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RNA silencing is induced by the expression of foreign recombinant products in transgenic rice

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ABSTRACT

RNA silencing plays important roles in the regulation of gene expression in eukaryotes. We previously reported that RNA silencing of a linked endogenous gene and a transgene in transgenic rice seeds can be induced by the expression of foreign recombinant mGLP-1, which acts as a silencing-inducible sequence through RNA interference. In this study, we found that the induction of RNA silencing by foreign transgenes is not restricted to mGLP-1 but is observed in many other genes as a relatively general phenomenon, as several foreign genes were involved in inducing RNA silencing in the same manner as mGLP-1 in transgenic rice. We detected 21–24-nt siRNAs using both sense and antisense probes specific to the silenced genes in both the leaves and endosperm of transgenic rice plants. Moreover, read-through transcripts were consistently observed in silenced transgenic rice plants. Taken together, these results suggest that proper transcription termination was prevented in these plants, and the highly divergent 3′-end transcripts served as templates for double-stranded RNA synthesis, resulting in the degradation of the target genes via siRNA.

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1. Introduction

RNA silencing is a eukaryotic gene repression mechanism that plays important roles in the regulation of endogenous gene expression and defense against the invasion of nucleic acids such as transgenes and viruses [1,2]. In this process, double-stranded RNA (dsRNA) induces homology-dependent degradation of the messenger RNA (mRNA) of a target gene into small interfering RNAs (siRNAs) [3]. The biogenesis of siRNA is triggered by the presence of dsRNA precursors, which are cleaved by dicer-like (DCL) protein, a dsRNA-specific RNase-III family ribonuclease, to yield single-stranded siRNAs ranging in size from 20 to 24 nucleotides [4]. Gene expression is inhibited once these siRNAs become associated with members of the AGO family of proteins to form RNA-induced silencing complexes (RISC); transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) occur in both the cytoplasm and nucleus of the plant cell [5].

Plant production systems for the generation of bio-products have potential advantages in terms of cost-effectiveness, safety, and scalability [6,7]. In particular, rice seeds have been used as an ideal bioreactor for the production of high-value recombinant proteins, such as pharmaceuticals, due to their advantages, including high accumulation levels, long-term stability, minimal risk of outcrossing by self-pollination, and direct oral delivery [8,9]. Many biopharmaceutical proteins and peptides have been successfully produced in transgenic rice seeds, and many have shown clinical effectiveness in animal models [10–19]. However, sometimes no recombinant proteins accumulate in the seeds of transgenic plants due to transgene silencing and co-suppression of endogenous rice storage proteins [20,21]. With the advancement of our understanding of the mechanism of plant RNA silencing through RNA interference (RNAi), we now know that RNAi plays a key role in controlling the magnitude of several biological activities, including development, metabolism, chromatin structure, transposon movement, defense against pathogens, and abiotic stress tolerance. RNAi is widely used in plant biotechnology research as a tool for discovering or validating gene functions and as a means of engineering specific reductions in the expression of chosen genes [14,22–24].

We first reported that in addition to the transgene, the endogenous glutelin B-1 (*GluB-1*) gene(s) are co-suppressed in the

Abbreviations: AGO, argonaute; dsRNA, double stranded RNA; siRNA, small interfering RNA; RISC, RNA-induced silencing complex; TGS, transcriptional gene silencing; PTGS, post-transcriptional gene silencing; RNAi, RNA interference; mGLP, modified glucagon-like peptide; UTR, untranslated region; RSIS, RNA silencing-inducible sequence; DCL, dicer-like enzyme; ER, endoplasmic reticulum; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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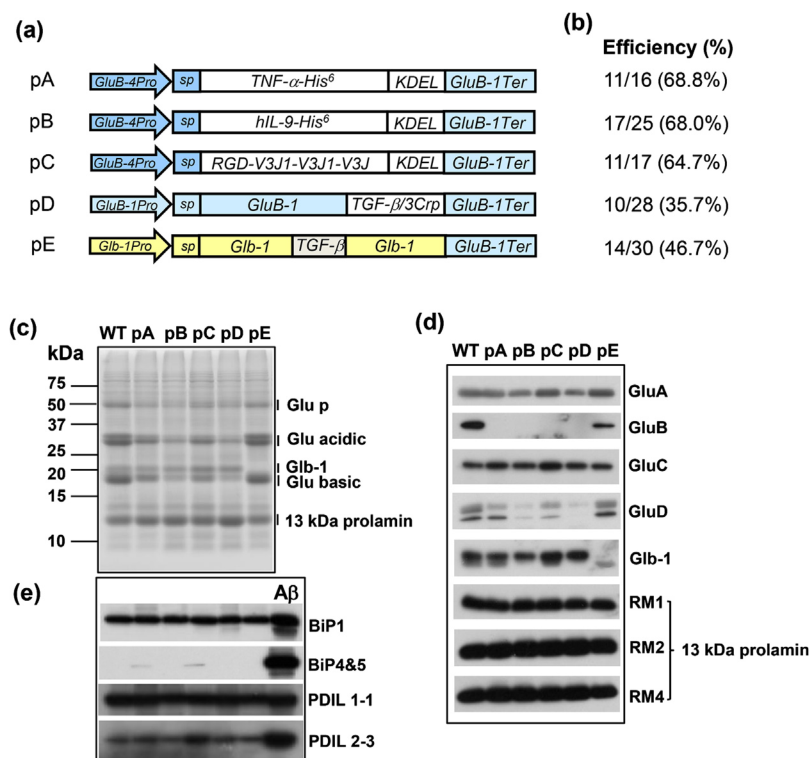

