Development of Gene Silencing Technology for Controlling Soil-Borne Fungal Diseases in Crops (Postprint)

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Abstract

Soil-borne fungal diseases of crops constitute one of the most critical challenges currently confronting agriculture, and due to the inherent difficulties in their control, they are increasingly becoming a significant factor limiting the sustainable development of agricultural production in China. Gene silencing (or RNA silencing, RNAi) represents an important pathway that is ubiquitously present in eukaryotes and regulates gene expression based on homologous sequences. The gene silencing technology developed from this mechanism, as a novel control strategy, has been extensively applied in research on controlling plant pests. This article provides a comprehensive overview of the occurrence and current control status of soil-borne fungal diseases of crops, the latest research advances in RNA silencing and its application in plant pest control, objectively analyzes the tremendous potential and major issues urgently requiring resolution for gene silencing technology in controlling soil-borne fungal diseases of crops, and elaborates on the importance of developing gene silencing technology for sustainable control of soil-borne fungal diseases of crops and its great application prospects.

Full Text

Development of Gene Silencing Technology for Controlling Crop Soil-borne Fungal Diseases

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Abstract

Soil-borne fungal diseases represent one of the most critical challenges facing modern agriculture. Due to the difficulties in effective control, these diseases

are increasingly becoming a major factor limiting the sustainable development of agricultural production in China. Gene silencing (or RNA silencing, RNAi) is a conserved pathway in eukaryotes that regulates gene expression based on sequence homology. The resulting gene silencing technology has been widely applied as a novel control strategy against plant pests and pathogens. This review comprehensively introduces the current status of soil-borne fungal disease occurrence and management, recent advances in RNA silencing research, and its applications in plant protection. We objectively analyze the tremendous potential and key challenges that must be addressed for gene silencing technology to control crop soil-borne fungal diseases, and discuss the importance and promising applications of developing RNAi-based strategies for sustainable management of these pathogens.

Keywords: crop, soil-borne fungal disease, gene silencing

1. Current Status of Soil-borne Fungal Disease Occurrence and Management

Soil-borne diseases are caused by pathogens that complete part or most of their life cycle in soil, infecting plants and inducing disease symptoms when conditions become favorable [1]. These pathogens include fungi, bacteria, nematodes, and actinomycetes, with fungi being the most predominant group. Fungal soil-borne diseases frequently cause root rot, wilting, and plant death, severely affecting crop growth and quality. In recent years, continuous cropping, excessive chemical fertilizer application, and climate change have altered the soil microenvironment and reduced soil fertility, leading to increasingly severe soil-borne fungal diseases. This has caused enormous economic losses and seriously constrained the sustainable development of agricultural production in China [2].

The major fungal genera causing soil-borne diseases include *Fusarium*, *Verticillium*, *Sclerotinia*, and *Gaeumannomyces*. *Fusarium* contains numerous plant pathogens, many of which are soil-transmitted and cause severe root and vascular diseases. For example, *Fusarium oxysporum* infects through the root system and colonizes the vascular tissue, causing devastating wilt disease. Infection at the seedling stage often results in seedling death, while infection in mature plants causes stunted growth, leaf scorching, and in severe cases, whole plant death. This pathogen has a broad host range, affecting over 100 plant species including cucurbits, solanaceous crops, legumes, ornamental flowers, economic crops, and fruit trees, frequently causing substantial economic losses [3].

All six plant pathogenic species in the genus *Verticillium* are soil-dwelling fungi, with *Verticillium dahliae* and *Verticillium albo-atrum* being the most destructive. *V. dahliae* alone causes Verticillium wilt in approximately 660 plant species [4]. In China, cotton Verticillium wilt affects 3 million hectares annually, causing economic losses of about 1.2 billion RMB [5]. Sclerotinia diseases caused by *Sclerotinia*, *Monilinia*, *Rhizoctonia*, and *Sclerotium* species primarily damage

dicotyledonous plants such as rapeseed, soybean, sunflower, and peanut. These pathogens mainly infect stems, leaves, and fruits, causing rot and necrosis, and produce abundant sclerotia within infected stems and pods [6,7]. In China, Sclerotinia diseases cause annual economic losses of 1-3 billion RMB, with yield reductions approaching 50% in severely affected areas [8].

