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REVIEW
Epimutant Induction as a New Plant Breeding Technology

Atsushi KASAI and Takeo HARADA*

Laboratory of Plant Breeding and Genetics, Faculty of Agriculture and Life Science, Hirosaki University (Hirosaki, Aomori 036-8561, Japan)

Abstract
A small interfering RNA (siRNA) can be custom-produced in plant cells when a transgene comprising an inverted-repeat sequence is used to form the corresponding double-stranded (ds)RNA. This siRNA induces methylation of the homologous DNA through the RNA-directed DNA methylation (RdDM) pathway. As methylation of the promoter region causes transcriptional gene silencing (TGS), dsRNA for the promoter sequence of a gene can induce TGS of the gene, accompanied by modification of chromatin. Such epigenetic variation provides a novel technique to induce silencing of a target gene. Furthermore, once epigenetic variation has occurred, the siRNA need not be present to maintain the stable repression of transcription even in the subsequent generation. Thus, the induction of epigenetic changes is a potentially new plant-breeding technology to improve crops.

Discipline: Plant breeding
Additional key words: DNA methylation, RNA silencing, transcriptional gene silencing, siRNA long-distance transport

Introduction

Napoli et al. (1990) and van der Krol et al. (1990) reported an RNA interference (RNAi) phenomenon for the first time. They overexpressed a transgene of chalcone synthase (*CHS*), a key enzyme in flavonoid biosynthesis and the rate-limiting enzyme in anthocyanin biosynthesis, to generate a darker violet petunia flower, but unexpectedly obtained a white flower. Because the *CHS* transcription level was 50-fold lower than that in the wild-type flower, they concluded that as well as the transgene, the endogenous *CHS* gene had also been silenced. A similar phenomenon in a fungus (*Neurospora crassa*), termed "quelling", was reported by Romano & Macino (1992). Furthermore, in an animal species (*Caenorhabditis elegans*), it was reported that introducing sense or antisense RNA of a gene to cells resulted in the mRNA degrading (Guo et al. 1995). Fire et al. (1998), Nobel Prize winner in 2006, clarified the RNAi mechanism by which dsRNA could cause endogenous mRNA to degrade. A number of subsequent studies revealed that dsRNA initiates gene silencing through inhibition at the post-transcriptional level (post-transcriptional gene silencing; PTGS) or the transcriptional level (transcriptional gene silencing; TGS). In the latter case, a small interfering RNA (siRNA) 24 nt long induces methylation of the fifth carbon cytosine residue within the region of sequence identity between the triggering RNA and the homologous DNA (Matzke et al. 2004).

Although cytosine methylation is mostly limited to CG dinucleotide sequence contexts in animal genomes, in plants the methylation occurs not only in the symmetrical CG context but also in the CHG and CHH sequence contexts (where H=A, C or T) throughout their genomes. The cytosine methylation of each context is performed by respective methylation enzymes. The process of *de novo* DNA methylation is triggered by 24 nt siRNAs produced by RdDM. Furthermore, two plant-specific RNA polymerases, PolIV and PolV and the RDR2, DCL3 and AGO4 proteins operate in this pathway (Matzke et al. 2009, Haag & Pikaad 2011). Chemical modifications of DNA influence chromatin structure through histone methylation, while the methylation of histone H3 lysine (H3K9) plays an important role in establishing and maintaining DNA methylation (Grewal & Jia 2007).

Epigenetic changes play pivotal roles in the developmental responses of plants. Recent studies have suggested

*Corresponding author: e-mail tharada@cc.hirosakiu.ac.jp
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that somaclonal variation (Strpid et al. 2013, Miguel & Marum 2011), heterosis (Chen 2013), sex determination (Martin et al. 2009) and graft hybrid (Wu et al. 2013) phenomena involve epigenetic changes. Therefore, heritable changes in phenotype are not only brought about by altered DNA base sequence but also by epigenetic changes. Consequently, when an siRNA produced artificially functions appropriately, gene expression can be suppressed by changes in the structure of chromatin without altering the DNA sequence. In this review, we briefly describe how epigenetic changes can be induced and discuss the relevant point of their application as a novel plant-breeding technique.

Transgene-induced epigenetic variation

A better understanding of the general RNAi process mechanisms has prompted plant researchers to use RNAi technology to modify various traits in plants. Although the phenomenon of co-suppression was discovered in petunia plants (Napoli et al. 1990) into which a transgene-expressing sense RNA was introduced, it became evident that efficient production of dsRNA for a target gene is associated with efficient RNAi induction. While we can transform plants independently with constructs that produce sense and antisense RNA and then cross these sense and antisense plants to obtain progeny expressing both these RNAs and thus induce RNAi, a widely used method to produce dsRNA in plant cells involves transforming plants with a construct comprising an inverted-repeat (IR) sequence of the target gene.

