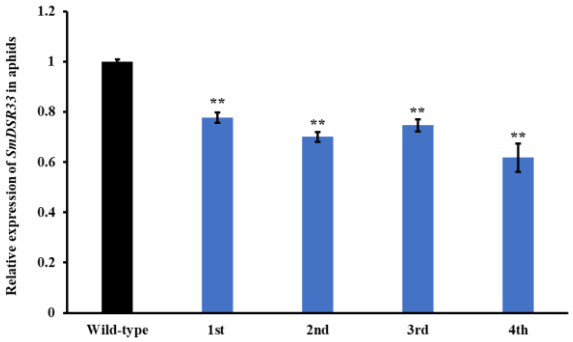
Figure 4-4 Effects of *SmDSR33* silencing on feeding behavior of *Sitobion miscanthi* based on EPG recordings

(A-F) Representative parameters of aphid feeding behavior. Non-probing (np), stylet probing (C), intracellular stylet puncture (pd), phloem salivation (E1), and phloem ingestion (E2). Data shown are mean ± SEM. Asterisks above bars indicate significant differences between controls and treatments (Student's *t*-test, * $p < 0.05$; ** $p < 0.01$). (G) Representative EPG waveforms of grain aphids feeding on dsGFP wheat plants and 33-592 transgenic wheat plants.

IV.3.5. Feeding on transgenic lines induces transgenerational silencing of SmDSR33 in aphids

Newborn nymphs produced in a parallel experiment were used to investigate potential transgenerational RNAi effects of *SmDSR33*. The expression levels of *SmDSR33* in the offspring of aphids fed on transgenic and wild-type plants was investigated subsequently. *SmDSR33* expression in grain aphids was suppressed in their offspring fed on wild-type plants (Figure 4-5). Aphid relative expression levels reached 77.71%, 70.04%, 74.63%, and 61.80% of control level in successive first to fourth generations (Figure 4-5A). Even after switching to wild-type plants, aphid offspring still exhibited higher mortality rates (Figure 4-5B).

(A)



(B)

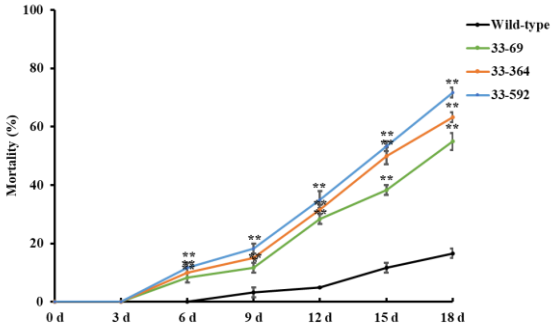


Figure 4-5 Transgenerational effect of *SmDSR33* silencing on aphid mortality rates

The one-day-old newborn nymphs of aphids feeding on transgenic wheat lines were transferred to fresh wild-type wheat plants and subsequently allowed to reproduce on wild-type.
(A) The *SmDSR33* transcript levels of adult aphids were determined in fourth successive aphid generations. (B) The mortality of the first generation of the offspring of aphids fed on transgenic lines at different time points after being switched to wild-type plants. Values and bars represent the mean ± SEM (Student's *t*-test, * $P<0.05$, ** $P<0.01$).

IV.4. Discussion

Aphids are phloem-feeding insects that secrete saliva effectors into plant cells to enable successful feeding (Wang et al., 2015b). Salivary proteins play important roles in the interaction of aphids with plants (Yang et al., 2018; Zhang et al., 2021). Engineering transgenic plants expressing dsRNA for insect pest management is an effective strategy in agricultural practice (Ghag, 2017). Plant-mediated RNAi has

been recognized as one of the most promising technologies to engineer insect-resistant crops, especially for wheat aphid control, which has great significance for food security, human health, and the agroecosystem in a global context (Sun et al., 2019; He, 2022).

Here, we identified a novel potential RNAi target gene (*SmDSR33*) from grain aphid, which had a high mortality due to the silencing of *SmDSR33* in grain aphid via artificial diet feeding assays (Wang et al., 2015a). *SmDSR33* was predicted as a gene encoding a secreted salivary protein which had a signal peptide and one predicted transmembrane helix (Figure 4-1A, Figure 4-S1). This result is in accordance with previous studies on salivary effectors. For example, ApC002 was predicted to be a signal peptide for an extracellular protein and the cleavage site was predicted between residues 23 and 24 (Mutti et al., 2008). A signal secretion peptide with cleavage sites either between Ala20 and Gln21 (SignalP) or between Ser22 and Arg23 (PSORT) was predicted in Armet (Wang et al., 2015b). A secretory signal peptide at the N-terminal of the protein ACYPI006346 was predicted, with the predicted cleavage site between residues 19 and 20 (Pan et al., 2015). The signal peptide of Sm9723 was constituted of the first 21 amino acids and the cleavage site was predicted between residues 21 and 22 (Zhang et al., 2022a). The signal peptide of Sg2204 was constituted of the first 25 amino acids and the cleavage site was predicted between residues 25 and 26 (Zhang et al., 2022b).

We then obtained stable transgenic wheat lines expressing dsRNA of *SmDSR33* in grain aphids. Significantly decreased fecundity, survival, and reproduction rates of aphids fed on transgenic wheat plants were observed than that of wild-type plants (Figure 4-3). Our results are in consistent with the plant-mediated RNAi experiments targeting salivary protein and effector encoding genes in aphids. For example, silencing the salivary protein gene *C002* reduced the reproduction and survival in the pea aphid (Mutti et al., 2006; Mutti et al., 2008). Silencing salivary proteins such as Mp10, Mp42, Mp56, Mp57, and Mp58 in tobacco caused reduced virulence and fecundity of green peach aphids (Bos et al., 2010; Elzinga et al., 2014; Rodriguez et al., 2014). Silencing *Sm9723* and *Sg2204* through a nanocarrier-mediated dsRNA delivery system negatively impacted aphid survival rates and fecundity of aphids (Zhang et al., 2022a; Zhang et al., 2022b).

We found that *SmDSR33* silencing increased the total duration of non-probing waveforms and C phases and decreased the duration of phloem ingestion (E2) (Figure 4-4). These results indicated that *SmDSR33* affected the feeding process and behavior of grain aphids. It was shown that interference of target genes could affect aphid feeding behavior. Knockdown of an effector protein Armet impeded the feeding behavior of pea aphids (Wang et al., 2015b). As an important multipeptide molecule, neuropeptide F (NPF) had been discovered in numerous insect species and regulated a variety of physiological activities. The probing time and total duration of phloem activity on broad bean plants were decreased when wingless adult pea aphids were injected with NPF dsRNA (Li et al., 2018). When feeding on *A. thaliana*, *Mp1* silencing decreased the fitness of green peach aphids. However, aphid feeding ability with *Mp1* silences was still retained (Wang et al., 2021). Plastid-mediated RNAi was also an efficient approach for aphid control. *M. persicae* exhibited different feeding

behaviors on nuclear-mediated RNAi transgenic plants and transplastomic-mediated RNAi transgenic plants (Dong et al., 2022). Feeding behavior of *S. miscanthi* and *S. graminum* were significantly impaired after knockdown of *Sm9723* and *Sg2204* (Zhang et al., 2022a; Zhang et al., 2022b).

According to previous studies on environmental RNAi, transgenerational silencing, also known as parental RNAi, in which the silencing effects of the respective target genes and survival rates could be significantly impacted in the offspring of the treated organism (Marré et al., 2016; Rechavi and Lev, 2017; Wang and Hunter, 2017). Our data showed that *SmDSR33* relative expression levels reached from 78 to 62% of control level in the following first to fourth successive generations (Figure 4-5). This result indicated that RNAi effect was persistent in grain aphids. This type of effect could last for several days, many weeks, few months, and even for multiple generations. With time and successive generations, the silencing effect decreased (Amdam et al., 2003; Jaubert-Possamai et al., 2007; Miller et al., 2012; Abdellatef et al., 2015). According to a previous study, parental RNAi may result from a specific dsRNA uptake mechanism or small amounts of incidentally incorporated dsRNA secondary amplification (Bucher et al., 2002). The phenomenon of telescoping generations existed in grain aphids, which means that the developing grandchildren are already carried by a parthenogenetic adult, may facilitate the transfer of siRNA/dsRNA to the subsequent generations in aphids (Abdellatef et al., 2015). Transgenerational silencing could also induce by small RNAs mediated epigenetic modifications (Castel and Martienssen, 2013). We observed the decreased silencing effect in 4th generation compared to that of 1st to 3rd generation. Our result was consistent with the study that the duration of the RNAi impact was doubled in nymphs whose mothers had been exposed to dsRNA-producing transgenic plants (12-14 days), which indicated that the RNAi effect may persist longer in nymphs than in their mothers (Coleman et al., 2015). This could be due to the fact that the stability of dsRNA in insects may be affected by the quantities of dsRNA, the lengths of the dsRNA fragments, the activities that degrade dsRNA, and the life stages of the target species (Griebler et al., 2008; Huvenne and Smagghe, 2010; Bolognesi et al., 2012; Miller et al., 2012; Abdellatef et al., 2015). Transgenerational gene silencing exhibited significant potential in RNAi-mediated pest control, although the molecular mechanisms in insect species remained to be elucidated.

In conclusion, we not only identified and characterized a novel RNAi target gene *SmDSR33*, which is a putative salivary secretion protein in grain aphids, but also revealed that targeted silencing of *SmDSR33* via plant-mediated RNAi significantly decreased the survival, fecundity, and total production of grain aphids, which consequently reduced aphid infestation on wheat plants. The altered feeding behavior and transgenerational RNAi silencing effects also minimized aphid infestation. As a result, our study demonstrated the significant potential of plant-mediated RNAi of an important putative salivary protein gene as a promising strategy for aphid control in crop plants in agricultural practice.

IV.5. References

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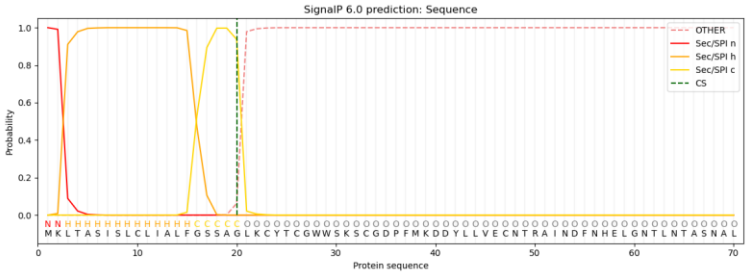
Supplementary Data

Contents

(A)



(B)

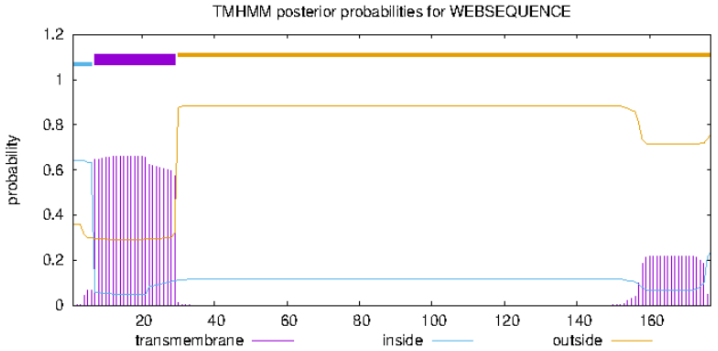


Figure 4-S1. The predicted signal peptide and transmembrane helices of SmDSR33

(A) The predicted signal peptide of SmDSR33. The cleavage site is between residues $S^{18}A^{19}G^{20}$-$L^{21}K^{22}$.
(B) The predicted transmembrane helix of SmDSR33.

Figure 4-S2. Phenotype of the aphids fed on *SmDSR33*-dsRNA expressing transgenic wheat line.

Phenotype of aphid resistance for wild-type (control) and 33-592 transgenic wheat plants. 10 synchronized one-day-old nymphs were placed on the leaf of plants. Photos were taken 12 days later.

Table 4-S1. The primer sets used in this study

Primer	Sequence (5′ to 3′)	Use of PCR products	Annealing (°C)	Fragment size (bp)
SmDSR33c-F	ATGAAGTTAACTGCATCAATATCGTTATG	Full length cDNA cloning	56	534
SmDSR33c-R	TTAGTGCTGTAGCCTGTAGAACAG			
SmDSR33S-F	ATCCCCGGGGGATCCACTAGTGTTTGGATCG AGTGCTGGTCT	Construct vector for RNAi	59	483
SmDSR33E-R	GCCTCCCAGATCGATTCGATATCGACCGCCG AAGACTTCAACG			
SmDSR33H-F	GGGGCAGACTCCCGTTTGTTAACGACCGCCG AAGACTTCAACG	Construct vector for RNAi	59	483
SmDSR33S-R	CGATCGGGGAAATTCGAGCTCGTTTGGATCG AGTGCTGGTCT			
qactin-F	CGGTTCAAAAACCCAAACCAG	Internal control of qRT-PCR for aphid	56	260
qactin-R	TGGTGATGATTCCCGTGTTC			
Rps27-F	TGTGAAGACGTTGACTGGGAAA	Internal control of qRT-PCR for aphid	57	114
Rps27-R	CGTTGCTGATCCGGAGGAATAC			
SmDSR33S-F	CGACGAGTCTAACGGACACC	Southern blot	58	456
SmDSR33S-R	CCCTAACCATGGACCGGAAC			
SmDSR33q-F	CCCTGTTTGGATCGAGTGCT	Detect *SmDSR33* in aphids	57	150
SmDSR33q-R	GGGTGTTTCCTAGTTCGTGGT			

Ubi-F ADH1-R	GCCTTCATACGCTATTTATTTGCTTGGTAC GGAGTCTGCCCCTAAGACAGATAAGC	Detect *SmDSR33* dsRNA in transgenic wheat	60	687
ADH1-F Nos-R	CTGGGAGGCCAAGGTATCTAATCAGC CGCAAGACCGGCAACAGGATTC	Detect *SmDSR33* dsRNA in transgenic wheat	60	647

5

Chapter V Increasing yield potential through manipulating of an ARE1 ortholog related to nitrogen use efficiency in wheat by CRISPR/Cas9

This work is an original contribution, adapted from:

Zhang, J., Zhang, H., Li, S., Li, J., Yan, L., & Xia, L. (2021). Increasing yield potential through manipulating of an ARE1 ortholog related to nitrogen use efficiency in wheat by CRISPR/Cas9. Journal of Integrative Plant Biology, 63(9), 1649-1663.

Abstract

Wheat (*Triticum aestivum* L.) is a staple food crop consumed by more than 30% of world population. Nitrogen (N) fertilizer has been applied broadly in agriculture practice to improve wheat yield to meet the growing demands for food production. However, undue N fertilizer application and the low N use efficiency (NUE) of modern wheat varieties are aggravating environmental pollution and ecological deterioration. Under nitrogen-limiting conditions, the rice (*Oryza sativa* L.) *abnormal cytokinin response1 repressor1* (*are1*) mutant exhibits increased NUE, delayed senescence and consequently, increased grain yield. However, the function of *ARE1* ortholog in wheat remains unknown. Here, we isolated and characterized three *TaARE1* homoeologs from the elite Chinese winter wheat cultivar ZhengMai 7698. We then used CRISPR/Cas9-mediated targeted mutagenesis to generate a series of mutant lines either with partial or triple-null *taare1* alleles. All mutant lines showed enhanced tolerance to N starvation, and showed delayed senescence and increased grain yield in field conditions. In particular, the *AABBdd* and *aabbDD* mutant lines exhibited delayed senescence and significantly increased grain yield without growth defects compared to the wild-type control. Together, our results underscore the potential to manipulate *ARE1* orthologs through gene editing for breeding of high-yield wheat as well as other cereal crops with improved NUE.

