Molecular Plant • Volume 2 • Number 5 • Pages 1084–1094 • September 2009 RESEARCH ARTICLE

Virus-Induced Gene Silencing in the Culinary Ginger (*Zingiber officinale*): An Effective Mechanism for Down-Regulating Gene Expression in Tropical Monocots

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ABSTRACT Virus-induced gene silencing (VIGS) has been shown to be effective for transient knockdown of gene expression in plants to analyze the effects of specific genes in development and stress-related responses. VIGS is well established for studies of model systems and crops within the Solanaceae, Brassicaceae, Leguminaceae, and Poaceae, but only recently has been applied to plants residing outside these families. Here, we have demonstrated that barley stripe mosaic virus (BSMV) can infect two species within the Zingiberaceae, and that BSMV–VIGS can be applied to specifically down-regulate phytoene desaturase in the culinary ginger *Zingiber officinale*. These results suggest that extension of BSMV–VIGS to monocots other than cereals has the potential for directed genetic analyses of many important temperate and tropical crop species.

Key words: Barley stripe mosaic virus; virus-induced gene silencing; VIGS; *Zingiber officinale*; Monocot.

INTRODUCTION

Virus-induced gene silencing (VIGS) is a technique that utilizes the RNA interference (RNAi) pathway to down-regulate endogenous gene expression (Dinesh-Kumar et al., 2003; Burch-Smith et al., 2004; Godge et al., 2008). This process begins by abrading leaves with modified viral transcripts that express a plant cDNA sequence of a gene to be targeted for degradation (Kumagai et al., 1995; Ruiz et al., 1998). Once the transcripts begin replicating *in vivo*, double-stranded RNAs (dsRNAs) are generated by a viral RNA-dependent RNA polymerase, and the dsRNA intermediates are recognized by the plant's defense system and targeted for degradation into small interfering RNAs (siRNAs) by DICER-like enzymes (Benedito et al., 2004; Robertson, 2004). Highly specific silencing of gene expression subsequently occurs as the amplified siRNAs are incorporated into RNA-induced silencing complexes (RISC) that degrade complementary endogenous plant mRNAs (Baulcombe, 2004).

VIGS is a relatively new approach to down-regulate gene expression in plants. The technique was first applied with tobacco mosaic virus (TMV) to interfere with chlorophyll synthesis in *Nicotiana tabacum* L. (Kumagai et al., 1995). Later potato virus X (PVX–VIGS) was used to silence phytoene desaturase (PDS) in wild-type *Nicotiana benthamiana* Domin and to express green fluorescence protein (GFP) in transgenic *N. benthamiana* (Ruiz et al., 1998). However, tobacco rattle virus (TRV) has become the most widely used VIGS vector for members of the Solanaceae and Brassicaceae (Ratcliff et al., 2001; Burch-Smith et al., 2004; Chen et al., 2004; Fu et al., 2005; Burch-Smith et al., 2006; Dong et al., 2007; Godge et al., 2008), and the related pea early browning virus (PEBV) has been applied for developmental analysis of legumes (Constantin et al., 2004, 2008). TRV–VIGS has also recently been used for genetic analyses of the non-model basal eudicots, *Papaver somniferum* L. (Hileman et al., 2005; Drea et al., 2007), *Aquilegia* (Gould and Kramer, 2007), and *Eschscholzia californica* Cham. (Wege et al., 2007). Among the cereal crops,

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doi: 10.1093/mp/ssp033, Advance Access publication 19 June 2009
Received 19 March 2009; accepted 24 April 2009

VIGS using barley stripe mosaic virus (BSMV–VIGS) has been applied for barley (*Hordeum vulgare* L.) (Holzberg et al., 2002; Bruun-Rasmussen et al., 2007) and wheat (*Triticum aestivum* L.) (Scofield et al., 2005), but application of VIGS for monocots other than cereal grass species has not been described.

Because BSMV–VIGS has been very valuable for analysis of gene function in its natural host *Hordeum* (Hein et al., 2005; Oikawa et al., 2007; Shen et al., 2007) and in the closely related *Triticum* (Scofield et al., 2005; Cloutier et al., 2007; Fu et al., 2007; Zhou et al., 2007; Sindhu et al., 2008), we sought to determine whether the technology could be applied to tropical plants of the order Zingiberales. The Zingiberales (tropical gingers and bananas) exhibit a wide range of flower forms, making them an interesting system for investigating the role of specific gene families in the evolution of floral development (Figure 1). The order also exhibits substantial differences in growth habit; hence it is ideal for developmental studies on shoot, rhizome, and root systems. For this purpose, we designed a BSMV–VIGS vector to suppress PDS in the culinary ginger, *Zingiber officinale* Roscoe, using strategies similar to those successfully applied to barley (Holzberg et al., 2002) and wheat (Tai et al., 2005). Our results suggest wild-type (wt) BSMV is able to establish systemic infections of *Z. officinale* and *Costus spicatus* (Jacq.) Sw. We found that in *Z. officinale*, silencing of endogenous PDS (ZoPDS) results in white striations or fully photobleached leaves in systemically infected plants. We propose using *Z. officinale* as a model for studying gene function in non-grass monocots.