Sijen et al. (2001) were the first to report transcriptional silencing of an endogenous gene in petunia, thus indicating that dsRNA-induced TGS of endogenous genes offers an additional approach for study of the gene function, particularly when analyzing gene family members with promoters that are more divergent than their coding regions. In maize, TGS technology has been used to identify functions and regulatory components unique to transcriptional gene control (Cigan et al. 2005). Okano et al. (2008) demonstrated in rice that siRNA rarely induces chromatin inactivation or changes in patterns of histone modification, particularly H3K9 methylation, within most genome regions.

In potato, the granule-bound starch synthase I (GBSSI) gene has been efficiently silenced by PTGS. However, although transformants obtained using antisense or inverted-repeat constructs show strong silencing of *GBSSI*, *GBSSI* mRNA remained present (Kuipers et al. 1994, Heilersig et al. 2006a). To obtain a transformant with no *GBSSI* mRNA, TGS technology has been used, while to induce allele-specific silencing, TGS could also be a good approach, since the four classes of *GBSSI* alleles are highly homologous in the coding region but vary in their promoter sequences (van de Wal et al. 2001). A promoter sequence specific to the three *GBSSI* alleles, but absent in one allele, was selected to design an allele-specific promoter inverted-repeat construct. Two other promoter inverted-repeat constructs targeting different regions of the *GBSSI* promoter were also made. The results obtained indicated that the silencing efficiency of the different promoter sequences varied. Maximal efficiency was induced with an inverted-repeat construct containing the full promoter sequence (Heilersig et al. 2006b).

Virus-induced epigenetic modification

Virus-induced gene silencing (VIGS) is a technology that exploits an RNA-mediated antiviral defense mechanism, which plants possess intrinsically to cope with viruses (Covey et al. 1997). Infected plants can attenuate the replication of many plant viruses by RNA silencing via production of siRNA from the invading virus genome. In addition to the dsRNA produced from the replication intermediate of the virus RNA (Lu et al. 2003), dsRNA is also formed by intramolecular pairing of the virus RNA (Ruiz et al. 1998, Molnar et al. 2005). In both cases, the dsRNA is recognized by Dicer, resulting in the production of viral siRNA and leading to degradation of the corresponding viral mRNA. Therefore, with virus vectors carrying inserts derived from host genes, the process can be additionally targeted against the corresponding mRNAs of the gene. VIGS has been widely used in plants to analyze gene function (Burch-Smith et al. 2006). Therefore, VIGS avoids the inefficient and cumbersome process of genetic transformation, making it an ideal system for rapid and high-throughput functional characterization of genes. The potato virus X (PVX), tobacco rattle virus (TRV) and cucumber mosaic virus (CMV) have been frequently used to induce VIGS (Kanazawa et al. 2008, Senthil-Kumar and Mysore 2011).

Kanazawa et al. (2011) reported that TGS of an endogenous gene, *CHS*, can be induced by targeting dsRNA to the endogenous gene promoters in petunia using CMV as a vector. They also demonstrated that this system induced TGS for an endogenous tomato gene, *LeSPL-CNR*, which is related to the ripening phenomenon. Furthermore, the heritability of both TGS was revealed through manifestation of the DNA methylation status and histone modification. Because CMV is eliminated during meiosis, the transgene is not carried into the progeny. Accordingly, CMV-based epigenetic variation could be a useful tool for artificially modified DNA methylation of crop genomes.

However, VIGS has certain constraints as far as crop improvement is concerned. Most viruses infect a specific host, thus limiting their extensive application to non-host-range plants. Furthermore, although the viruses are eliminated during reproduction with the exception of seed-transmissible ones, the elimination technique is not applicable to plants that propagate vegetatively, such as most

fruit trees and some flowers and vegetables. Finally, there is a risk of potential generation of new infectious viruses through recombination and mutation generated by errors during replication of the genome (Allison et al. 1990). This underlines the need to derive a method other than VIGS for TGS when breeding horticultural crops.

Epimutant induction by grafting

PTGS signals such as siRNA can be delivered over extended distances through the sieve tube (Mourrain et al. 2007), usually from source to sink, following the direction of phloem flow (Tournier et al. 2006). Short-distance spreading of PTGS signals via plasmodesmata is limited to within 10-15 cells (Himber et al. 2003) unless the target transcript works as a template for RNA-dependent RNA polymerase 6 (RDR6), in which case the PTGS can spread through the entire plant by transitivity (Brosnan et al. 2007).