V.1. Introduction

Bread wheat (*Triticum aestivum* L., 2n = 42, AABBDD) is a staple food crop consumed by more than 30% of the world's population. Nitrogen (N) fertilizer is applied broadly in agriculture to improve wheat yield to meet the growing demands for food production. However, the widely cultivated semi-dwarf 'Green Revolution' wheat varieties with increased yield potential resulted in contingent effects of reduced N-use efficiency (NUE); in fact, most modern wheat varieties only absorb less than 40% of the supplied N (Raun and Johnson, 1999; Good and Beatty, 2011; Kant et al., 2011). The remaining N is released to the environment through leaching and volatilization (Good and Beatty., 2011). In consequence, the low NUE of wheat and undue N fertilizer applications are aggravating environmental pollution and ecological deterioration (Li et al., 2021a). Moreover, the additional N inputs increased the fecundity of aphid on rice and wheat, and stimulated the development of English grain aphid and bird cherry-oat aphid (Jahn et al., 2005; Khan and Port, 2008; Gash, 2012; Carreras Navarro et al., 2020). Therefore, improving NUE will helpful to the sustainability of wheat production as well as food safety and security.

Ongoing studies have made some impressive progresses in understanding and increasing NUE in crop plants (Hu et al., 2015; Li et al., 2018; Wang et al., 2018; Gao et al., 2019; Tang and Ye, 2019; Zhang et al., 2021). NUE depends on multiple physiological processes including N uptake, assimilation, metabolism, allocation, and remobilization. Plants take up nitrate (NO_3^-) and ammonium (NH_4^+) via the plasma membrane-localized nitrate transporters (NRTs) and ammonium transporters (AMTs) (Williams and Miller, 2001; Miller et al., 2007). Nitrate is converted to ammonium by nitrate reductase (NR) and nitrite reductase (NiR). Then, ammonium is assimilated into amino acids via the glutamine 2-oxoglutarate amidotransferase (GOGAT) cycle and glutamine synthetase (GS) (Yang et al., 2016; Lv et al., 2021). Ferredoxin-dependent glutamate 2-oxoglutarate aminotransferase (Fd-GOGAT) and nicotinamide adenine dinucleotide-dependent glutamate 2-oxoglutarate aminotransferase (NADH-GOGAT) are two different forms of GOGAT that use ferredoxin and NADH as the electron donors, respectively. Fd-GOGAT is primarily located in leaf chloroplasts, whereas NADH-GOGAT is predominantly localized in non-photosynthetic plastids (Temple et al., 1998; Forde and Lea, 2007). During N remobilization, *Fd-GOGAT* plays an important role in ammonium reassimilation in photosynthetic tissues and *NADH-GOGAT* assimilates ammonium in non-photosynthetic cells (Lea & Miflin. 2003; Quraishi et al., 2011).

Genetic studies in rice have identified additional factors involved in NUE. The *abnormal cytokinin response1 repressor1* (*are1*) mutant was identified as a suppressor of a rice *fd-gogat* mutant defective in N assimilation. Loss-of-function mutations in rice *ARE1* result in delayed senescence, enhanced NUE, and increased grain yield under N-limiting condition (Wang and Nian, 2018). The rice transcription factor *Grain number*, *plant height*, and *heading date 7* (*Ghd7*) directly represses the expression of *ARE1* to positively regulate NUE and grain yield. The *Ghd7-ARE1* regulatory module has undergone diversifying selection. Combining the elite alleles of *Ghd7* and *ARE1* significantly improves NUE and grain yield under low N conditions (Wang et al.,

2021). These results indicate that *ARE1* orthologs play important roles in NUE and might be a good candidate to improve NUE in wheat and other major cereal crops. However, the *ARE1* ortholog in wheat has not been functionally characterized.

Although the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) has been extensively applied for functional genomics studies and crop improvement (Ma et al., 2015; Li et al., 2019; Biswas et al., 2020; Liu et al., 2020; Zhan et al., 2021), generating targeted mutations in common wheat remains challenging due to its complex hexaploid genome. So far, only a few agronomically important traits in wheat have been successfully improved through CRISPR/Cas9 such as grain size and weight (Wang et al., 2018c; Zhang et al., 2018; Wang et al., 2019; Zhang et al., 2019), decreased gliadin content (Sánchez-León et al., 2018), increased resistant starch (Li et al., 2021b), increased powdery mildew resistance (Wang et al., 2014), and improved tolerance to pre-harvest sprouting (Abe et al., 2019). CRISPR/Cas9 has also been used in haploid induction (Liu et al., 2020; Lv et al., 2020), male sterility (Okada et al., 2019), and multiplex gene editing (Luo et al., 2021) in wheat. To date, increasing wheat NUE through manipulating key genes involved in N uptake and assimilation by genome editing has not been documented.

In the context of global climate change, increasing population, and diminishing farmland, it is essential to resilient wheat production by improving NEU, decreasing the N application and maintaining the yield stability. Here, we isolated and functionally characterized a rice ARE1 ortholog in the elite Chinese wheat variety cultivar ZhengMai 7698 (ZM). We analyzed the subcellular localization and spatial expression patterns of the three *TaARE1* homoeologs in wheat. We then generated a series of mutant wheat lines through CRISPR/Cas9-mediated targeted mutagenesis of *TaARE1*. We observed the development of roots in different mutant lines in response to N starvation and supply at different concentrations under hydroponic conditions. Among these mutant lines, the *AABBdd* and *aabbDD* mutant lines, rather than the *aabbdd* triple null mutant line, exhibited significantly enhanced NUE, delayed senescence, and increased grain yield without growth penalty compared to the wild-type control in field conditions. Thus, we successfully generated novel wheat germplasm with enhanced NUE and improved grain yield. Our results demonstrated that harnessing *ARE1* orthologs through gene editing could be an alternative way to improve NUE and thus enhance grain yield of wheat and other major cereal crops.

V.2. Materials and methods

V.2.1. Identification of ARE1 genes in the wheat genome databases and phylogenetic analysis

Based on blast analysis of the *ARE1* gene in rice (Os08g0224300), the *ARE1* ortholog in wheat variety cultivar ZM was identified from the databases of the International Wheat Genome Sequencing Consortium, EnsemblPlants (http://plants.ensembl.org/) and a BAC library of an elite Chinese wheat variety AiKang 58 (AK). The full-length cDNA sequences of the three homoeologs of *TaARE1* were obtained by PCR using

ZM cDNA as template with primer set cTaARE1-F/cTaARE1-R. The genomic structure of the *TaARE1* was illustrated by the Gene Structure Display Server (GSDS; http://gsds.cbi.pku.edu.cn/). The deduced ARE1 amino acid sequences of TaARE1-A (KAF7095826.1), TaARE1-B (KAF7101772.1), TaARE1-D (KAF7108895.1), HvARE1 (AK375792), OsARE1 (BAT04386), GmARE1 (XM_021451333.1), ZmARE1 (NM_001139043.2) and AtARE1 (AEE85850) were aligned by Clustal W (http://www.clustal.org), and then submitted to MEGAX constructing the phylogenetic tree using the maximum-likelihood method. The number of bootstrap replications was 100.

V.2.2. Subcellular localization of TaARE1 in wheat protoplast

In order to analysis the subcellular localization of TaARE1, pC1390-TaARE1-A-YFP vector was constructed. Firstly, the primer set YTaARE1-F/ YTaARE1-R was used to obtain a 1305 bp PCR fragment using ZM cDNA of *TaARE1-A* as a template. Then the fragment was inserted into *Pst* I sites of pC1390-YFP vector to obtain pC1390-TaARE1-A-YFP vector. The primer sets are listed in Table 5-S1.

The common wheat cultivar ZM was used for protoplast isolation. Wheat seedlings were grown at 25°C with a 16 h/8 h light/dark condition for 10 d. Stem tissues were collected and cut into 0.5-1.0-mm strips. Then, the strips were transferred into 0.6 M mannitol for 10 min, followed by incubation in an enzyme solution for 4 h at 28°C with gentle shaking (40-60 rpm) in darkness. Protoplasts were washed with W5 solution, and collected using a 150 μm mesh filter, followed by centrifugation at 150 *g* for 3 min. The collected protoplasts were re-suspended in W5 solution, followed by centrifugation at 150 *g* for 3 min. Afterwards, protoplasts were resuspended in MMG solution at a final concentration of 1-5×10^6 cells/mL. For wheat transformation, 5 μg plasmid DNA pC1390-YFP and pC1390-TaARE1-A-YFP were transformed into 100 μL fresh protoplasts by PEG-mediated transformation. The transformation process was stop by gently adding 1.5 mL W5 solution. The protoplasts were centrifuged at 150 *g* for 3 min, followed by gently re-suspended in W5 solution. Finally, the protoplasts were incubated at 25°C for 12-16 h, and then were visualized and scanned under a confocal laser scanning microscope (LSM700).

V.2.3. Reverse transcription quantitative PCR analysis of TaARE1 homoeologs at the grain-filling stage

Total RNAs were extracted from root, peduncle, top second leaf, flag leaf, flag leaf sheath, spike and grain at the grain-filling stage using TransZol Up (TransGen Biotech, Beijing, China). Roots of seedlings under different treatments were also extracted using TransZol Up (TransGen Biotech, Beijing, China). The cDNA was synthesized using TransScript®First-Strand cDNA Synthesis SuperMix (Tiangen, Beijing, China) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) assay was performed using SuperReal PreMix Plus (SYBR Green) (Tiangen, Beijing, China) in a CFX96TM Real-Time System (BIO-RAD, US). The wheat cell division control protein (CDC) gene was selected as an internal control for normalization. Sequences of all primers used in qRT-PCR are listed in Table 5-S1. All qRT-PCR

experiments were repeated in triplicate, and the mean value generated from the reference gene was used to calculate the relative gene expression of the respective target gene using the $2^{-\Delta\Delta CT}$ method.

V.2.4. Knockout vectors construction

The knockout vectors used in this study were based on pCXUN-Ubi-Cas9-Nos-35S-hptII-Nos vector which contained a codon-optimized Cas9 driven by the maize (*Zea mays L.*) *ubiquitin* gene promoter. The backbone of pCXUN-Ubi-Cas9-Nos-35S-hptII-Nos contained a *hptII* for callus selection. The *Aar* I and *Sac* I sites in pCXUN-Cas9 were used for introducing the annealed gRNA oligos and the gRNA expression cassettes, respectively.

Two primer sets KTaARE1-F1/ KTaARE1-R1 and KTaARE1-F2/ KTaARE1-R2 were used to obtain gRNA1 and gRNA2 oligos by annealing PCR. The annealed gRNA1 and gRNA2 oligos were cloned into the linearized pCXUN-Cas9 with *Aar* I, by using pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China). Then the vectors pCXUN-Ubi-Cas9-Nos-TaU6-gRNA1-35S-hptII-Nos and pCXUN-Ubi-Cas9-Nos-TaU6-gRNA2-35S-hptII-Nos were constructed. For the construction containing two gRNAs, the gRNA2 cassette was amplified by the primer set SacI-U6-F/ SacI-U6-R using the plasmid of pCXUN-Ubi-Cas9-Nos-TaU6-gRNA2-35S-hptII-Nos as template. The gRNA2 cassette was cloned into the *Sac* I site of the linearized pCXUN-Ubi-Cas9-Nos-TaU6-gRNA1-35S-hptII-Nos using pEASY-Uni Seamless Cloning and Assembly Kit, and the vector pCXUN-Ubi-Cas9-Nos-TaU6- gRNA1-TaU6-gRNA2-35S-hptII-Nos was constructed. All primer sets used in this study are listed in Table 5-S1.

V.2.5. Wheat transformation

The immature wheat embryos were selected for wheat transformation. Three constructs were introduced into the immature embryos of ZM via biolistic transformation, respectively, following the previous protocol (Altpeter et al., 1996). Afterwards, the embryos were put on resting medium for 5 d and then selected on medium containing 15 mg/L and 30 mg/L hygromycin for 10 d, respectively. The vigorously grown calli were transferred to the regeneration medium (15 mg/L hygromycin) to generate green plants. Finally, the regenerated green plants were transferred to rooting medium.

V.2.6. Molecular characterization of different mutant lines

Genomic DNA from leaf tissue was extracted using a DNA Quick Plant System (Tiangen, Beijing, China). PCR amplification was performed using FastPfu (TransGen Biotech, Beijing, China) with 50 ng of genomic DNA as template. The primer set Cas9-F/Cas9-R was designed for *Cas9* detection, and the genome specific primer sets TaARE1-A-F/TaARE1-A-R was designed to amplified gRNA1 and gRNA2 of A genome, TaARE1-B1-F/TaARE1-B1-R and TaARE1-D1-F/TaARE1-D1-R were designed to amplified gRNA1, the genome specific primer sets TaARE1-B2-F/TaARE1-B2-R and TaARE1-D2-F/TaARE1-D2-R were designed to amplified

for gRNA2. The genome specific primer sets TaARE1-A-F/TaARE1-A-R, TaARE1-B1-F/TaARE1-B2-R, TaARE1-D1-F/TaARE1-D2-R were designed to amplify for gRNA1 and gRNA2 to detect whether there was large fragment deletion. For the *Cas9* positive wheat lines, the PCR products amplified for gRNA1 were digested with T7 Endonuclease I (T7E1) and the PCR products amplified for gRNA2 were digested with *Bts* CI. Then the undigested bands with *Bts* CI were recovered and directly sequenced to screen mutant plants in *TaARE1*. And the PCR products which can be digested by T7EI were also sequenced to screen *TaARE1* mutant plants. The sequence chromatograms were analyzed by a web-based tool (http://dsdecode.scgene.com/) to check the genotype and zygosity of the tested plants (Liu et al., 2015). Some PCR products were also cloned into the pEasy-Blunt zero vector (TransGen Biotech, Beijing, China), and at least 10 positive colonies for each sample were sequenced. The primer sets are listed in Table 5-S1.

V.2.7. Off-target analysis

We selected two and three potential off-target sites to investigate the off-target effects based on the prediction of the WheatCrispr (https://crispr.bioinfo.nrc.ca/WheatCrispr/) for target 1 and target 2, individually (Table 5-S2). Site-specific genomic PCR and Sanger sequencing were used to determine the off-target effects. The primer sets are listed in Table 5-S1.

V.2.8. Hydroponic culture

taare1 mutant lines in T_2 generation and wild-type were used for N treatment in hydroponic culture. For hydroponic culture, seeds were surface-sterilized with 70% ethanol for 30 s and then washed with sterile water four times. Sterilized seeds were germinated in petri dishes for 3 d, and then the seeds with residual endosperm removed were transferred to the hydroponic culture solution and fresh solution was changed every 2 d. The hydroponic culture solution contained variable concentrations of NH_4NO_3 (0 mM, 0.5 mM, 1.0 mM or 1.5 mM), 2.5 mM K_2SO_4, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 0.5 mM KI, 4 mM $CaCl_2$, 0.1 mM Fe-EDTA, 0.1 mM H_3BO_3, 0.1 mM $MnSO_4$, 0.03 mM $ZnSO_4$, 1×10^{-4} mM $CuSO_4$, 0.01 mM Na_2MoO_4, and 1×10^{-4} mM $CoCl_2$, pH 6.0. Wheat seedlings were cultured in a growth chamber under a 16 h/8 h light/dark and 22°C/18°C day/night conditions. When transferring between hydroponic culture solutions containing different concentrations of NH_4NO_3, seedlings were washed in distill water for 3-5 times.

