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Virus-induced gene silencing in two novel functional plants, *Lycium barbarum* L. and *Lycium ruthenicum* Murr.

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ABSTRACT

Two species of Goji, *Lycium barbarum* L. and *Lycium ruthenicum* Murr., are novel functional vegetables and functional fruits widely used in China and other Asian countries. Both species possess complex secondary metabolic pathways and show high tolerance and adaptability to saline-alkali stress, making them novel targets for functional genetic analysis of the biochemical pathways involved. Although stable transgenic Goji lines have been produced, the process is very labor-intensive and time-consuming. Virus-induced gene silencing (VIGS) presents an effective and rapid alternative for creating targeted gene knock-outs to study gene function in plants. In this study, the first application of VIGS in *Lycium* species is presented, using the *Tobacco rattle virus* (TRV) vectors. A number of vector delivery methods were trialed, including leaf syringe-infiltration, agrodrench, seedling vacuum-infiltration and sprout vacuum-infiltration (SVI). Vacuum-infiltration was the most effective method and was used to successfully silence two reporter genes, *phytoene desaturase* (*PDS*) and *Mg-chelatase H subunit* (*Chl H*), concomitant with photobleaching and yellow leaf phenotypes, respectively. The proven application of VIGS to these *Lycium* sp. will expedite the functional characterization of novel genes involved in the biosynthesis of functional components both in leaves and fruits, as well as the abiotic stress tolerances.

1. Introduction

"Goji" refers to the Chinese species of genus *Lycium* (family Solanaceae). *Lycium barbarum* L. (generally called Goji berry or Wolfberry) and *Lycium ruthenicum* Murr. have been used as functional fruits and functional vegetables, as well as traditional Chinese herbs (Potterat, 2010). *L. barbarum*, in particular, is a crop of high economic importance in Northwest China, with the fruits (*Gouqizi* in Chinese) having high contents of *L. barbarum* polysaccharides (LBP), flavonoids and carotenoids that can improve eyesight, liver and kidney function (Wang et al., 2010; Yao et al., 2011). Young leaves of *L. barbarum* have also been used as functional vegetables with high contents of microelement, alkaloid, carotenoids and flavonoids (Potterat, 2010; Zhang and Yang, 2010). *L. ruthenicum* is another traditional herb used for treating heart disease, abnormal menstruation and menopause. Functional compounds in *L. ruthenicum* fruit have been recently investigated and include various pigments, essential oils and polysaccharides (Altintas et al., 2006; Li et al., 2006; Peng et al., 2012; Zheng et al., 2011). In addition to their nutritive values, *L. barbarum* and *L. ruthenicum* have high saline-alkali resistance, and can be functional specialty crops with a high economic value on harsh marginal lands (Wei et al., 2006; Zhang and Zhang, 2004).

Despite the importance of Goji in traditional Chinese herbs, its rapidly increasing popularity in the global novelty and functional foods market (Potterat, 2010), and its tolerance of abiotic stress, little information is currently available on the genetic pathways involved in the biosynthesis of secondary metabolites and

Abbreviations: VIGS, virus-induced gene silencing; TRV, *Tobacco rattle virus*; PDS, phytoene desaturase; Chl H, Mg-chelatase H subunit; SVI, sprout vacuum-infiltration; TCM, traditional Chinese medicine; LBP, *L. barbarum* polysaccharide; EST, expressed sequence tag; PTGS, post-transcriptional gene silencing; RISC, RNA-induced silencing complex; GOI, gene of interest.

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abiotic adaption. Sequence information for Goji is being rapidly generated, including expressed sequence tags (ESTs) and genomic sequences (Zeng et al., unpublished); the challenge now is to relate this large body of data to gene activity and function. Although stable transformation protocols for *L. barbarum* are available for gene function analyses (Chen et al., 2009; Hu et al., 2002; Zhu et al., 2010), they are considerably difficult and time-consuming. It is therefore important to develop a reliable, efficient, and high-throughput approach for studying gene function in these novel functional plant species. The advantages of virus-induced gene silencing (VIGS) provide an alternative tool to investigate gene functions in Goji.

VIGS is based on RNA-mediated post-transcriptional gene silencing (PTGS), which targets viral RNA in a sequence-specific manner, and has become one of the most widely used tools for plant functional genomics during the last decade (Burch-Smith et al., 2006; Gouldi and Kramer, 2007; Liu et al., 2002a; Senthil-Kumar and Mysore, 2011). Although the precise VIGS mechanism is still being studied, a clear outline of the VIGS processes has been defined (Llave, 2010). Upon infection of plant tissues, DNA and RNA viruses produce viral double-stranded RNA (dsRNA), which is then cut by a Dicer-like nuclease into viral small interfering RNAs (siRNAs) varying in length from 21 to 23 nt (Ding and Voinnet, 2007). At the same time, viral single-stranded RNAs (ssRNA) can be used as templates to produced dsRNA by plant RNA-dependent RNA polymerase (Donaire et al., 2008), which amplifies the initial silencing responses. The viral siRNAs associate with the RNA-induced silencing complex (RISC), which then targets homologous RNAs for cleavage and degradation (Llave, 2010).