Gaeumannomyces graminis is an important root-parasitic fungus of Poaceae plants, causing take-all disease in numerous cereal crops, turf grasses, and forage grasses worldwide. Based on differences in pathogenicity to various Poaceae hosts, four varieties of *G. graminis* have been identified: wheat, cereal, maize, and oat varieties [9-11]. The wheat variety exhibits strong virulence, rapid spread, and devastating impact on wheat production, and is listed as a quarantine pathogen in major Chinese wheat-producing regions.

Soil-borne fungal diseases are difficult to control for three main reasons. First, unlike airborne diseases, most soil-borne fungal diseases are polycyclic epidemics with large initial inoculum. Since these pathogens persist long-term in soil and primarily infect root tissues, they cannot be controlled through large-scale chemical applications during the optimal pre-infection and early infection stages as with airborne diseases. Second, soil-borne pathogens have strong survival capabilities; for example, the sclerotia formed by *Verticillium* and *Sclerotinia* species exhibit high stress tolerance, making eradication difficult once established. Third, soil contains diverse microbial species that often cause mixed infections with complex pathogenic mechanisms, and resistant germplasm resources are relatively scarce. Consequently, soil-borne diseases have become a major challenge in disease management, making the search for new control methods urgent.

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2. Mechanisms of RNA-Mediated Gene Silencing and Research Progress on Fungal Silencing Pathways

RNA silencing is a biological phenomenon that causes sequence-specific suppression of gene expression at the transcriptional, post-transcriptional, or translational level [12]. This process is present in nearly all eukaryotes, with conserved mechanisms that widely participate in organism development, heterochromatin formation, and responses to biotic and abiotic stresses [13]. RNA silencing is triggered by double-stranded RNA (dsRNA), which is recognized and cleaved by RNase III-type Dicer proteins into small RNAs (sRNAs) of 21-30 nucleotides. These sRNAs are loaded into Argonaute (AGO) proteins to form the RNA-induced silencing complex (RISC), which guides sequence-specific regulation of target sequences at both transcriptional and post-transcriptional levels [13-15].

RNA silencing was first discovered in plants in 1989, when researchers observed transgene inactivation in tobacco [16]. The following year, studies on petunia flower color modification revealed that transgene-derived RNA could act

as an elicitor to cause sequence-specific co-suppression of homologous endogenous genes [17]. After a decade of research, plant scientists proposed in 1998 that dsRNA could induce sequence-specific post-transcriptional gene silencing (PTGS) [18]. In the same year, Fire and Mello discovered that dsRNA triggers gene silencing in *Caenorhabditis elegans*, earning them the 2006 Nobel Prize in Physiology or Medicine [19]. In 1999, Baulcombe and colleagues detected small RNAs as key determinants of the RNA silencing process in plants [20]. Subsequently, the mechanisms and functions of RNA silencing have been extensively investigated in both animals and plants.

Research on fungal RNA silencing pathways lags far behind that in plants and animals, with in-depth studies reported only in a few model fungi. In the filamentous fungus *Neurospora crassa*, a gene silencing phenomenon similar to plant RNA silencing, termed "quelling," was discovered [21]. Subsequent studies identified a series of quelling-deficient mutants (*qde*) and cloned the corresponding genes [22]. In *N. crassa*, the quelling pathway primarily involves small RNAs induced by DNA stress or damage (QDE-2-interacting sRNA, qiRNA) [23,24] and microRNA-like small RNAs (milRNA) [25]. The four major milRNAs are synthesized through distinct pathways with unknown functions [25]. In another model fungus, *Mucor circinelloides*, functional studies have been conducted on RNA silencing pathway components including DCL (Dicer-like), RdRP (RNA-dependent RNA polymerase), and AGO proteins [26-31].