Fig. 1. Characterization of transgenic rice transformed with various transgenes. (a) Diagrams of the constructs used in this study. GluB-1Pro-SP, GluB-4Pro-SP, and Glb-1Pro-SP represent the *GluB-1*, *GluB-4*, and *Glb-1* promoters and their signal sequences, while GluB-1 Ter represents the GluB-1 terminator and KDEL is the ER retention signal. (b) Silencing efficiency is expressed as the number of silenced lines/the total number of generated independent lines. GluB-4 is silenced in pA, pB and pC, while GluB-1 and Glb-1 are silenced in pD and pE, respectively. (c) CBB stained 12% SDS-PAGE analysis of the suppression of GluB and Glb-1 in transgenic rice seeds. Glu p, Glu acidic, Glu basic, and Glb-1 represent glutelin precursor, glutelin acidic subunit, glutelin basic subunit, 26 kDa globulin, while RM1, RM2 and RM4 are 13 kDa prolamins, respectively. Molecular size markers are indicated on the left. (d) and (e) Western blot analysis of storage proteins and chaperones in transgenic rice seeds. Aβ is β-amyloid accumulating transgenic rice line which showed ER-stress related phenotype. BiP1, BiP4&5, PDIL 1–1 and PDIL 2–3 are chaperone proteins of binding protein 1, binding protein 4&5, protein disulphide isomerase-like 1–1 and protein disulphide isomeras-like 2–3, respectively.

endosperm of transgenic rice plants when codon-optimized modified glucagon-like peptide-1 (mGLP-1, which promotes insulin secretion depending on the blood glucose concentration) is expressed under the control of the *GluB-1* promoter containing its 5′ UTR, signal peptide, and terminator [20]. Furthermore, even when the 5′ UTR or terminator is substituted with that of other genes, expression of linked genes used for the production of mGLP-1 is depressed in the tissues when these genes are driven by a specific promoter, indicating that the *mGLP-1* sequence functions as an RNA silencing-inducible sequence (RSIS) [21]. We have subsequently taken advantage of RSIS to specifically silence target genes by linking unique sequence(s) derived from the target gene to the 5′ or 3′ end of the RSIS [14,21,25]. Furthermore, when RNA sequencing of small RNAs was performed to elucidate the underlying molecular mechanisms of RSIS-mediated RNA silencing, read-through transcripts were consistently observed for transcripts containing RSIS, most of which were not polyadenylated [21]. This finding suggests that RSIS inhibits proper termination, resulting in the production of aberrant dsRNA, leading to siRNA-mediated sequence-specific mRNA degradation.

In the present study, to further understand transgene silencing that occurs in transgenic rice plants used as bioreactors for the production of recombinant proteins, we analyzed several transgenic rice plants that exhibited the phenomenon of silenced transgenes as well as suppressed endogenous storage proteins, which were generated in our laboratory. Notably, gene silencing observed in the endogenous and transgenes occurred in a similar manner to that observed in transgenic rice expressing RSIS (mGPL-1). Furthermore, we employed artificial genes encoding 5Chao1 (a hybrid T cell epitope peptide derived from cypress pollen allergen Cha o 1 [26]) and its variants to examine their roles in RNA silencing in transgenic rice plants.

2. Materials and methods

2.1. Plasmid construction and plant transformation

DNA sequences encoding cytokines, including human TNF-α, mouse hIL-9, and human TGF-β, as well as artificial peptides 3Crp [27] and V3J1 (partial amino acid sequence, residues 274–292 of envelope surface glycoprotein gp120 of HIV) and 5Chao1 (five linked human major T cell epitopes derived from the cypress [*Chamaecyparis obtusa*] pollen allergen Cha o 1), were optimized for translation based on codon usage in rice seed storage protein genes by avoiding rare codons; those sequences were subsequently synthesized by GenScript Corporation (South Plainfield, NJ, USA).

Genes encoding TNF-α, hIL-9, and V3J1x3 were ligated downstream of the 1.4-kb *GluB-4* promoter containing a DNA sequence encoding a signal peptide. The coding sequence of the KDEL ER retention signal was attached to the C-termini of the genes, followed by 0.65-kb of the *GluB-1* terminator; the resulting plasmids were termed pA, pB and pC, respectively (Fig. 1a) [28]. After ligation of the in-frame fused TGF-β/3Crp gene to the C-terminal region of GluB-1 and ligation of the TGF-β gene into the 26 kDa globulin (Glb-1) variable region, the genes were further linked with 2.3 kb *GluB-1* or 1 kb *Glb-1* promoters at their 5′ termini and with the *GluB-1* terminator at the 3′ ends; the resulting plasmids were designated pD and pE (Fig. 1a).

Four sets of 5Chao1 (1–282-nt)-related plasmids encoding five linked major T cell epitopes were constructed as follows.

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