V.2.9. RT-qPCR analysis of key genes involved in N transport and assimilation

taare1 mutant lines in T_2 generation and wild-type were cultured in different hydroponic solutions as described above. After two weeks, seedlings in 1.5 mM NH_4NO_3 hydroponic culture solutions were transferred into N-free solution for 24 h of a N deprivation treatment. Roots of *taare1* mutant lines and wild-type were collected at 0 h, 0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h and 24 h after N deprivation treatment. Total RNAs extraction, first-strand cDNA synthesis and qRT-PCR assay were

performed as described above. Four genes *TaAMT1*;*3b*, *TaNRT2.1, TaGS1;1* and *TaNADH-GOGAT* involved in N transport and assimilation were selected to evaluate the expression patterns of these genes in the roots of mutant lines and wild-type upon N deprivation.

V.2.10. Root morphological analysis

Wheat seedlings grown for 5 days in a nutrient solution that contained 0 mM NH_4NO_3, 0.5 mM NH_4NO_3, 1.0 mM NH_4NO_3 and 1.5 mM NH_4NO_3. The primary root tip (1.0 cm) was taken manually from each individual root, using a razor blade to investigate the morphology and the cross-section structure of the elongation zone in primary roots. Root cross sections of 0.5 cm thickness (1.0-1.5 cm distant from the primary root tip) were also taken to observe the diameter of root cross sections. The cross sections were stained for 1 min with Methylene blue solution (0.1% [w/v] in distilled water) before being imaged with Zeiss Axio Imager Z2 microscope.

Roots were collected from *taare1* mutant and wild type wheat seedlings, which grown for 12 d in a nutrient solution containing 0 mM and 1.5 mM nitrate, individually. The root morphological parameters were analyzed with WinRHIZO software developed by Regent Instruments Canada Inc. (Ottawa, ON, Canada). In terms of root and shoot ratio, dry weight of roots and shoots were measured under different hydroponic culture solution for wild-type and *taare1* mutant seedlings after 20 d.

V.2.11. Measurement of chlorophyll content

The total chlorophyll contents in the first leaf of 20-d-old wild-type and *taare1* mutant lines under different hydroponic culture solutions were measured according to a published protocol (Lichtenthaler, 1987). Chlorophyll was extracted with 100 % ice-cold acetone and the chlorophyll content was calculated spectrophotometrically based on the absorbance of the supernatant at 644.8 nm and 661.6 nm. Samples were diluted to an optimal concentration before measuring using a spectrophotometer (Ultrospec 7000, Biochrom).

V.2.12. Field experiments

The field experiment was conducted at the experimental station of the Chinese Academy of Agricultural Sciences (CAAS) in Shunyi District, Beijing. The wild-type and T_2 wheat mutant lines derived from the homozygous T_1 plants were used in the field experiment. Seeds were sown at the beginning of October, and plants were harvested in early June next year. For each replicate of a mutant line, 45 seeds were sown in three 1.5-m-long rows, and the rows were spaced 25 cm apart. In general, 115 kg ha^{-1} of diamine phosphate and 150 kg ha^{-1} of urea were applied before sowing, and 150 kg ha^{-1} of urea was applied at the jointing stage. Other field managements were in consistent with local cultivation practices of wheat varieties in the field. For each line, the 1,000-grain weight was determined according to the dry weight of 500 dried grains with at least three replicates. Grain dimensions of the matured seeds from the primary spike were measured using an electronic digital display Vernier caliper in millimeter. In addition, plant heights were recorded using a ruler in centimeter. The

tiller number, spikelet number and grain number of per main spike were recorded for 10 representative plants for each mutant line. Spike lengths were also measured using a ruler in centimeter from the base of the ear to the tip of the apical spikelet (excluding awns). The relative chlorophyll content in flag leaves of plants grown in the field was determined by a soil-plant analysis development (SPAD) value measured by an SPAD-502 Plus chlorophyll meter (Konica Minolta, Japan) followed by the manufacturer's instructions.

V.2.13. Statistical analysis

The two-tailed Student's *t*-test was used to assess the differences between the wild-type and *taare1* mutant lines. Results with a corresponding probability value of $P < 0.05$ and $P < 0.01$ were considered to be statistically significant and very significant, respectively. For each treatment, the standard deviation (SD) of the mean was calculated based on at least three biological replicates. For the $\chi 2$ test, $P > 0.05$ were considered to be very good agreement with expected segregation ratio.

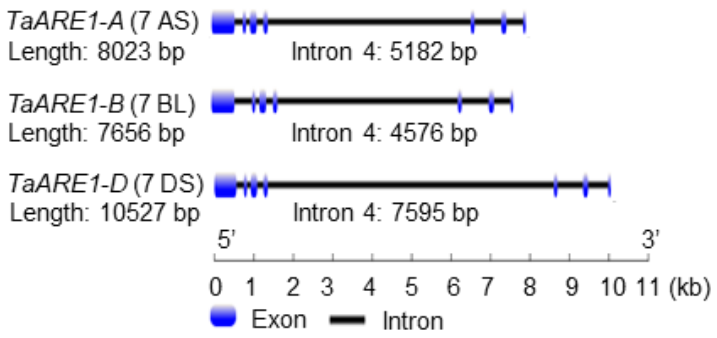
V.3. Results

V.3.1. Cloning and characterization of TaARE1

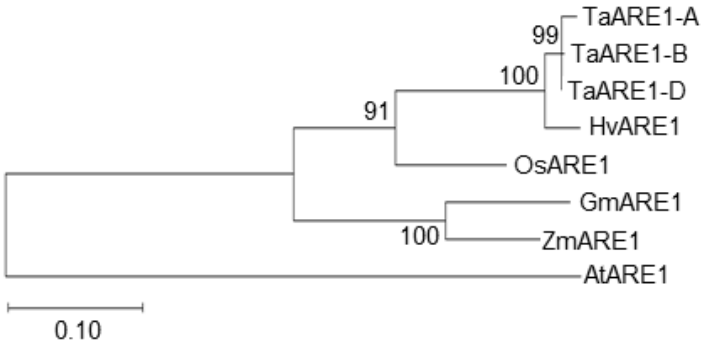
We identified a TaARE1 ortholog in the elite Chinese winter wheat cultivar ZM using the amino acid and nucleotide sequences of OsARE1 (Os08g0224300) to conduct BLAST searches against the databases of the International Wheat Genome Sequencing Consortium, EnsemblPlants (http://plants.ensembl.org/), and a BAC library of the elite Chinese wheat variety AiKang 58 (AK). The three homoeologs *TaARE1* genes are located on chromosomes 7AS (*TaARE1-A*, A subgenome short arm), 7BL (*TaARE1-B*, B subgenome long arm) and 7DS (*TaARE1-D*, D subgenome short arm). We also isolated their full-length cDNA sequences. The genomic sequences of the three *TaARE1* homoeologs consisted of 7 exons and 6 introns (Figure 5-1A). The cDNA sequences of the three *TaARE1* homoeologs were all 1266 bp in length, sharing a common conserved domain. Only 19 single-nucleotide polymorphisms (SNPs) differences exist among the three homoeologs (Figure 5-S1). We constructed a phylogenetic tree based on the full-length predicted amino acid sequences of the three TaARE1 homoeologs and other plants, and the phylogenetic analysis showed that TaARE1-A, TaARE1-B, and TaARE1-D are closely related to HvARE1 in barley (*Hordeum vulgare*) (Figure 5-1B). To examine the subcellular localization of the TaARE1 proteins, we transiently expressed a fusion of TaARE1-A to yellow fluorescent protein (YFP). When transiently expressed in wheat protoplasts, TaARE1-A-YFP localized in chloroplasts, indicating that TaARE1-A is a chloroplast-localized protein (Figure 5-1C). To analyze the expression profile of the three *TaARE1* homoeologs, we designed specific primers for *TaARE1-A*, *TaARE1-B* and *TaARE1-D* and used reverse transcription quantitative PCR (RT-qPCR) to measure their transcript levels in different tissues at the grain-filling stage. The result showed that *TaARE1-A* had relatively higher expression level compared with *TaARE1-B* and *TaARE1-D* (Figure 5-1D). In addition, the three *TaARE1* homoeologs were expressed in all tissues;

however, relatively higher expression levels of *TaARE1-B* and *TaARE1-D* were detected in roots and grains at the grain-filling stage, respectively (Figure 5-1D).

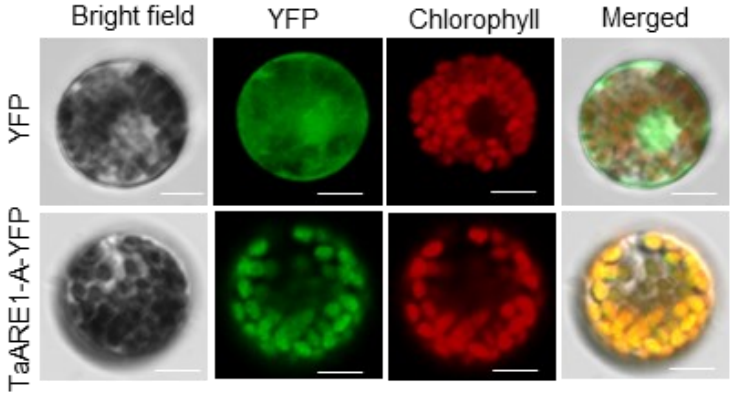
(A)



(B)



(C)



(D)

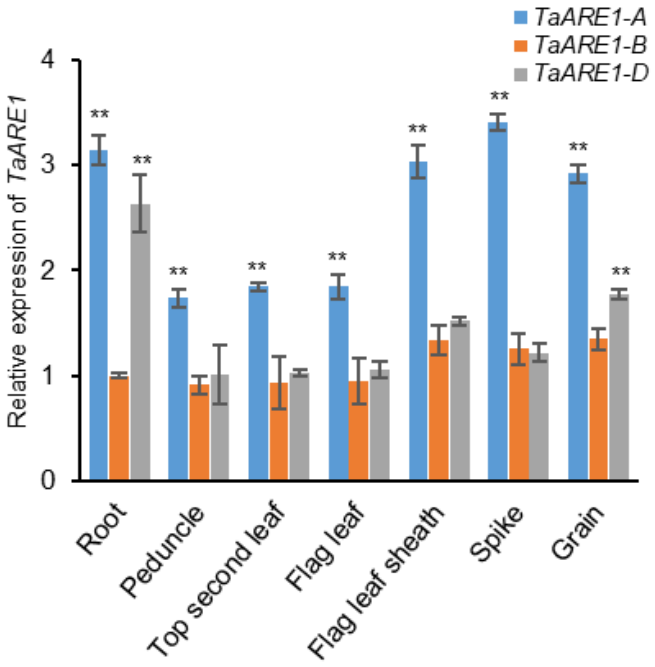


Figure 5-1 Characterization of *TaARE1*

(A) Gene structure of *TaARE1-A*, *TaARE1-B* and *TaARE1-D*. Exons and introns are shown as blue boxes and black lines, respectively. The full-length genomic sequence of TaARE1-A is 8023 bp, containing 7 exons (exon 1: 588 bp, exon 2: 86 bp, exon 3: 174 bp, exon 4: 115 bp, exon 5: 98 bp, exon 6: 136 bp, exon 7: 69 bp) and 6 introns (intron 1: 206 bp, intron 2: 98 bp, intron 3:169 bp, intron 4: 5182 bp, intron 5: 680 bp, intron 6: 422 bp). The full-length genomic sequence of TaARE1-B is 7656 bp, containing 7 exons (The length of each exon is same as TaARE1-A) and 6 introns (intron 1: 444 bp, intron 2: 98 bp, intron 3:169 bp, intron 4: 4576 bp, intron 5: 683 bp, intron 6: 420 bp). The full-length genomic sequence of TaARE1-D is 10527 bp, containing 7 exons (The length of each exon is same as TaARE1-A) and 6 introns (intron 1: 191 bp, intron 2: 98 bp, intron 3:166 bp, intron 4: 7595 bp, intron 5: 677 bp, intron 6: 534 bp). (B) Phylogenetic tree of ARE1 and its homoelogs in plants. GenBank accession number and transcript assembly number for each sequence used in the tree are listed below: TaARE1-A (TraesCS7A02G286400, KAF7095826.1), TaARE1-B (TraesCS7B02G196800, KAF7101772.1), TaARE1-D (TraesCS7D02G283700, KAF7108895.1); HvARE1 (HORVU7 Hv1G063720, AK375792), OsARE1 (Os08g0224300, BAT04386), GmARE1 (SORBI_3001G536901, XM_021451333.1), ZmARE1 (Zm00001d048524, NM_001139043.2) and AtARE1 (AT4G31040, AEE85850). Sequences of the ARE1 proteins were aligned with Clustal W, and then submitted to MEGAX to construct the

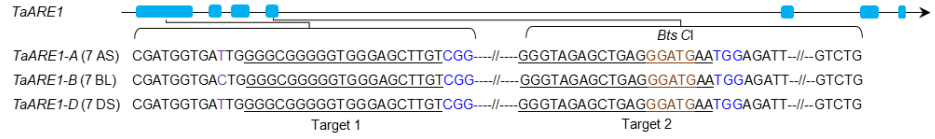
phylogenetic trees based on the maximum-likelihood method. The number of bootstrap replications was 100. (C) Subcellular localization of TaARE1. Analysis of subcellular localization of TaARE1- A-YFP (yellow fluorescent protein) protein in wheat protoplasts (Scale bars=10 μm). (D) The relative expression levels of TaARE1. Analysis of the TaARE1 expression in various organs at the grain-filling stage by qRT-PCR. Data presented are mean values of 3 technical replicates with SD (n = 3). The data was compared to that of TaARE1-B using the two-tailed Student's t test; *significant at $P < 0.05$, **significant at $P < 0.01$.

V.3.2. CRISPR/Cas9-mediated targeted mutagenesis of TaARE1 in wheat

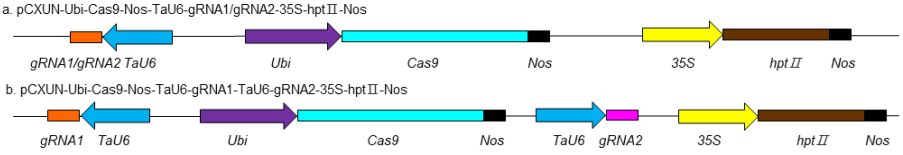
To examine the function of the TaARE1 homoeologs, we used CRISPR/Cas9 to generate loss-of-function mutants. To this end, we designed two gRNAs targeting the conserved sequences in the first exon and the fourth exon of the *TaARE1-A*, *TaARE1-B*, and *TaARE1-D* (Figure 5-2A). The gRNA2 contained a *Bts* CI restriction site that was used for screening mutants through a polymerase chain reaction-based restriction enzyme (PCR/RE) digestion assay (Figure 5-2A). The gRNA1 and gRNA2 cassettes driven by the *TaU6* promoter were cloned into the vector pCXUN-Ubi-Cas9-Nos-35S-hptII-Nos either alone or simultaneously (Figure 5-2B). Then, these three CRISPR/Cas9 vectors were separately transformed into immature embryos of ZM via particle bombardment. The regenerated wheat plants were recovered after two rounds of hygromycin selection and one round of regeneration. We identified one edited plant from eleven transgenic plants for gRNA1, one edited plant from nine transgenic plants for gRNA2 and one edited plant from eight transgenic plants for gRNA1 and gRNA2 (Figure 5-2C). The genome-specific primers are listed in Table 5-S1.

To investigate the off-target effects in these mutant lines, we identified 2-3 predicted potential off-target sites of these two gRNAs based on the WheatCrispr (https://crispr.bioinfo.nrc.ca/WheatCrispr/). We then used genome-specific PCR combined with Sanger sequencing to detect the potential off-target mutations. No mutations were detected at the tested putative off-target sites in these mutant lines (Table 5-S2).