In *N. crassa*, the quelling pathway serves as an important mechanism for silencing transposable elements in the stable genome [32]. Meiotic silencing by unpaired DNA (MSUD) is another form of homologous RNA silencing [22,23]. In fission yeast, heterochromatin formation is also associated with the RNA silencing pathway [33]. In pathogenic fungi such as *Colletotrichum higginsianum*, *Cryphonectria parasitica*, and *Aspergillus nidulans*, RNA silencing mechanisms are crucial for antiviral defense [34-36]. Most other reports on fungal RNA silencing focus on small RNA sequencing and data analysis without in-depth mechanistic studies. Therefore, the molecular mechanisms and biological functions of fungal RNA silencing pathways require further investigation.

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3. Application of Gene Silencing Technology for Plant Pest Control

With deeper understanding of RNA silencing mechanisms, this technology has become a modern gene regulation tool widely used to control plant pests and pathogens, achieving remarkable results and demonstrating excellent prospects. Insects and pathogenic microorganisms are major biological constraints on plant growth and development. In plants, transgenic expression of inverted repeat sequences derived from viral genomes can induce antiviral RNA silencing. For example, papaya transformed with papaya ringspot virus fragments exhibits resistance to the virus [37]. Since plants are naturally co-infected by multiple

pathogens, artificial miRNA precursors designed to mimic endogenous miRNA structures have been widely applied to express efficient targeting sites for single or multiple genes [38-41].

RNAi can also be induced by direct injection or oral ingestion of exogenous dsRNA to reduce target gene expression in pests. Feeding insects with transgenic plants expressing target-specific RNAi constructs has proven effective in retarding the development of root-knot nematodes and insects from Lepidoptera and Coleoptera orders. Transgenic cotton expressing RNAi targeting the *CYP6AE14* gene of *Helicoverpa armigera* has been successfully applied in field conditions to resist this pest [42,43]. This technology, where host plants express RNAi constructs targeting pathogen genes to silence them and confer resistance, is called Host-Induced Gene Silencing (HIGS).

Over the past decade, HIGS has shown conceptual promise in controlling airborne fungal pathogens. For instance, expressing dsRNA in plants targeting fungal glucan transferase genes or fungal effector genes reduced haustorium formation in powdery mildew and enhanced plant resistance [44]. Silencing genes encoding mitogen-activated protein kinases and calcineurin regulatory subunits in wheat leaf rust fungus enhanced wheat resistance to this pathogen [45]. Expressing RNAi constructs targeting the fungal DCL gene in *Arabidopsis* and tomato reduced the growth and pathogenicity of *Botrytis cinerea* [46]. For soilborne fungal diseases, expressing RNAi targeting *Fusarium* genes in *Arabidopsis* and banana effectively enhanced resistance to Fusarium wilt [35,36].

Recently, our laboratory achieved breakthrough progress in using HIGS technology to control cotton Verticillium wilt, a typical soil-borne disease caused by *V. dahliae* that is notoriously difficult to manage and known as "cotton cancer." We first identified the hydrophobin gene *VdH1* in *V. dahliae* as a potential virulence factor; knockout mutants of *VdH1* showed significantly reduced Verticillium wilt symptoms. We then generated RNAi cotton stably expressing small RNAs targeting *VdH1*. Disease assays demonstrated that RNAi cotton exhibited highly effective resistance to Verticillium wilt under both laboratory and field conditions [35], indicating that cotton can utilize HIGS to silence pathogen virulence genes and reduce disease incidence. Molecular analysis of pathogens isolated from infected RNAi cotton revealed downregulation of the target *VdH1* gene and accumulation of corresponding small RNAs [35]. This represents the first molecular evidence of host-to-pathogen small RNA transfer and target gene silencing in fungi, and the first successful case of HIGS controlling a soil-borne fungal disease under natural field conditions. This work provides an important foundation for sustainable control of cotton Verticillium wilt.