Several autonomous plant RNA and DNA viruses have been developed as VIGS vectors (Huang et al., 2012; Senthil-Kumar et al., 2008), such as *Tobacco rattle virus* (TRV) (Ratcliff et al., 2001), *Cotton leaf crumple virus* (CLCrV) (Tuttle et al., 2008), and *Barley stripe mosaic virus* (BSMV) (Holzberg et al., 2002). In particular, TRV-based VIGS has become an important tool in the last decade because the virus has a broad host range, results in efficient silencing and produces mild symptoms on the plants (Liu et al., 2002b; Ratcliff et al., 2001). Another distinct advantage of TRV-based VIGS is the ability of this virus to spread rapidly throughout the entire plant, and it has been used to study gene functions in leaves (Ratcliff et al., 2001), flowers (Gouldi and Kramer, 2007), fruits (Fu et al., 2005) and roots (Dubreuil et al., 2009). Several methods have been developed to introduce the virus into plants, commonly involving *Agrobacterium* and including syringe-infiltration (Orzaez et al., 2006; Ratcliff et al., 2001), spray infiltration (Liu et al., 2002a), seedling vacuum-infiltration (Ekengren et al., 2003), agrodrench (Ryu et al., 2004), and sprout vacuum-infiltration (SVI) (Yan et al., 2012).

Although VIGS can be highly efficient for characterizing gene function in plants, there have been no reports on gene silencing in Goji using VIGS. The aim of this study was to develop a TRV-based gene silencing method for seedlings of *L. barbarum* and *L. ruthenicum*. Two recognized reporter genes, *phytoene desaturase* (*PDS*) and *Mg-chelatase H subunit* (*Chl H*) were selected as the target genes to assess the efficiency of the gene silencing protocol. *PDS* encodes an enzyme that catalyzes an important step in the carotenoid biosynthesis pathway (Hirschberg, 2001), and is commonly as a marker gene for visual examination of VIGS-induced silencing (Kumagai et al., 1995). Silencing of *PDS* in green tissues results in a photobleaching phenotype due to the blocked accumulation of Xanthophylls. The *Chl H* gene encodes the H subunit of magnesium-protoporphyrin chelatase, which is involved in chlorophyll biosynthesis. Silencing of *Chl H* in green tissues causes an easily recognizable yellow-colored phenotype as a result of a reduction in chlorophyll synthesis (Hiriart et al., 2002). This study examined different *Agro*-infiltration methods and provides a detailed protocol for successful silencing of gene function in two valuable *Lycium* sp. using VIGS.

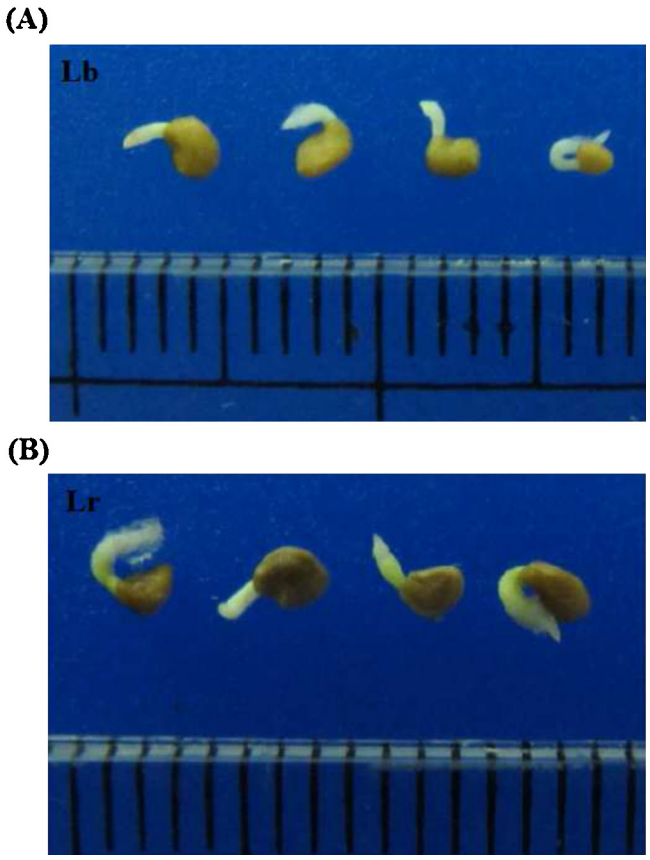

Fig. 1. Sprouts of *L. barbarum* (A) and *L. ruthenicum* (B) used for sprout vacuum-infiltration (SVI). Five days after germination (1–2 mm in length).

2. Materials and methods

2.1. Plant materials and growth condition

L. barbarum L. and *L. ruthenicum* Murr. seeds were immersed in distilled water for 10–12 h at 40–50 °C, then sown into tray substrate (Klasmann-Deilmann, Geeste, Germany) mixed with vermiculite at a 2:1 ratio. Plants were grown in a growth room at 23 °C with a photoperiod of 16 h/8 h of white light. Seedlings with 2–4 euphylla (about one-month old) were used for seedling vacuum-infiltration and agrodrench. For sprout vacuum-infiltration (SVI), seeds were germinated on filter paper moistened with sterile water and grown at 23 °C in a growth room until the sprouts reached 1–2 mm in length (5 days; Fig. 1). Sprouts were infiltrated, sown into the aforementioned soil mixture and returned to the growth room at 23 °C.

2.2. Isolation of the PDS and Chl H genes

Total RNA was isolated from the leaves of the two Goji species using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using SuperScript II™ (Invitrogen) according to manufacturer's instructions. Nucleotide sequences of *PDS* and *Chl H* were identified from a *L. ruthenicum* EST database using SlPDS (ABR57230) from tomato and NtChl H (AAB97152) from tobacco as queries in Blast searches. The full-length *PDS* and partial *Chl H* coding sequences (approximately 2140 bp and 1600 bp, respectively) were then PCR amplified from *L. barbarum* and *L. ruthenicum* cDNA