(A)



(B)



(C)

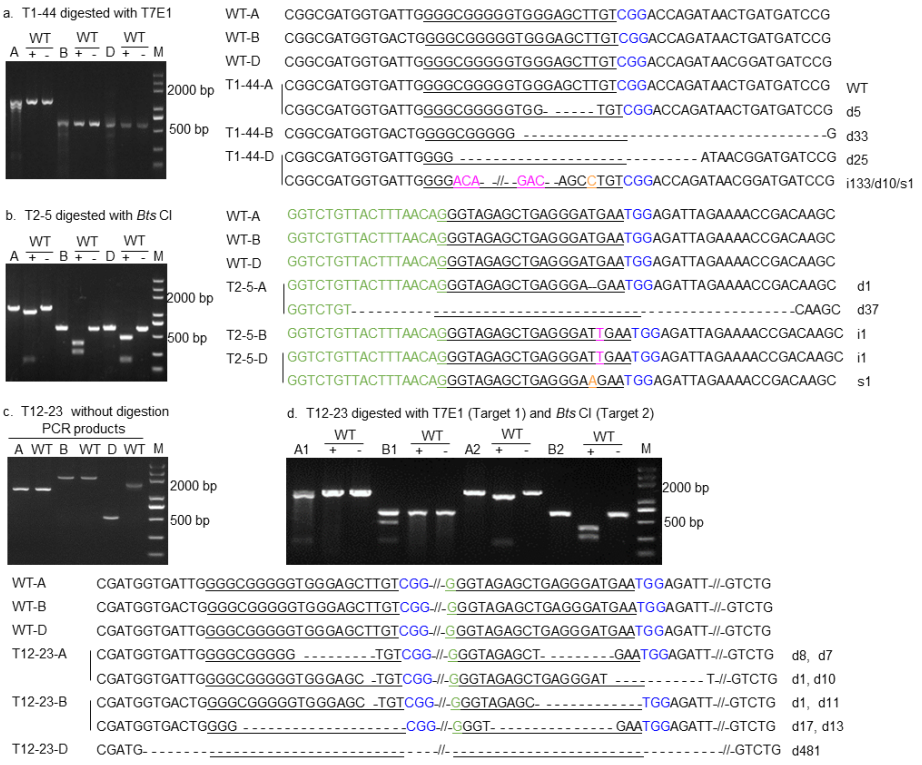


Figure 5-2 CRISPR/Cas9 induced targeted mutagenesis of *TaARE1* gene in wheat

(A) The gene structure of *TaARE1*. Exon regions are shown as blue boxes. The SNPs among *TaARE1* homoelogs are highlighted in purple. The detail underneath shows partial sequences of *TaARE1* and its homoelogs. The sequences of target 1 (locating on exon 1 of *TaARE1*) and target 2 (locating on exon 4 of *TaARE1*) are underlined, respectively. PAM sites (5'-NGG-3') are highlighted in blue. *Bts* CI restriction enzyme site is highlighted in brown. (B) Schematic presentation of the linearized CRISPR/Cas9 vectors. (a) The linearized CRISPR/Cas9 vector of gRNA1/gRNA2. (b) The linearized CRISPR/Cas9 vector of gRNA1 and gRNA2. (C) Detection of mutations in *TaARE1*-gRNA1 and *TaARE1*-gRNA2 via PCR/RE assay in T_0 generation. "WT", wild-type, "+", with digestion, "-", without digestion. (a) Detection of mutations in line T1-44 via PCR/T7EI assay. The PCR products of TaARE1-gRNA1 mutant lines can be digested with T7 Endonuclease I (T7EI). Line T1-44 had mutations around target 1. It contained heterozygous mutation in A genome (WT; a deletion of 5 bp), homozygous mutation in B genome (a deletion of 33 bp) and bi-allelic mutations in D genome (a deletion of 25 bp; an insertion of 133 bp followed

with a deletion of 10 bp and a substitution of 1 bp). (b) Detection of mutations in line T2-5 via PCR/RE assay. The PCR products of TaARE1-gRNA2 mutant lines are resistant to *Bts* CI. Line T2-5 had mutations around target 2. It contained bi-allelic mutations in A genome (a deletion of 1 bp; a deletion of 37 bp), homozygous mutation in B genome (an insertion of 1 bp) and bi-allelic mutations in D (an insertion of 1 bp, a substitution of 1 bp (T-A, Asp-Glu)). (c) Detection of large fragment deletions in line T12-23 via PCR. It contained large fragment deletion in D genome. (d) Detection of mutations in line T12-23 via PCR/RE assay. The PCR products around target 1 and target 2 in line T12-23 were digested with T7EI and *Bts* CI, respectively. Line T12-23 had mutations around target1 and target 2. It contained bi-allelic mutations in A genome (a deletion of 8 bp around target 1, a deletion of 7 bp around target 2; a deletion of 1 bp around target 1, a deletion of 10 bp around target 2), bi-allelic mutations in B genome (a deletion of 1 bp around target 1, a deletion of 11 bp around target 2; a deletion of 17 bp around target 1, a deletion of 13 bp around target 2), homozygous mutation in D genome (a deletion of 481 bp). The PAM motifs are highlighted in blue, target sequences are underlined, insertions are highlighted in pink, substitutions are highlighted in orange, intron sequences are highlighted in green. “M”, DL2000; “WT”, wild-type; “d”, deletion; “i”, insertion; “s”, substitution.

V.3.3. Inheritance and stability of the mutations, and generation of taare1 mutant lines

We used genome-specific primers (Table 5-S1) to genotype individual T_1 progeny to further investigate whether the CRISPR/Cas9 generated mutations could be transmitted to the next generation. We randomly selected 15 to 34 T_1 progenies derived from each T_0 plant for further genotyping analysis (Table 5-1). All of the detected mutations in the T_0 generation were transmitted to the T_1 progeny without generating new mutations. The homozygous mutation transmission rates were 100%, and heterozygous mutations were inherited according to the Mendelian ratios (homozygous/heterozygous/wild-type = 1:2:1) in the T_1 generation derived from line T2-5 (Table 5-1). However, the segregation patterns of lines T1-44 and T12-23 did not fit a Mendelian ratio, probably because of aberrant gamete or seed formation (Table 5-1).

We then used primer sets designed to specifically amplify *Cas9*, the *gRNA* cassette, and *hptII* sequences to determine whether plasmid DNA was present in these mutant lines (Figure 5-S2 and Table 5-1). Any lines carrying transgenes were excluded from subsequent analyses. As a result, we successfully recovered *Cas9*, *gRNA* cassette and *hptII* free plants in the T_1 generation following segregation (Figure 5-S2). Any alleles with small deletions or insertions that caused no frame shift in the coding region were treated as wild-type alleles. Finally, we obtained one *AABBdd* line (T1-44-7) and one *aaBBdd* line (T1-44-3) derived from the T_0 line T1-44; two *aabbdd* lines (T2-5-37 and T2-5-38), and one *aabbDD* line (T2-5-63) from the T_0 line T2-5 in the T_1 generation; and one *aabbdd* line (T12-23-49) from the T_0 line T12-23 in the T_1 generation. The *a*, *b*, and *d* in these genotypes indicate null mutations of *TaARE1* in

the A, B, and D subgenomes, respectively. The genotypes of these mutant lines are listed in Table 5-S3. The different mutant lines enabled us to evaluate the effects of single, double, or triple null alleles of the three *TaARE1* homoeologs from the A, B, and D subgenomes on NUE in a sole genetic background. Due to laborious works that needed to be done next, we chose the representative T1-44-3 (*aaBBdd*), T1-44-7 (*AABBdd*), T2-5-63 (*aabbDD*), and T12-23-49 (*aabbdd*) lines for subsequent analyses (Table 5-S3).

Table 5-2 Transmission and segregation of CRISPR/Cas9-mediated target mutagenesis at multiple loci and transgenes from T_0 to T_1 generation

Analysis of T_0 plants					**Mutation segregation in T_1 population**				
Line ID	**Target sites**	**Geno-type**	**Induced mutations (bp)**	**No. of T_1 plants detected**	**Genotype (Ho1:He: Ho2)**	**Expected segregation ratio (Ho1:He: Ho2)**	**χ2**	***P*-Value**	***Cas9*, *hptII*, *gRNA***
T1-44	*gRNA1*	Aa	wt; d5	34	4:25:5	1:2:1	7.588	0.023	28+: 6-
		BB	d33		34	1:2:1	0	1.000**	
		dd	d25; i133/d10/s1		7:24:3	1:2:1	6.706	0.036	
T2-5	*gRNA1*	aa	d1; d37	20	5:11:4	1:2:1	7.533	0.861**	14+: 6-
		bb	i1		20	1:2:1	0	1.000**	
		Dd	i1; s1		4:12:4	1:2:1	0.8	0.670**	
T12-23	*gRNA1*	aa	d8; d1	15	1:12:2	1:2:1	5.533	0.063	12+: 3-
		bb	d1; d17		2:11:2	1:2:1	3.267	0.195*	
	gRNA2	aa	d7; d10		1:12:2	1:2:1	5.533	0.063	
		bb	d11; d13		2:11:2	1:2:1	3.267	0.195*	
	gRNA12	dd	d481		15	1:2:1	0	1.000**	

Notes: The capital letters A, B, D and the lower-case letters a, b, d represent the wild-type and mutated alleles of a specific target gene in the A, B and D subgenomes, respectively. A slash indicates that both knockout and insertion occur at the same allele and

after the semicolon is the genotype of another allele. wt, wild-type; “d” indicates deletion of the indicated number of nucleotides, “i” indicates insertion of indicated number of nucleotides, “s” indicates substitution of indicated number of nucleotides.

Ho1, homozygote; He, heterozygote; Ho2, homozygote.

$\chi 2$ results are shown for the closest Mendelian ratios to be observed. For transgene analysis, “+” represents that *Cas9*/*hptII*/*gRNA* are detected, and “–” shows *Cas9/hptII/gRNA* are not detected.

** $P > 0.5$, in very good agreement with 1:2:1. *$0.1 < P < 0.5$, in good agreement with 1:2:1.

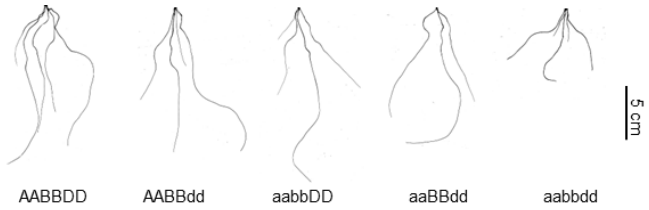
V.3.4. Root phenotypes of the mutant lines under different N conditions

The root phenotypes of the different *taare1* mutant lines and the wild-type under N deficiency (0 mM NH_4NO_3) and supply (1.5 mM NH_4NO_3) hydroponic conditions are as shown in Figure 5-3A, 5-3B, and Table 5-S4. Upon N starvation, all mutant lines exhibited decreased total root length (TRL), total root surface area (TRSA), and total root volume (TRV) compared to the wild-type, while the *aabbdd* triple null line exhibited a significantly increased root average diameter (RAD) (Table 5-S4). However, the root-to-shoot ratio was increased in all mutant lines under N deficiency (0 mM NH_4NO_3) or supply conditions (0.5 mM, 1.0 mM, and 1.5 mM NH_4NO_3) compared to the wild-type (Figure 5-3C), indicating that these mutant lines were tolerant to N starvation. Moreover, the chlorophyll content in all mutant lines retained at a higher level than that of the wild-type under all conditions, with the highest level observed in the *aabbdd* triple null line (Figure 5-3D).

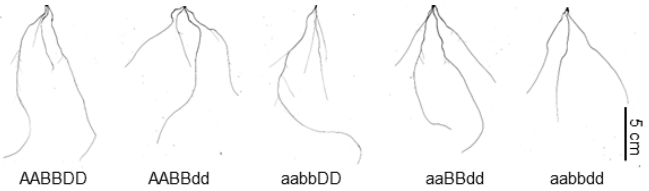
To further investigate the morphology of primary roots, we observed the structure of root tips and root cross sections in the different lines after 5 d of N starvation or supply treatments. For wild-type seedlings, the diameter of root cross sections in the elongation zone was smaller under N deficiency (0 mM NH_4NO_3) hydroponic condition (Figure 5-4A). The diameters increased under low N (0.5 mM and 1.0 mM NH_4NO_3) hydroponic condition, demonstrating that low N concentration could promote the development of roots (Figure 5-4B, 5-4C). Under the high N (1.5 mM NH_4NO_3) hydroponic condition, the root diameter showed no or minor changes, indicating that the wild-type seedlings were not sensitive to high levels of N. This phenomenon is consistent with a previous report showing that most modern wheat varieties cultivated after green revolution are insensitive to high N applications (Li et al., 2018). Under N starvation, the diameter of the elongation zone in the roots of the mutant lines was much larger than that of the wild-type (Figure 5-4A). Combined with the chlorophyll content in all mutant lines (Figure 5-3D), these results indicated that the mutant lines were tolerant to N starvation.

However, the response of the different lines exhibited different response to N treatments. When the plants were supplied with N at different concentrations, the elongation zone in the roots of the *aaBBdd* and *aabbdd* lines maintained a similar morphology as when grown under N starvation (Figure 5-4A-D), whereas under the low N (0.5 mM NH_4NO_3) treatment, the diameter of the primary roots from the *AABBdd* and *aabbDD* lines was significantly increased compared to when grown without N (Figure 5-4B). We also observed significantly enlarged root cortical cells and increased cell numbers in the different mutant lines, especially in the *AABBdd* and *aabbDD* lines, as compared to the wild-type either under N starvation or supply conditions (Figure 5-4A-D). An increased root cortical cell diameter corresponded with reduced energy costs of root growth in adapting to various abiotic stresses (Colombi et al., 2019). These results indicated that root phenotypes, especially the size and number of cortical cells in the elongation zone, upon exposure to N starvation may be harnessed to improve wheat performance under N-limiting conditions.