RESULTS

BSMV Is Able to Infect Members of the Zingiberales

BSMV has a very broad host range and infects several graminaceous hosts as well as some non-monocot species (Jackson and Lane, 1981). Although there is a single report of *Commelina communis* L. (Commelinaceae; Commelinales) susceptibility (Jackson and Lane, 1981), extensive studies have not been carried out on monocots belonging to families other than Poaceae, and, to the best of our knowledge, BSMV host range studies with the Zingiberales have not been conducted. Leaves of young *Z. officinale* shoots were inoculated with extracts of leaves from *H. vulgare* harboring the wt ND18 strain of BSMV. At 10 d after inoculation, newly emerging leaves developed a lightly striated mosaic phenotype (Figure 2B), and infection was confirmed with a Western blot for viral coat protein (CP) (Figure 3A) and by RT–PCR using primers targeting a 734-nt fragment within ORFs 3 and 4 of RNAβ (Figure 3B). In addition to *Z. officinale*, we tested the susceptibility of the closely related *C. spicatus* to BSMV. We were able to confirm the presence of the BSMV in all inoculated plants by Western blotting (Figure 3A) and RT-PCR (Figure 3B) in all *C. spicatus*-inoculated individuals.

Interestingly, new shoots of *Z. officinale* that developed from growing apices of rhizomes of plants previously infected with BSMV also developed symptoms of the viral infection. These shoots typically emerged 14–20 d post infection and do not appear to be delayed compared with uninfected plants. This observation supports past seed transmission and VIGS studies showing that BSMV is able to infect meristematic tissue of grasses (Jackson and Lane, 1981; Benedito et al., 2004). Our results also indicate that in *Z. officinale*, BSMV can move systemically from the inoculated leaves of a shoot into the rhizome system and infect new shoots arising from the rhizome. Because of the growth habit of *Z. officinale*, in which many genetically identical shoots can be generated from the same rhizome, only one shoot may need to be infected to obtain a large number of genotypically identical infected plants bearing terminal flowering shoots.

BSMV Can Elicit VIGS of ZoPDS in Ginger

To determine whether *Z. officinale* endogenous plant mRNAs can be silenced via a BSMV–VIGS approach, a fragment of the coding region of ZoPDS (GenBank accession number AF049356) was amplified by RT–PCR from *Z. officinale* mRNA. Once amplified, ZoPDS was sequenced and inserted at the 5′ terminus of the γb gene to create an infectious BSMV–VIGS vector unable to express the γb protein (Tai et al., 2005). The ZoPDS fragment is an excellent gene for VIGS assays because it encodes for an enzyme involved in the biosynthesis of carotenoids and, once silenced, PDS is unable to protect chlorophyll from photo-oxidation, resulting in photobleaching due to decreased carotene content (Kumagai et al., 1995; Benedito et al., 2004). Silencing of PDS in *H. vulgare* (Holzberg et al., 2002) and *T. aestivum* (Tai et al., 2005) has been shown to reduce levels of carotene content and to result in an obvious photobleached phenotype.

Endogenous gene silencing by BSMV–VIGS was accomplished by inoculating leaves of eight young *Z. officinale* shoots through leaf abrasion with a combination of BSMV RNA transcripts designated BSMVγ–ZoPDS. This combination consisted of RNAα, a modified BSMV RNAβ derivative (B7) that is deficient in expression of the coat protein (CP) (Petty and Jackson, 1990), and BSMV RNAγ–ZoPDS transcripts. The RNAβ and RNAγ modifications were introduced previously to enhance VIGS expression in barley and wheat (Holzberg et al., 2002; Tai et al., 2005). The 'B7' RNAβ mutant was originally engineered to eliminate CP expression by mutagenesis of the AUG initiation codon of the CP ORF (Petty and Jackson, 1990), and was used by Holzberg et al. (2002) to enhance BSMV–VIGS. Expression of the γb silencing supressor protein was also disrupted by creation of a *Bam*HI site to eliminate the γb AUG (Petty et al., 1990) and to provide a site for insertion of cloned DNA fragments (Bragg and Jackson, 2004).

Thirty days post inoculation with BSMVγ–ZoPDS, a silenced PDS photobleached phenotype appeared in the systemic leaves of all eight inoculated plants. Photobleaching was easily visible as partially or fully bleached sectors following the parallel veination along the length of the leaf blades (Figure 2C

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