Although it has been considered that the TGS triggered by transgene-derived siRNA is not graft-transmissible (Mlotshwa et al. 2002, Mourrain et al. 2007), Molnar et al. (2010) demonstrated that transgene-derived siRNA moved across the graft union using an *Arabidopsis* mutant in which siRNA biogenesis was blocked. They provided evidence that a 24-nucleotide mobile siRNA from an endogenous gene could direct epigenetic DNA methylation in the genome of the recipient cells. Since the mobile signal is the siRNA itself, grafting transmission of PTGS and TGS can be unified as the transmission of the siRNA with a different target (the coding region for PTGS and the promoter for TGS).

Bai et al. (2011) successfully achieved TGS of the 35S:GFP (Green Fluorescent Protein) locus in a *Nicotiana benthamiana* transgenic line in the scion using siRNAs derived from the hairpin RNA of the 35S promoter sequence in the rootstock. In this case, the hairpin mRNA was controlled by a companion cell-specific promoter, the CoYMV (commelina yellow mottle virus) promoter, to increase the potential level of siRNA in the phloem (Matsuda et al. 2002). When the scion shoot was grafted onto the stock, the newly developed leaves exhibited GFP loss around the veins. The success of TGS was confirmed by the presence of minimal *GFP* transcript and a high level of methylation of the target region. Conversely, when reverse grafting of the siRNA donor as the scion onto 35S-GFP rootstock was performed, *GFP* silencing was clearly evident in the root, particularly lateral roots, including the root apical meristem, while no TGS of the parental root apical meristem was observed. The difference of TGS between the two meristems was considered attributable to the fact that lateral roots in angiosperms are initiated from the cells of the pericycle, which is bound to phloem cells (Lloret & Casero 2002, Smet 2011). Therefore, lateral roots originate endogenously from tissues lying inside the parent root and the founder cells that undergo TGS become the lateral root primordium, which eventually form a TGS lateral root. Complete silencing in lateral roots means the TGS plant can be regenerated through the tissue culture. Some fruit trees or shrubs can readily form adventitious shoots (root sucker) from the root. Moreover, a plant that has acquired the ability to transmit siRNA could be grafted onto appropriate cultivars as stock or scion provided there is mutual grafting compatibility and transmission of the siRNA would consequently induce TGS in the graft partners, suggesting that several cultivars could be improved using a single transgenic plant.

Perspective

Epigenetic mutation can be applied as a new technology for crop breeding (Fig. 1). However, to use the epigenetic changes for crop improvement, their maintenance is crucial.

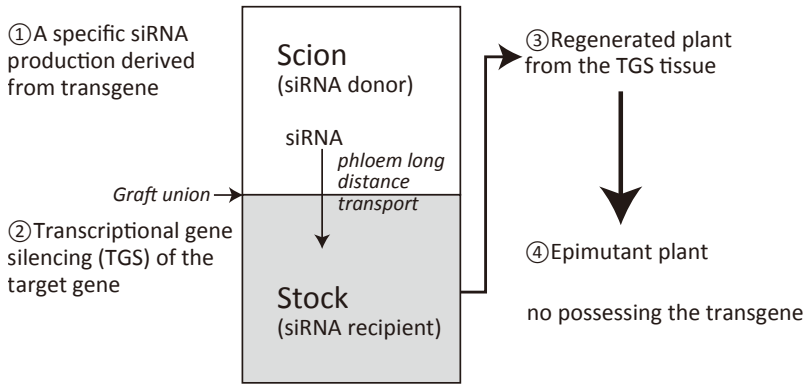

Fig. 1. Schematic presentation of epimutant plant production by grafting between siRNA donor scion and the siRNA recipient stock. 1, Production of siRNA from transgene in scion. 2, Transportation of siRNA from scion to the stock plant, which targets improvement. 3, Induction of TGS in lateral roots. 4, Production of the regenerated plant from the TGS lateral roots, resulting acquisition of the TGS plants.

A recent study indicated that a minimum length of dsRNA above a certain threshold and the frequency of cytosines at symmetrical sites in the region targeted by dsRNA are the major factors allowing induction of heritable epigenetic variation (Otagaki et al. 2011). Stable epigenetic maintenance of almost all genes would be enforced by entertaining these factors. Originally, it was proposed that systemically delivered siRNA signals are excluded from meristem tissues and thus do not silence tissues that are undergoing meiosis. Some recent reports, however, have suggested that siRNA signals can penetrate meristems (Liang et al. 2012) and floral organs (McGarry & Kragler 2013). When a more efficient operating system for handling of siRNA has been developed, it should prove useful for heritable epimutant induction in crops, particularly vegetative propagation crops because their propagation has no germ cell stage in which reprogramming of DNA methylation occurs (Calarco et al. 2012).

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