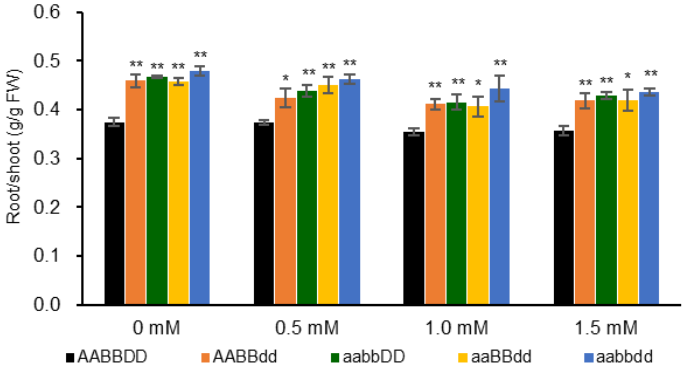
(A)



(B)



(C)



(D)

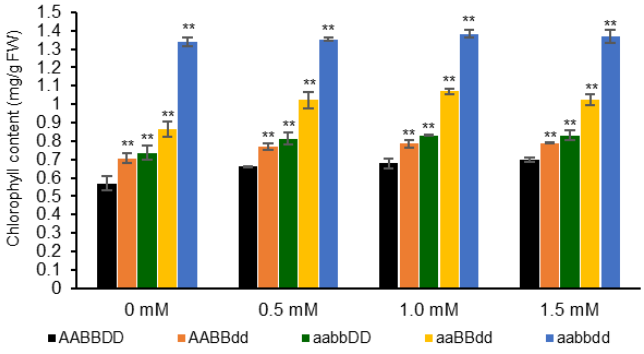
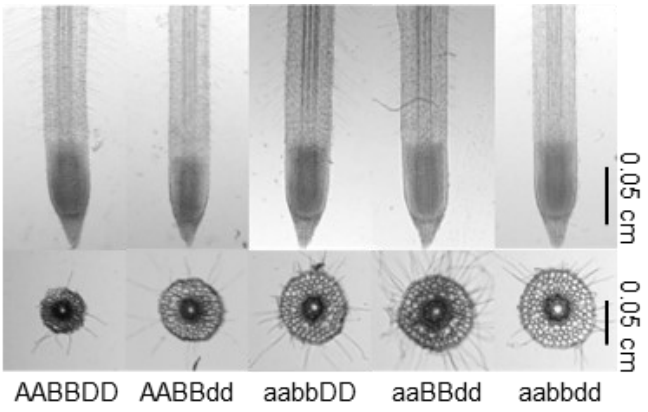


Figure 5-3 Root morphologies, the root/shoot ratios and chlorophyll contents of *taare1* mutant lines compared to the wild-type control

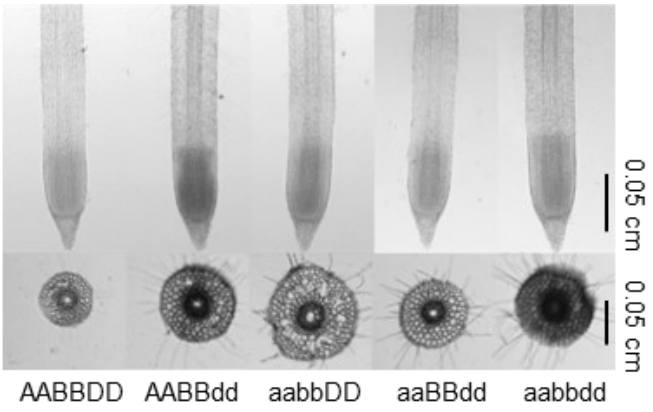
(A) Root morphologies of wild-type and different *taare1* mutant lines under N deficiency (0 mM NH_4NO_3) hydroponic conditions (Scale bars =5 cm). (B) Root morphologies of wild-type and different *taare1* mutant lines under N supply (1.5 mM NH_4NO_3) hydroponic conditions (Scale bars =5 cm). (C) Root/shoot ratios of wild-type and different *taare1* mutant lines under different concentration of N (0 mM NH_4NO_3, 0.5 mM NH_4NO_3, 1.0 mM NH_4NO_3, 1.5 mM NH_4NO_3) hydroponic conditions. (D) Quantification of chlorophyll contents in wild-type and different *taare1* mutant lines under different concentration of N (0 mM NH_4NO_3, 0.5 mM NH_4NO_3, 1.0 mM NH_4NO_3, 1.5 mM NH_4NO_3) hydroponic conditions.

For (C) and (D), data presented are mean values with SD (n=3). The data was compared to that of *AABBDD* using the two-tailed Student's t test; *significant at $P < 0.05$, **significant at $P < 0.01$.

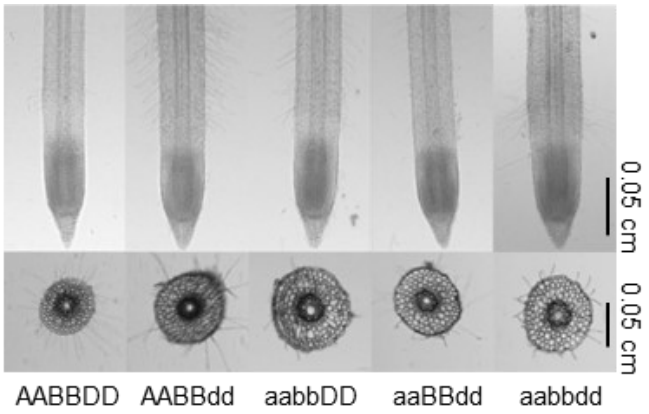
(A)



(B)



(C)



(D)

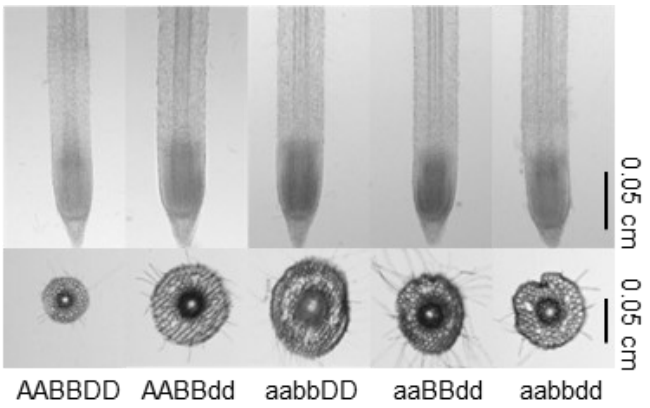


Figure 5-4 Morphologies of root tips and root cross sections of wild-type and different *taare1* mutant lines

The primary root tip (1.0 cm) was taken from each individual root of 5-d-old seedling, using a razor blade. Root cross sections of 0.5 cm thickness (1.0-1.5 cm distant from the primary root tip) taken from each individual root were stained for 1 min with Methylene blue solution (0.1% [w/v] in distilled water). (A) Morphologies of root tips and root cross sections of wild-type and different *taare1* mutant lines under N deficiency (0 mM NH_4NO_3) hydroponic conditions (Scale bars =0.05 cm). (B) Morphologies of root tips and root cross sections of wild-type and different *taare1* mutant lines under N supply (0.5 mM NH_4NO_3) hydroponic conditions (Scale bars =0.05 cm). (C) Morphologies of root tips and root cross sections of wild-type and different *taare1* mutant lines under N supply (1.0 mM NH_4NO_3) hydroponic conditions (Scale bars =0.05 cm). (D) Morphologies of root tips and root cross

sections of wild-type and different *taare1* mutant lines under N supply (1.5 mM NH_4NO_3) hydroponic conditions (Scale bars =0.05 cm).

V.3.5. Expression patterns of key N transport and assimilation genes upon N deprivation

We next selected four genes involved in N transport and assimilation and evaluated their expression patterns in the mutant and wild-type roots upon N deprivation. *TaAMT1*;*3b* and *TaNRT2.1* are involved in ammonium transport (Li et al., 2017) and nitrate transport (Yin et al., 2007), respectively. The cytosolic glutamine synthetase *TaGS1.1* modulates N assimilation and remobilization (Wang et al., 2020). *TaNADH-GOGAT* plays important roles in ammonium assimilation during N remobilization (Lea and Miflin. 2003; Quraishi et al., 2011).

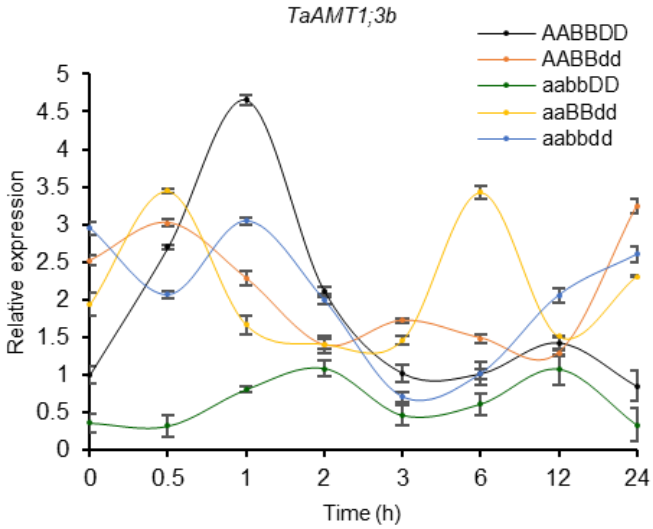
In 2-week-old seedlings that we transferred from a N-containing solution (1.5 mM NH_4NO_3) to a N-free solution, the expression of key genes involved in N transport and assimilation was rapidly induced in the roots of the wild-type and the *taare1* mutant lines (Figure 5-5). The different mutant lines showed different expression patterns for *TaAMT1*;*3b*. *TaAMT1*;*3b* expression peaked at 1 h in the wild-type and the *aabbdd* line, but its expression was lower overall in the *aabbdd* line than in the wild-type (Figure 5-5A). The *aabbDD* line showed the lowest *TaAMT1*;*3b* expression level, with no large fluctuation observed upon N deprivation (Figure 5-5A). These results indicated that the mutant lines were tolerant to N deprivation.

The expression of *TaNRT2.1* was quickly induced in the wild-type and the *aabbdd* line from 0.5 h and peaked at 1 h, but the wild-type had lower expression overall compared to the *aabbdd* line (Figure 5-5B). The expression of *TaNRT2.1* was induced in lines *AABBdd*, *aabbDD* and *aaBBdd* from 0.5 h and peaked at 2 h, but the *aabbDD* line had a relatively lower expression level. Interestingly, the expression of *TaAMT1*;*3b* and *TaNRT2.1* exhibited quite different profiles in the *aaBBdd* line upon N deprivation compared with other lines; for example, two expression peaks were observed (Figure 5-5B). These results implied that knock-out of one, two, or all three *TaARE1* homoeologs resulted in more active nitrate transport than ammonium transport upon N deprivation, and the different *TaARE1* homoeologs have diverged in this function (Figure 5-5A, 5-5B).

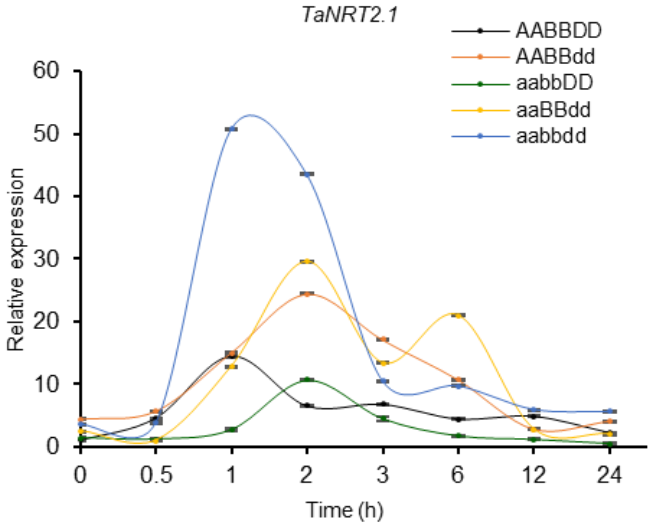
For *TaGS1;1* which is involved in ammonium assimilation, the relative expression levels were much higher in all mutant lines than in the wild-type (Figure 5-5C). The expression of *TaGS1;1* was quickly induced in the *aabbdd* triple null line from 1 h and peaked at 2 h, whereas its expression was induced in lines *AABBdd*, *aaBBdd*, and *aabbDD* from 2 h and peaked at 3 h (Figure 5-5C). This result implied that knock-out of one, two or all three *TaARE1* homoeologs resulted in more active ammonium assimilation. For *TaNADH-GOGAT* which is involved in ammonium assimilation during N remobilization, its expression was induced in the wild-type and the *aabbdd* line from 1 h and peaked at 2 h, whereas its expression in the other mutant lines peaked at 3 h (Figure 5-5D). In particular, the *AABBdd* line had a relatively higher *TaNADH-GOGAT* expression among all the plants, whereas the *aabbDD* line had the lowest expression level (Figure 5-5D). This result implied that knock-out of *TaARE1-D*

homoeologs significantly improved the assimilation of ammonium during N remobilization.

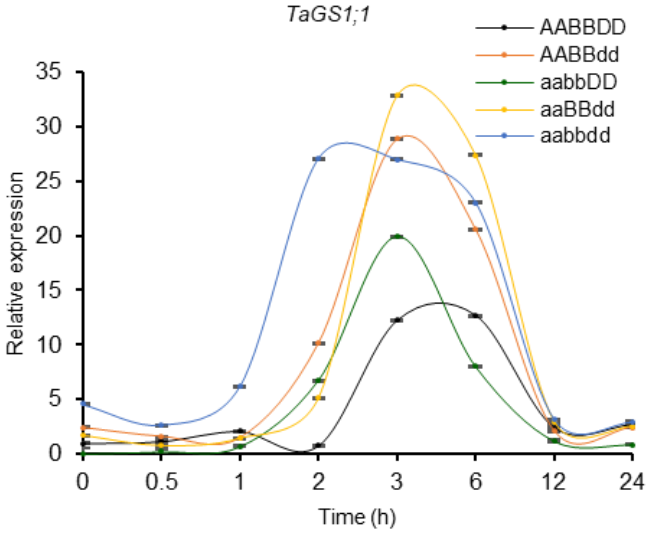
(A)



(B)



(C)



(D)

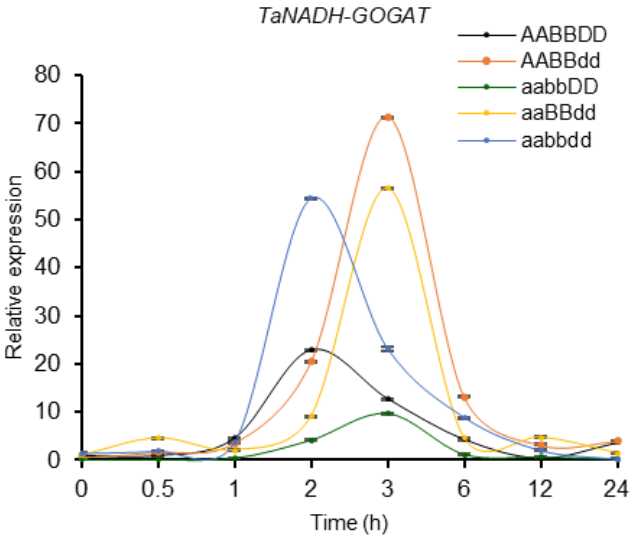


Figure 5-5 Expression of genes involved in N transport and assimilation in response to N deprivation

Two-week-old seedlings grown in N supply (1.5 mM NH_4NO_3) solution were transferred to N deficiency (0 mM NH_4NO_3) solution, and then cultured for the indicated times. (A), (B) *TaAMT1;3b*, *TaNRT2.1* were key genes involved in N uptake. (C), (D) *TaGS1;1* and *TaNADH-GOGAT* were key genes involved in N assimilation.

Total RNA was prepared from roots and used for qRT-PCR analysis. Data presented are mean values of 3 technical replicates with SD (n = 3).

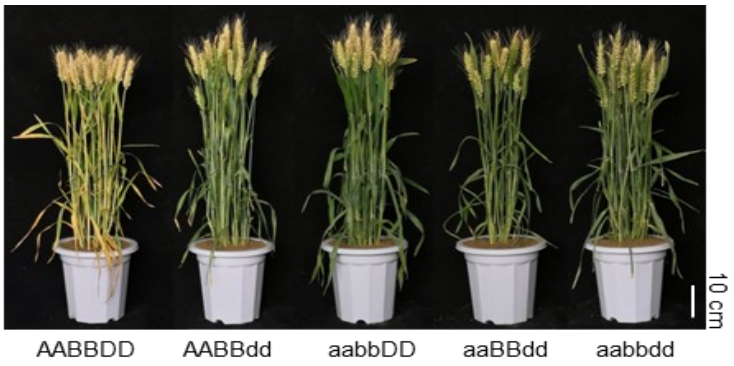
V.3.6. Field performances of the different mutant lines

We evaluated the field performances of the different *taare1* mutant lines and the wild-type under normal field conditions in a confined environment (Figure 5-6). During the early vegetative growth stages, all mutant lines showed a similar phenotype to that of the wild-type. At later growth stages, the *AABBdd* and *aabbDD* lines had a slightly increased plant height (Figure 5-6A and Figure 5-6D, Table 5-2).