More importantly, our laboratory recently demonstrated that cotton, tomato, and *Arabidopsis* can transport their endogenous miRNAs into pathogenic fungal cells, mediating cleavage of target genes and degrading virulence factors to reduce *V. dahliae* pathogenicity [36]. This study is the first to reveal at the molecular level the fundamental principle of cross-kingdom RNA silencing induced by host-derived small RNAs, providing crucial theoretical support for

HIGS applications. The successful application of HIGS technology in disease control [35,36] further corroborates the existence of this natural cross-kingdom RNA silencing pathway as a plant defense mechanism.

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4. Prospects and Future Directions for Gene Silencing Technology Against Soil-borne Fungal Diseases

Traditional chemical control methods are largely ineffective against root-infecting pathogens, making HIGS an increasingly attractive strategy for controlling soil-borne fungal diseases [35,36,46]. However, the journey from laboratory to practical field application remains long. The soil microenvironment is highly complex, and crops frequently face mixed infections from multiple pathogens. Selecting efficient and specific RNAi targets to confer effective resistance requires deeper understanding of plant-pathogen interactions in soil.

Current mechanistic studies of HIGS are largely limited to model organisms, and theoretical questions regarding RNAi differences among species and cross-kingdom regulatory mechanisms remain to be fully elucidated. Plant roots harbor diverse microbial communities including fungi, bacteria, and oomycetes that interact closely with the root system and co-evolve with the host. Increasing evidence demonstrates that interspecies exchange of water, nutrients, viruses, proteins, and RNAs is widespread. Soil microbiome research will help elucidate interactions among microorganisms in the microecosystem and their collective effects on maintaining ecological balance and influencing plant growth and disease resistance.

Understanding these interspecies exchange mechanisms and microbial community-plant interactions will guide the selection of efficient targets for HIGS applications. Furthermore, comprehensive studies of the microecosystem will help protect beneficial microbes while controlling pathogens during HIGS implementation, promoting sustainable agriculture. Microbiome research will provide a more comprehensive theoretical foundation for HIGS technology.

The efficiency of HIGS may vary among different host plants, and the mechanisms by which plant-derived small RNAs function in different fungal cells may also differ. How to design appropriate RNAi vectors to produce effective small RNAs against various soil-borne pathogens depends on understanding and utilizing specific fungal RNA silencing pathways. The diversity of fungal small RNA synthesis pathways and the divergence of RNA silencing mechanisms [22,36], combined with the complex life cycles and infection mechanisms of soil-borne fungi, present research challenges. However, the increasing availability of fungal genome sequences and deeper understanding of gene functions and infection processes will accelerate elucidation of fungal RNA silencing mechanisms. Perhaps leveraging the diversity and divergence of fungal RNA silencing pathways will enable scientists to design more effective and specific HIGS vectors, developing green and precise RNAi technologies against soil-borne fungal diseases.

Based on research into pathogenic mechanisms and identification of virulence genes, designing multiple RNAi constructs and/or polycistronic RNAi vectors targeting several virulence genes will effectively reduce the probability of resistance breakdown due to pathogen variation, solving the challenge of durable and broad-spectrum disease resistance. In summary, HIGS-based RNAi technology is demonstrating tremendous potential as a novel control strategy against soil-borne fungal diseases.

In conclusion, gene silencing technology represents an important supplement to traditional control strategies for soil-borne fungal diseases that are currently difficult to manage. This is particularly true for crops lacking resistance resources, where HIGS shows unique advantages. Developing gene silencing technology for sustainable control of crop soil-borne fungal diseases will be a key research direction in future plant protection.

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