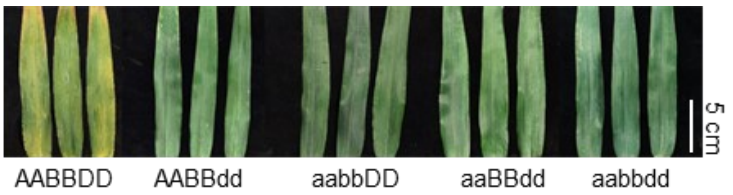
At the dough and kernel ripe stage, all *taare1* mutant lines exhibited a stay-green phenotype and prolonged photosynthetic activity compared to the wild-type (Figure 5-6A-D). While the relative chlorophyll content of the wild-type and the mutant lines was maintained almost at the same level at the heading and early grain-filling stages, the relative chlorophyll content of the mutant lines was much higher than that of the wild-type at the dough stage (Table 5-S5).

The tiller number and spike length of the *AABBdd*, *aabbDD* and *aabbdd* lines were significantly higher than wild-type (Figure 5-6D and Figure 5-6E, Table 5-2). However, although the largest spike length was observed in the *aabbdd* line, some of the top spikelets were infertile (Figure 5-6E), indicating the occurrence of growth defects when all three *TaARE1* homoeologs were knocked out. As a result, only the spikelet number and grain number per spike were higher than wild-type in the *AABBdd* and *aabbDD* lines (Table 5-2). While the grain length of all *taare1* mutant lines was longer than that of the wild-type (Table 5-S5, Figure 5-6F-a), the grain width increased significantly only in lines *AABBdd*, *aabbDD* and *aaBBdd* (Figure 5-6F-b). Consequently, the 1000-grain weights of all tested mutant lines increased in comparison with the wild-type, and in particular, the *AABBdd* and *aabbDD* lines showed significantly increased 1000-grain weights (Table 5-2). Together, the field performances of the obtained mutant lines demonstrated that lines *AABBdd* and *aabbDD* could be used as novel germplasm in breeding high-yield wheat varieties.

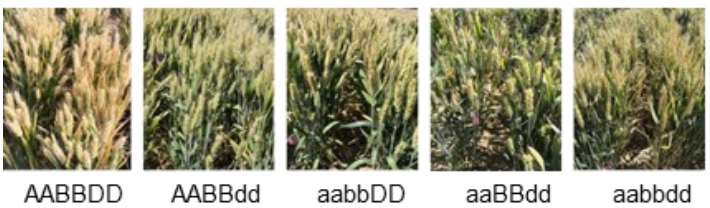
(A)


10 cm
AABBDD
AABBdd
aabbDD
aaBBdd
aabbdd

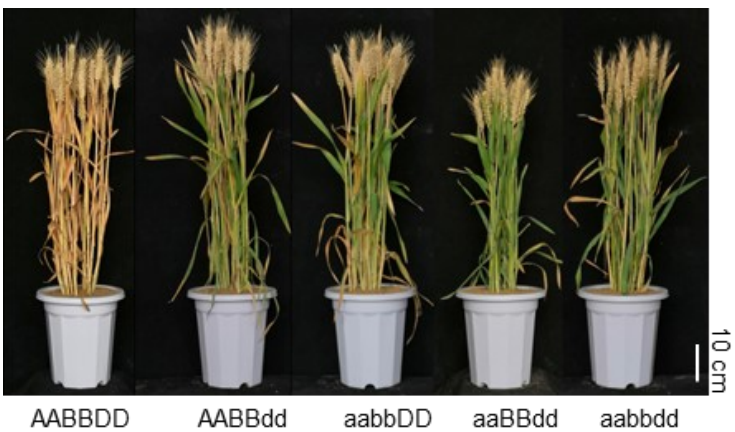
(B)


5 cm
AABBDD
AABBdd
aabbDD
aaBBdd
aabbdd

(C)


AABBDD
AABBdd
aabbDD
aaBBdd
aabbdd

(D)


10 cm
AABBDD
AABBdd
aabbDD
aaBBdd
aabbdd

(E)

(F)

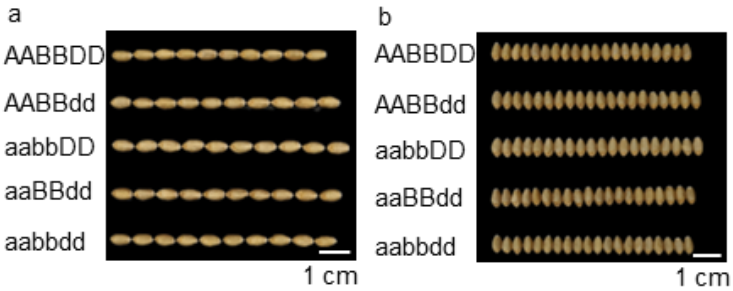


Figure 5-6 Phenotypes of wild-type and different *taare1* mutant lines in the field

A. Plant phenotypes of wild-type and different *taare1* mutant lines at the dough stage (Scale bars =10 cm). B. Phenotypes of flag leaves of wild-type and different *taare1* mutant lines at the dough stage (Scale bars =5 cm). C. Plant phenotypes of wild-type and different *taare1* mutant lines at the dough stage in the field. D. Plant phenotypes of wild-type and different *taare1* mutant lines at the kernel ripe stage (Scale bars =10 cm). E. Spike phenotypes of wild-type and different *taare1* mutant lines at the kernel ripe stage (Scale bars =5 cm). F. Grain size and appearance in wild-type and different *taare1* mutant lines at the kernel ripe stage. The grains were aligned to illustrate grain length (a) and grain width (b) between wild-type and mutant lines (Scale bars =1 cm).

Table 5-2 Field performance and major yield components of wild-type and different *taare1* mutant lines

Yield components	*taare1* mutant lines				
	AABBDD	AABBdd	aabbDD	aaBBdd	aabbdd
1,000-grain weight (g)	47.6±0.3	54.1±0.4**	56.4±0.6**	53.6±0.6**	53.6±0.4**
Length (mm)	6.7±0.1	7.1±0.1**	7.3±0.2**	7.1±0.2**	7.0±0.1**
Width (mm)	3.8±0.1	4.0±0.1**	4.1±0.1**	3.9±0.1*	3.8±0.1
Plant height (cm)	68.0±1.5	72±1.1**	70.2±1.6**	63.0±2.7**	69.2±1.3
Tiller number	15.7±1.4	17.9±2.5*	20.6±1.6**	15.0±2.8	19.5±1.8**
Spikelet number	22.2±1.6	23.8±0.9*	24.4±1.0**	21.0±2.0	23.6±2.1
Grain number of per main spike	65.4±2.2	68.5±3.3*	70.1±3.9**	67.0±6.4	68.9±4.7*
Spike length (cm)	8.5±0.3	9.1±0.3**	9.3±0.2**	8.8±0.4*	10.8±0.4**

Notes: Data presented are mean values with s.d. (n=10); for grain length and width, n=20. The data was compared to that of AABBDD using the two-tailed Student's t test; *significant at $P < 0.05$, **significant at $P < 0.01$.

V.4. Discussion

Although wheat production has significantly increased over the past few decades, future wheat production will face unprecedented challenges in the face of increasing world population, global climate change, decreased arable land, and deteriorating environment due to intensive applications of fertilizers and pesticides. In fact, nearly all modern wheat varieties are semi-dwarf varieties with increased yield potential but reduced NUE (Li et al., 2018). Therefore, breeding of high-yield wheat varieties with improved NUE is essential for sustainable agriculture development. However, the complex genome of hexaploid wheat and gene redundancy complicate forward genetic research and wheat improvement by conventional breeding. Although various traits have been improved in wheat through genome editing (For review, please see Li et al., 2021a), improvement of both NUE and yield potential in wheat via genome editing has not been documented.

ARE1 is a negative regulator of N assimilation in rice, and its loss of function can result in enhanced NUE and increased grain yield (Wang and Nian., 2018). The *ARE1* ortholog in wheat has not been functionally characterized. In this study, we isolated and characterized three *TaARE1* homoeologs in the elite Chinese winter wheat cultivar ZM (Figure 5-1). We then generated a series of *taare1* mutant lines with partial or triple null alleles (Figure 5-2). All mutant lines showed enhanced tolerance to N deficiency or deprivation under hydroponic conditions (Figure 5-3 and Figure 5-4) and showed delayed senescence and increased grain yield in a field experiment under normal growth conditions (Figure 5-6). The *AABBdd* and *aabbDD* mutant lines exhibited significantly enhanced NUE, delayed senescence, and increased grain yield without growth defects compared to the wild-type control (Figure 5-6). Thus, we generated novel wheat germplasm with improved NUE and yield potential through manipulating *TaARE1* orthologs by genome editing for the first time. Our results demonstrate the potential to manipulate *ARE1* orthologs through gene editing for breeding of high-yield wheat as well as other cereal crops with improved NUE for sustainable agricultural development.

Root system architecture (RSA) could be harnessed for increasing water and nutrient use efficiencies as well as yield potential in crop plants. The nutritional status of plants and external nutrient availability will affect root morphology. Low N stimulated primary root and lateral root elongation but not lateral root initiation. However, under severe N deprivation, lateral root formation was almost completely absent (Krouk et al., 2010; Sinha et al., 2015; Maccaferri et al., 2016). N deficiency can result in accumulation of carbohydrates in leaves and allocation of carbon to the roots, resulting in an increase in the root-to-shoot biomass ratio (Sinha et al., 2015). In this study, although all *taare1* mutant lines showed decreased TRL, TRSA, and TRV compared to the wild-type when cultured under N deficiency hydroponic condition, both the root-to-shoot ratio and the chlorophyll content were increased in all mutant lines under all conditions compared to the wild-type (Figure 5-3C and 5-3D). These results indicated that the mutant lines were more tolerant to N starvation and less sensitive to external N availability than the wild-type control, especially under N-limiting conditions.

Root plasticity allows plants to optimize their root phenotypes upon exposure to abiotic stresses. High energy costs and low resource accessibility in root growth can reduce shoot growth and eventually lead to yield losses. The root cortical cell diameter was shown to be associated with the energy costs of root maintenance (Colombi et al., 2019). In this study, we observed significantly enlarged root cortical cells and increased cell numbers in the different mutant lines compared to the wild-type under N deficiency or supply conditions (Figure 5-4). The increased root cortical cell size may result in decreased energy costs of root elongation to adapt to unfavorable soil condition and nutrient availability including N-limiting conditions.

To dissect the potential underlying mechanism of the differences observed among the mutant lines and the wild-type control, we examined the expression patterns of N transport and assimilation genes in the roots upon N deprivation. The expression pattern of *TaAMT1*;*3b* differed among the mutants, but all mutants exhibited a relatively low expression level (Figure 5-5A), indicating that the expression of *TaAMT1*;*3b* is less sensitive to N starvation than as observed in rice (Wang et al., 2018b). *TaNRT2.1* is a member of the HATS family and is a nitrate transporter that can act either as a nitrate sensor or a signal transducer to coordinate NO_3^- uptake and transport (Yin et al., 2007). When the seedlings of the mutant lines were transferred to a N-free solution, the expression level of *TaNRT2.1* was higher in the *aabbdd* line than in the wild-type control, whereas its expression differed among the *AABBdd*, *aabbDD*, and *aaBBdd* lines (Figure 5-5B). The results of *TaAMT1*;*3b* and *TaNRT2.1* expression indicated that knock-out of *TaARE1* homoeologs could stimulate nitrate transport rather than ammonia transport upon N deprivation. Furthermore, *TaARE1-D* may play an important negative role in nitrate transport since a higher level of *TaNRT2.1* expression was observed in the *AABBdd* line than in the *aabbDD* line (Figure 5-5B).

The relative expression level of *TaGS1;1* was much higher in all mutant lines than in the wild-type (Figure 5C), which is consistent with a previous report showing that *TaGS1.1* is the major *GS1* gene in wheat and is up-regulated by low N (Wang et al., 2020). The expression of *TaNADH-GOGAT* in the wild-type and the *aabbdd* line was similar as that for *TaAMT1*;*3b* and *TaNRT2.1* upon N deprivation (Figure 5-5A and 5-5B). However, the expression of *TaNADH-GOGAT* differed among the *AABBdd*, *aabbDD* and *aaBBdd* lines (Figure 5-5D), probably because these lines contain at least one functional copy of the *TaARE1* homoeologs.

Delayed leaf senescence at the grain-filling stage in crops could increase grain yield by prolonging leaf photosynthesis (Wang et al., 2018b). In this study, we observed obviously delayed senescence in different mutant lines but not in the wild-type at the dough and kernel ripe stages (Figure 5-6 A-D; Table 5-S5). This resulted in significantly increased spike length in *AABBdd*, *aabbDD* and *aabbdd* lines (Figure 5-6D and Figure 5-6E, Table 5-2). However, we noted the occurrence of growth defects when all three *TaARE1* homoeologs were knocked out (Figure 5-6E). Thus, only the spikelet number and grain number per spike of lines *AABBdd* and *aabbDD* were significantly increased (Table 5-2). In terms of grain appearance, while the grain length of all *taare1* mutant lines was longer than that of the wild-type (Figure 5-6F-a), only the grain width of lines *AABBdd*, *aabbDD* and *aaBBdd* increased significantly (Figure 5-6F-b). Consequently, the 1000-grain weight of all tested mutant lines

increased compared to the wild-type, in particular, the *AABBdd* and *aabbDD* lines showed significantly increased 1000-grain weights (Table 5-2). The increased yield potential of the *AABBdd* and *aabbDD* lines may be attributed to the fact that *TARE1-A* and *TARE1-D* homoeologs had relatively higher expression levels in roots and grains than *TARE1-B* at the grain-filling stage (Figure 5-1D), and that the *AABBdd* and *aabbDD* lines had significantly enlarged root cortical cells and increased cell numbers under all hydroponic conditions (Figure 5-4A). This suggests that at least one functional copy of either *TARE1-A* or *TARE1-D* is necessary for this improved yield performance (Figure 5-6). The field performance of the obtained mutant lines demonstrated that the *AABBdd* and *aabbDD* lines could be used as novel germplasm in breeding for high-yield wheat varieties. Meanwhile, study on CRISPR/Cas9-mediated targeted mutagenesis of *TaARE1* gene in winter wheat variety Kenong 199 (KN199) also demonstrates that *taare1* mutants can boost NUE and grain yield (Guo et al., 2021), indicating the immense potentials of *TaARE1* gene for the crop genetic improvement.

V.5. Conclusion

In conclusion, we isolated and characterized three *TaARE1* homoeologs in the elite Chinese winter wheat cultivar ZM. We then obtained a series of *taare1* mutant lines with partial or triple null alleles. All mutant lines showed enhanced tolerance to N deficiency under hydroponic conditions. We observed significantly enlarged root cortical cells and increased cell numbers in the different mutant lines, especially in lines *AABBdd* and *aabbDD* under all hydroponic conditions. Upon N deprivation, a set of genes involved in N transport and assimilation were induced with different expression patterns in different mutant lines and the wild-type control. The potential mechanism underlying these phenomena remains to be investigated in future. All mutant lines showed delayed senescence and increased grain yield in a field experiment under normal growth conditions. In particular, lines *AABBdd* and *aabbDD* exhibited significantly enhanced NUE, delayed senescence, and increased grain yield without growth defects when compared to the wild-type control. Thus, we here generated novel wheat germplasm with improved NUE and yield potential through manipulating *TaARE1* by genome editing for the first time. Our results demonstrate the potential to manipulate *ARE1* orthologs through genome editing for breeding of high-yield wheat as well as other cereal crops with improved NUE to improve agricultural sustainability and global food security.

V.6. References

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Supporting information

Contents

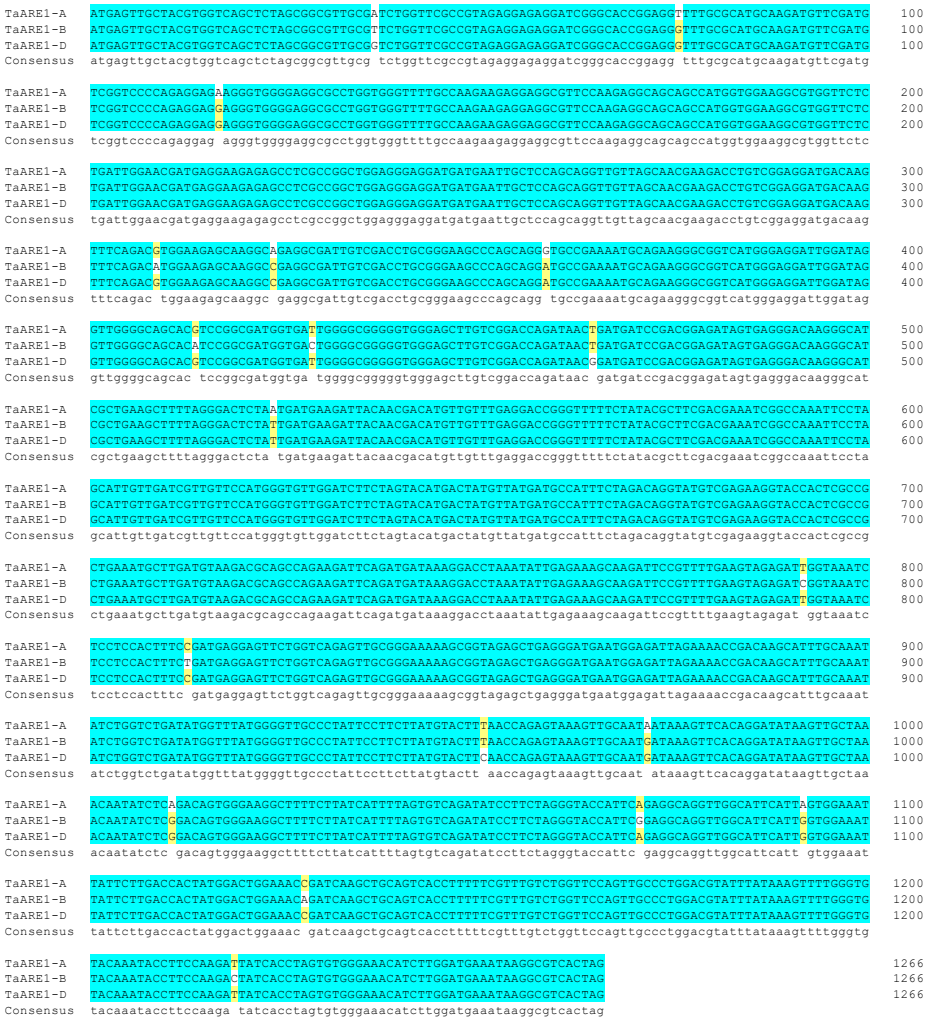

Figure 5-S1 cDNA sequences alignment of three *TaARE1* homoeologs

TaARE1-A, *TaARE1-B* and *TaARE1-D* indicated the cDNA sequences of A, B and D genome. The consensus sequences were highlighted in blue. The homology level over 50% were highlighted in yellow. The similarity of *TaARE1-A*, *TaARE1-B* and *TaARE1-D* were 99.45%.

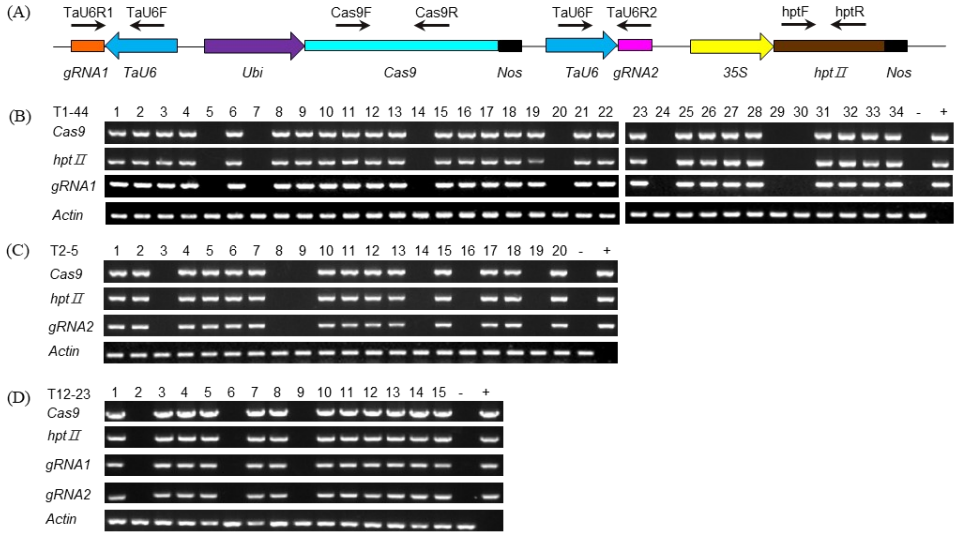

Figure 5-S2 Isolation of *Cas9*, *sgRNAs*, and *hptII* free T_1 plants from T1-44, T2-5 and T12-23 mutant lines

(A) A schematic description of the location/direction of the primer sets used for the analysis for the presence of T-DNA-gRNA1 sequences in T_1 plants of T1-44 (B), T-DNA-gRNA2 sequences in T2-5 (C) and T-DNA-gRNA1-gRNA2 sequences in T12-23 mutant lines (D). T_0 lines containing T-DNA in their genome, self-pollination allows the removal of the T-DNA in the T_1 generation plants. DNA fragments of *Cas9*, *hptII* and *gRNAs* were not detected by PCR in genomic DNA isolated from some of the T_1 plants. The control PCR product was amplified from the endogenous *Actin* gene, indicating that the genomic DNA used have sufficient quality for PCR. "-", wild-type DNA control, "+", plasmid positive control.

Table 5-S1. The primer sets used in this study

Primer	Sequence (5′ to 3′)	Use of PCR products	Annealing (℃)	Fragment size (bp)
KTaARE1-F1	GCTTGCTGCATCAGACTTGGGGCGGGGGTGG GAGCTTGTGTTTTAGAGCTAGAAATAGC	Construct vector for gRNA1	60	59
KTaARE1-R1	GCTATTTCTAGCTCTAAAACACAAGCTCCCA CCCCCGCCCCAAGTCTGATGCAGCAAGC			
KTaARE1-F2	GCTTGCTGCATCAGACTTGGGGTAGAGCTGA GGGATGAAGTTTTAGAGCTAGAAATAGC	Construct vector for gRNA2	60	59
KTaARE1-R2	GCTATTTCTAGCTCTAAAACTTCATCCCTCAG CTCTACCCCAAGTCTGATGCAGCAAGC			
SacI-U6-F	CTTTCGCCAGGGGTACCGAGCTCCGATATCA AGCTTGACCAAGC	Construct vector for gRNA1 and gRNA2	60	573
SacI-U6-R	GCTATGACCATGATTACGAATTCAGCTATGT TTAAACCAATACG			
YTaARE1-F	CTGCACTAGGTACCTGCAGATGAGTTGCTAC GTGGTCAGC	Construct vector subcellular localization	58	1305
YTaARE1-R	ACCATGGATCCGTCGACCTGCAGGTGACGCC TTATTTCATCCAAG			
TaARE1-A-F	CAGACGTGGAAGAGCAAGGCA	Detect mutations at ARE1 target sites (A genome)	57	1201
TaARE1-A-R	TCAGCGATGCCCTTGTCCC			
TaARE1-B1-F	CTCTAGCGGCGTTGCGT	Detect mutations at ARE1 target site 1(B genome)	58	645
TaARE1-B1-R	CGCATTGGAAAGCGGTGCAT			

TaARE1-B2-F	GATCGGTAAATCTCCTCCACTTTCT	Detect mutations at ARE1 target site 2 (B genome)	58	597
TaARE1-B2-R	GGAAAACTTAGATCAGTTGACCTCTTCA			
TaARE1-D1-F	CTCTAGCGGCGTTGCGG	Detect mutations at ARE1 target site 1 (D genome)	58	641
TaARE1-D1-R	CATTGGAAAGCGGTGCACGT			
TaARE1-D2-F	TGGCGTTCCATATCTTCCCTTC	Detect mutations at ARE1 target site 2 (D genome)	58	604
TaARE1-D2-R	AATGGCACACTTTTACAATTGAATACC			
Cas9-F	CGACCTCGACAATCTCCTCG	Detect *Cas9* transgene	58	535
Cas9-R	GTAGTACGGGATGCGGAAGG			
hpt-F	CAATGACCGCTGTTATGCGG	Detect *hptII* transgene	58	563
hpt-R	CTCGGAGGGCGAAGAATCTC			
TaU6-F	GGAGCACATTGTTACTCACTGC	Detect gRNA1 sequence	58	332
TaU6-R1	AACACAAGCTCCCACCCCC			
TaU6-F	GGAGCACATTGTTACTCACTGC	Detect gRNA2 sequence	58	329
TaU6-R2	TTCATCCCTCAGCTCTACCC			
TaActin-F	GTTCCAATCTATGAGGGATACACGC	Detect *Actin* gene	57	205
TaActin-R	TAGCCGTTTCCAGCTCCTGC			
T1-OFF1-F	CAAGGAAAGGCTATGATTTAGCG	Detect mutations on putative off-target site of *ARE1* target 1	57	576
T1-OFF1-R	GCGTCACCAGACGTCAACGA			

T1-OFF2-F	GGAACACTCCAAGTAAGATGAGG	Detect mutations on putative off-target site of *ARE1* target 1	57	403
T1-OFF2-R	CTTGCCCTGCTTCGACAC			
T2-OFF1-F	TCATACTCTGTTAGCACCATCATG	Detect mutations on putative off-target site of *ARE1* target 2	55	343
T2-OFF1-R	CGTTGTTTAGTAAATGGAATATAGTGG			
T2-OFF2-F	GGAAGTGAGCCACTTTGGAG	Detect mutations on putative off-target site of *ARE1* target 2	56	332
T2-OFF2-R	TCAACATCTGCAACAACTGATCC			
T2-OFF3-F	GTCGCAACAGATGGCCAGAG	Detect mutations on putative off-target site of *ARE1* target 2	57	356
T2-OFF3-R	TATAAAACCTTGTGCAGGGCAC			
qARE1-A-F	CTTTAACCAGAGTAAAGTTGCAATA	qPCR of ARE1 (A genome)	58	170
qARE1-A-R	CATAGTGGTCAAGAATAATTTCCACT			
qARE1-B-F	TCGGAGGATGACAAGTTTCAGACA	qPCR of ARE1 (B genome)	58	146
qARE1-B-R	CAGTCACCATCGCCGGAT			
qARE1-D-F	GACGTGGAAGAGCAAGGCC	qPCR of ARE1 (D genome)	58	183
qARE1-D-R	CTCACTATCTCCGTCGGATCATCC			
qCDC-F	CAGCTGCTGACTGAGATGGA	qPCR of *TaCDC* (cell division control protein)	58	77
qCDC-R	ATGTCTGGCCTGTTGGTAGC			
qNADH-F	GGACACGCAGACCATGTTCTT	qPCR of *TaNADH-GOGAT*	58	198
qNADH-R	CACAGCAACATCTCTACCAGTT			

qAMT1;3b-F	TTCTTCGGCCTCAAGGACATGC	qPCR of *TaAMT1;3b*	58	180
qAMT1;3b-R	GGACACGACCGGGTAGACG			
qNRT2.1-F	TCCTTCAGGCTCTTCTCCTTCG	qPCR of *TaNRT2.1*	58	140
qNRT2.1-R	TTGGCGAGGTTGAGGTTGTCG			
qGS1;1-F	CACTACAAGGCCTGCCTCTT	qPCR of *TaGS1;1*	58	176
qGS1;1-R	TGACGACAACTCCGGCGAT			

Table 5-S2. Analysis of potential off-target effects

Target	Name of putative off-target site	Putative off-target locus	Sequence of the putative off-target site	No. of mis-matching bases	No. of plants sequenced	No. of plants with mutations
TaARE1-gRNA1	OFF1	Chr07B: 510754179-510754198	GGGCGCAGATGGGAGCTTGTGGG	3	3	0
	OFF2	Chr04B: 372112192-372112214	GGGCGGGGGTGGGAGCTTGTCGG	3	3	0
TaARE1-gRNA2	OFF1	Chr05A: 40721089-40721111	GGGGAGAGCTAAGGGATGATTGG	3	3	0
	OFF2	Chr03B: 124744800-124744822	GGGAATAGCTGAAGGATGAAAGG	3	3	0
	OFF3	Chr04A: 637362640-637362662	GGGCATATCTGAGGAATGAAGGG	4	3	0

Note: The PAM motifs are underlined and highlighted in blue; mismatching bases are shown in red.

Table 5-S3. Genotypes of different homozygous T_1 mutant lines

Line	Genotype of *TaARE1* homologs	Sequence	T_1 lines
AABBDD (Target 1)	AA	CGGCGATGGTGATTGGGGCGGGGGTGGGAGCTTGTCGGACCAGATAACTG ATGATCCG wt	
	BB	CGGCGATGGTGACTGGGGCGGGGGTGGGAGCTTGTCGGACCAGATAACTG ATGATCCG wt	
	DD	CGGCGATGGTGATTGGGGCGGGGGTGGGAGCTTGTCGGACCAGATAACGG ATGATCCG wt	
aaBBdd (Target 1)	aa	CGGCGATGGTGATTGGGGCGGGGGTGG - - - - -TGTCGGACCAGATAACTG ATGATCCG d5	T1-44-3
	BB	CGGCGATGGTGACTGGGGCGGGGG- -G d33	
	dd	CGGCGATGGTGATTGGGG- ATAACGG ATGATCCG d25	
AABBdd (Target 1)	AA	CGGCGATGGTGATTGGGGCGGGGGTGGGAGCTTGTCGGACCAGATAACTG ATGATCCG wt	T1-44-7
	BB	CGGCGATGGTGACTGGGGCGGGGG -G d33	
	dd	CGGCGATGGTGATTGGGG - ATAACGG ATGATCCG d25	
AABBDD (Target 2)	AA	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGATGAATGGAGATTAGAAA ACCGACAA wt	
	BB	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGATGAATGGAGATTAGAAA ACCGACAA wt	

	DD	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGATGAATGGAGATTAGAAA ACCGACAA wt	
aabbdd (Target 2)	aa	GGTCTGT - CAA d37	T2-5- 37
	bb	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGATTGAATGGAGATTAGAA AACCGACAA i1	
	dd	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGATTGAATGGAGATTAGAA AACCGACAA i1	
aabbdd (Target 2)	aa	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGA- GAATGGAGATTAGAAAACCG ACAA d1	T2-5- 38
	bb	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGATTGAATGGAGATTAGAA AACCGACAA i1	
	dd	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGATTGAATGGAGATTAGAA AACCGACAA i1	
aabbDD (Target 2)	aa	GGTCTGT- -CAA d37	T2-5- 63
	bb	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGATTGAATGGAGATTAGAA AACCGACAA i1	
	DD	GGTCTGTTACTTTAACAGGGTAGAGCTGAGGGAAGAATGGAGATTAGAA AACCGACAA s1	
AABBDD (Target 1+2)	AA	CGATGGTGATTGGGGCGGGGGTGGGAGCTTGTCGG-//-GGGTAGAGCTGAG GGATGAATGGAGATT-//-GTCTG wt	
	BB	CGATGGTGACTGGGGCGGGGGTGGGAGCTTGTCGG-//-GGGTAGAGCTGAG GGATGAATGGAGATT-//-GTCTG wt	
	DD	CGATGGTGATTGGGGCGGGGGTGGGAGCTTGTCGG-//-GGGTAGAGCTGAG GGATGAATGGAGATT-//-GTCTG wt	

aabbdd (Target 1+2)	aa	CGATGGTGATTGGGGCGGGGGTGGGAGC -TGTCGG-//-GGGTAGAGCTGAG GGAT- - - - - - - - - - - - - T-//-GTCTG	d1, d10	T12-23-49
	bb	CGATGGTGACTGGGGCGGGGGTGGGAGC -TGTCGG-//-GGGTAGAGC - - - - - - - - - - - - - - -TGGAGATT-//-GTCTG	d1, d11	
	dd	CGATG- //- -//-GTCTG	d481	

Note: The PAM motifs are highlighted in blue, target sequences are underlined. Target 1 and target 2 are shadowed in light blue and yellow, respectively. Insertions are highlighted in pink, substitutions are highlighted in orange, intron sequences are highlighted in green. “WT”, wild-type; “d”, deletion; “i”, insertion; “s”, substitution.

Table 5-S4. Root morphological parameters of *taare1* mutant lines compared to the wild-type control

Line	TRL/ cm	TRSA/ cm^2	RAD/ mm	TRV/ cm^3	TRL/ cm	TRSA/ cm^2	RAD/ mm	TRV/ cm^3
	0 mM NH_4NO_3				1.5 mM NH_4NO_3			
AABBDD	59.934± 5.158	6.779± 0.706	0.360± 0.007	0.061± 0.007	54.701± 2.105	4.515± 0.359	0.263± 0.0140	0.030± 0.004
AABBdd	39.610± 2.671**	4.553± 0.172**	0.365± 0.014	0.042± 0.002*	44.532± 3.263**	4.422± 0.445	0.316± 0.009**	0.035± 0.004
aabbDD	35.050± 4.578**	3.980± 0.039**	0.363± 0.012	0.036± 0.002**	40.644± 2.605**	3.814± 0.276	0.299± 0.004*	0.028± 0.002
aaBBdd	37.400± 5.584**	4.379± 0.615*	0.374± 0.023	0.041± 0.006*	42.744± 5.863*	4.435± 0.648	0.330± 0.007**	0.037± 0.005
aabbdd	26.090± 2.321**	3.830± 0.311**	0.467± 0.004**	0.045± 0.003*	28.345± 2.660**	3.144± 0.476*	0.353± 0.038*	0.028± 0.006

Notes: 0 mM NH_4NO_3, 12-d-old seedlings of *taare1* mutant lines and wild-type in nitrogen deficiency (0 mM NH_4NO_3) hydroponics condition; 1.5 mM NH_4NO_3, 12-d-old seedlings of *taare1* mutant lines and wild-type in nitrogen supply (1.5 mM NH_4NO_3) hydroponics condition. TRL, total root length; TRSA, total root surface area; RAD, root average diameter; TRV, total root volume. Data presented are mean values with SD (n=3). The data was compared to that of AABBDD using the two-tailed Student's t test; *significant at $P < 0.05$, **significant at $P < 0.01$.

Table 5-S5. The relative chlorophyll content of wild-type and different *taare1* mutant lines at different developmental stages

Developmental stage	*taare1* mutant lines				
	AABBDD	AABBdd	aabbDD	aaBBdd	aabbdd
Heading stage	57.3±1.3	58.1±1.2	58.0±1.1	58.1±1.3	58.3±1.3
Grain-filling stage (18 DAP)	54.9±1.0	55.9±1.1	56.2±1.1	56.5±1.2*	56±1.2
Dough stage	36.6±1.1	51.7±0.9**	52.0±1.2**	51.9±1.0**	51.6±1.1**

Notes: 18 DAP, 18 days after pollination. Data presented are mean values of 3 technical replicates with SD (n=5). The data of each genotype was compared to that of AABBDD using the two-tailed Student's t test; *significant at $P < 0.05$, **significant at $P < 0.01$.

6

Chapter VI General discussion, conclusion and perspectives

VI.1. General discussion, conclusion and perspectives

Engineering novel germplasms for insect-resistance, disease-resistance, and efficient utilization of major nutrients (nitrogen, phosphorus, potassium, boron, and so on) would be an important direction in crop breeding.

RNAi technology has emerged as a promising strategy and has been widely used in crop plants for pest management. A growing number of studies indicate that HIGS-based or SIGS-based RNAi has been successfully applied to most aphid species. Transgenic plants-mediated RNAi seemed to be a more beneficial approach to increasing RNAi effects. Nonetheless, the lack of transformation technology in several crop species has limited the wide applicability of HIGS. Public concern about the biosafety of genetically modified organisms (GMOs) limits global applications of HIGS (Yan et al., 2020b; Zhao et al., 2021). In comparison to HIGS, SIGS does not produce GMOs. However, the instability of naked dsRNA is a significant limitation of SIGS, resulting in a relatively short period of protection. The target gene selection, the length of dsRNA, the delivery method of dsRNA, and the stability of dsRNA affect the efficiency of both HIGS-mediated or SIGS-mediated RNAi. The exclusion of potential off-target effects and effects on non-target organisms is another concern for RNAi application.

The identification and functional analysis of these salivary proteins provide the opportunity of understanding some aspects of plant-insect molecular interaction mechanisms and assessing possible pest management targets (Huang et al., 2016). In this thesis, we identified a novel potential RNAi target gene (*SmDSR33*) from grain aphid which had a signal peptide and one predicted transmembrane helix. Some candidate salivary effectors were also reported to have these structures, including ApC002 (Mutti et al., 2008), Armet (Wang et al., 2015b), ACYPI006346 (Pan et al., 2015), Sg2204 (Zhang et al., 2022a) and Sm9723 (Zhang et al., 2022b). When feeding on transgenic wheat lines expressing *SmDSR33*-dsRNA in grain aphids, significantly decreased fecundity, survival, and reproduction rates of aphids were observed compared to those of aphids feeding on wild-type plants. Our results were consistent with some studies focusing on targeting salivary protein and effector encoding genes through RNAi in aphids. Silencing the salivary protein gene *C002* achieved a reduction in feeding, reproduction, and survival in the pea aphid (Mutti et al., 2006; Mutti et al., 2008). Silencing salivary proteins such as Mp10, Mp42, Mp56, Mp57, and Mp58 in tobacco caused reduced virulence and fecundity of green peach aphids (Bos et al., 2010; Elzinga et al., 2014; Rodriguez et al., 2014). Silencing *Sm9723* and *Sg2204* negatively impacted aphid survival and fecundity of aphids via nanocarrier-mediated dsRNA delivery system (Zhang et al., 2022a; Zhang et al., 2022b). We also found that *SmDSR33* silencing increased the total duration of non-probing waveforms and C phases and decreased the duration of phloem ingestion (E2). These results indicated that *SmDSR33* affected the feeding process of grain aphids. Similarly, knockdown of the effector protein Armet impeded the feeding behavior of pea aphids (Wang et al., 2015b). The probing time and the total duration of phloem activity on broad bean plants were decreased when wingless adult pea aphids were

injected with NPF dsRNA (Li et al., 2018c). Plastid-mediated RNAi was also an efficient approach for aphid control. *M. persicae* exhibited different feeding behaviors on nuclear-mediated RNAi transgenic plants and transplastomic-mediated RNAi transgenic plants (Dong et al., 2022). Feeding behavior of *S. miscanthi* and *Schizaphis graminum* were significantly impaired after knockdown of *Sm9723* and *Sg2204* (Zhang et al., 2022a; Zhang et al., 2022b). Furthermore, feeding on transgenic lines could induce transgenerational silencing of *SmDSR33* in aphids, which indicated that RNAi effect was persistent in grain aphids.

Meanwhile, numerous studies have documented the impact of nitrogen fertilizer application on aphid development and performance. As a primary constituent of the nucleotides and proteins, nitrogen (N) is an essential element for all living organisms. N availability is a significant constraint for plant growth and development. In modern agriculture, large amounts of N fertilizer are applied each year to maximize yield, which caused severe environmental issues (Tilman et al., 2002; Gastal et al., 2015). The availability of vital nutrients in the phloem sap of host plants affects the growth and reproduction of aphids (Noma et al., 2010). The host nitrogen concentration is one of the most important dietary factors influencing the performance of herbivorous insects. Aphids and other piercing-sucking insects exhibit a particularly potent reaction to the nitrogen content of their host plants (Aqueel and Leather, 2011). High nitrogen fertilizer improves the nutritional content of plant tissues, making plants more susceptible to herbivorous insect infestations due to the increase in their population (Fallahpour et al., 2015). Salman et al. (2007) reported that an increase in N fertilizer levels resulted in a considerable rise in aphid population density. Nitrogen fertilization had a positive impact on the aphid population; the plants that had the most nitrogen fertilization had the highest abundance of aphids. Which may assist in understanding why aphid populations are higher on plants fertilized with higher nitrogen amounts (Sinha et al., 2018; Fallahpour et al., 2020). Studies have shown a correlation between the soybean aphid population growth rate and the availability of nitrogen in soybeans (Tilmon et al., 2011). Aphid population growth is accelerated when aphids are on plants with higher phloem nitrogen concentrations or at plant growth stages where nitrogen is more easily accessible (Noma et al., 2010; Walter and Difonzo, 2014). When utilized higher N fertilizer application rates, the aphid *Hysteroneura setariae* raised on rice had significantly greater fertility and longevity, (Jahn et al., 2005), the fecundity and intrinsic growth rate of the wheat-raised cereal aphid *Metopolophium dirhodum* increased (Gash, 2012). The English grain aphid and the bird cherry-oat aphid both had faster growth rates and greater fecundity after receiving additional N fertilizer (Khan and Port, 2008). This effect could be achieved directly through disrupted plant physiology and metabolite composition, or indirectly through alterations in plant defense mechanisms and decreased regulation of defense pathways (Fallahpour et al., 2015).

Moreover, excessive nitrogen use typically increases the population of herbivorous insects, which in turn changes the effectiveness of their natural enemies towards insect management (Daugherty, 2011). Nitrogen fertilization increases host plant quality in tritrophic systems (crop-insect pest-natural enemy), resulting in an alteration in insect natural enemy performance and abundance through an increase in herbivore quality

or population (Price et al., 1980; Francis et al., 2001; Karungi et al., 2006; Fallahpour et al., 2020). Furthermore, fertilization may alter the architecture and microclimate of plants, which may affect the behavior, ecology, and population characteristics of both herbivorous insects and their natural enemies (Alford, 2003).

Increasing N use efficiency (NUE) and decreasing N fertilizer consumption are necessary to create sustainable and productive agriculture in the future (Tilman et al., 2002; Gastal et al., 2015; Rakotoson et al., 2017). Green Revolution varieties (GRVs) has achieved great success in enhancing food-grain production but has poor utilization efficiency of nitrogen fertilizer (Khush, 1999). Innovative breeding techniques are required to reduce fertilizer consumption and maintain agricultural sustainability. Genome-editing technologies have revolutionized plant research and have enormous potential for crop improvement. The application of CRISPR/Cas in crop improvement is particularly important in the context of global climate change, as well as in the face of diverse agricultural, environmental and ecological challenges (Li et al., 2021).

Crop NUE and grain yield have been enhanced by targeting genes directly involved in N uptake, assimilation, translocation, and remobilization through plant transgenic engineering and precise genome editing (Hu et al., 2015a; Li et al., 2018a; Wang et al., 2018a; Han et al., 2020; Guo et al., 2021; Zhang et al., 2021a). In rice, *ARE1* is a negative regulator of N assimilation, and its loss of function can lead to an increase in NUE and grain yields (Wang et al., 2018a). In this thesis of chapter V, we isolated and characterized three *TaARE1* homoeologs in the elite Chinese winter wheat cultivar ZM7698. Then, we generated a series of *taare1* mutant lines with partial or triple-null alleles through CRISPR/Cas9 mediated genome editing. Our results indicated that all mutant lines showed enhanced tolerance to N deficiency or deprivation under hydroponic conditions and exhibited delayed senescence and increased grain yield in a field experiment under normal growth conditions. The expression patterns of N transport genes in the roots upon N deprivation showed that knock-out of *TaARE1* homoeologs could stimulate nitrate transport (*TaNRT2.1*) rather than ammonium transport (*TaAMT1*;*3b*) upon N deprivation. For N assimilation and remobilization gene (*TaGS1;1*), knock-out of one, two or all three *TaARE1* homoeologs resulted in more active ammonium assimilation. The expression patterns of *TaNADH-GOGAT* implied that knock-out of *TaARE1-D* homoeologs significantly improved the assimilation of ammonium during N remobilization. We also observed obviously delayed senescence in different mutant lines but not in the wild-type at the dough and kernel ripe stages. The statistical results of agronomic traits suggested that at least one functional copy of either *TaARE1-A* or *TaARE1-D* is necessary for this improved yield performance. Consistent with our results, study on CRISPR/Cas9-mediated targeted mutagenesis of *TaARE1* gene in winter wheat variety Kenong 199 (KN199) also demonstrated that *taare1* mutants can boost NUE and grain yield (Guo et al., 2021). Our results demonstrated the potential to manipulate *ARE1* orthologs through gene editing for breeding of high-yield wheat as well as other cereal crops with improved NUE for sustainable agricultural development.

Taken together, in this thesis, we engineered novel wheat germplasms with increased aphid-resistance and nitrogen-use efficiency through plant-mediated RNAi and CRISPR/Cas9-mediated genome editing by using the same winter wheat variety-

ZM7698. For further study, engineering a novel wheat germplasm pyramided with these two important agronomic traits would be helpful to speed up the breeding process, which will play a significant role in improving agricultural sustainability and guaranteeing global food safety and security.

VI.2. References

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Appendix-Publications

1. **Zhang, J**., Zhang, H., Li, S., Li, J., Yan, L., and Xia, L. (2021). Increasing yield potential through manipulating of an ARE1 ortholog related to nitrogen use efficiency in wheat by CRISPR/Cas9. *Journal of Integrative Plant Biology* 63: 1649-1663.

2. **Zhang, J**., Li, H., Zhong, X., Tian, J., Segers, A., Xia, L., and Francis, F. (2022). RNA-interference-mediated aphid control in crop plants: a review. *Agriculture* 12, 2108.

3. **Zhang, J**., Li, H., Zhong, X., Tian, J., Segers, A., Xia, L., and Francis, F. (2022). Silencing an aphid-specific gene *SmDSR33* for aphid control though plant-mediated RNAi in wheat. (Frontiers in Plant Science-Minor revision)

4. Li, S., Li, J., He, Y., Xu, M., **Zhang, J**., Du, W., Zhao, Y., and Xia, L. (2019). Precise gene replacement in rice by RNA transcript-templated homologous recombination. *Nature Biotechnology* 37:445-450.

5. Li, J., Luo, J., Xu, M., Li, S., **Zhang, J**., Li, H., Yan, L., Zhao, Y., and Xia L. (2019). Plant genome editing using xCas9 with expanded PAM compatibility. *Journal of Genetics and Genomics*, 20; 46 (5):277-280.

6. Li, S., Li, J., **Zhang, J**., Du, W., Fu, J., Sutar, S., Zhao, Y., and Xia, L. (2018). Synthesis-dependent repair of Cpf1-induced double strand DNA breaks enables targeted gene replacement in rice. *Journal of Experimental Botany* 69:4715-4721.

7. Li, J., Zhang, X., Sun, Y., **Zhang, J**., Du, W., Guo, X., and Xia, L. (2018). Efficient allelic replacement in rice by gene editing: a case study of the NRT1. 1B gene. *Journal of Integrative Plant Biology*, 60(7), 536-540.


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