*Journal of Integrative Agriculture* 2016, 15(9): 2040–2048

Available online at www.sciencedirect.com

ScienceDirect

RESEARCH ARTICLE

# A novel strategy to enhance resistance to *Cucumber mosaic virus* in tomato by grafting to transgenic rootstocks

**BAI Miao[1*], CHEN Wen-ting[1*], XIE Bing-yan[2], YANG Guo-shun[1]**

[1] *College of Horticulture and Landscape, Hunan Agricultural University, Changsha 410128, P.R.China*
[2] *Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, P.R.China*

**Abstract**

*Cucumber mosaic virus* (CMV) can infect a wide range of host species. For the lacking of CMV resistant varieties of tomato, RNA interference (RNAi) can be used as a fast and effective method for the generation of transgenic resistant varieties. In this current study, five intron-spliced hairpin RNA (ihpRNA) plant expression vectors aimed at five genes of CMV have been constructed. Transgenic tomatoes were obtained by *Agrobacterium tumefaciens*-mediated transformation with expression vectors. Highly resistant generations of transgenic plants were employed as rootstocks and grafted onto non-transgenic tomatoes that resulted in the successful transfer of resistance to the scions. Using a novel method of plant cuttings for rootstock propagation, we obtained large quantities of disease-resistant material. Further, this method produces scions that can remain undetectable for transgenic resistance marker genes that may provide novel approaches to evade collective concerns about genetically-modified organism (GMO) biosafety.

**Keywords:** *Cucumber mosaic virus* (CMV), graft, RNA silencing, *Solanum lycopersicum*, virus resistance

## 1. Introduction

RNA interference (RNAi) refers to double-stranded RNA (dsRNA)-mediated degradation or inhibition of homologous target mRNA (Eamens *et al.* 2008; Voinnet 2009), which is widely used to tune the expression of genes in eukaryotic organisms. In plants, RNAi affects the expression level of target genes through post-transcriptional gene silencing (PTGS). The RNAi pathway in plants can be divided into three critical stages. First, double-stranded RNAs, such as miRNA precursors, hairpin RNAs, viral RNAs or transgene are processed into small RNAs by the RNaseIII-type activities of Dicer-like (DCL) proteins. Then, one strand of these small RNAs is loaded into the RNA-induced silencing complex (RISC), which contains argonaute proteins (AGOs). Interaction of AGOs with sequences complementary to the small RNAs results in silencing by either cleavage or blocking translation of the target mRNA (Voinnet 2009). In plants, RNA-dependent RNA polymerase (RdRp) are responsible for the synthesis of dsRNAs, and then the dsRNAs are cut into secondary small interfering RNAs (siRNAs) by DCL and give rise to a new round of RNA silencing (Lipardi *et al.* 2001). These secondary siRNAs support systemic silencing (Molnar *et al.* 2010).

In nature, plants frequently encounter a variety of viral

Received 12 November, 2015 Accepted 5 February, 2016
BAI Miao, Mobile: +86-18374879885, E-mail: baimiao1984@qq.com; Correspondence YANG Guo-shun, Tel: +86-731-84618767, Fax: +86-731-84618171, E-mail: guoshunyang@foxmail.com
[*] These authors contributed equally to this study.


doi: 10.1016/S2095-3119(16)61330-8

infections. RNAi has evolved as an antiviral immune strategy against viruses, called virus-induced RNA silencing (or RNA-based antiviral immunity). Indeed, virus-induced small RNAs (vsRNAs) that triggered host RNA silencing have been detected in many plants infected with viruses (Ding 2010; Llave 2010). However, vsRNAs are usually not sufficient to resist viral infection; thus, it is common to use transgenic over-expression of the virus-source small RNA to confer resistance to virus infection in plants. Inverted repeat (IR) structural sequences can form hairpin double-stranded RNA (hpRNA) that is processed by DCL to produce siRNAs. Thus, plant expression vectors containing IRs will generate siRNAs *in vivo* that target the viral RNA as part of an antiviral process. Previous studies have found that hpRNA engineered within an intron, called ihpRNA, are most efficient in gene silencing than other structures (Helliwell and Waterhouse 2003). Numerous studies indicated that RNAi technology is feasible for conferring virus resistance to plants (Wang and Metzlaff 2005; Md Ali *et al.* 2013). In addition, some studies have indicated that, siRNAs can be transported throughout the plant vascular system for a long-distance to direct mRNA silencing, and that process was called as systemic RNA silencing (Sonoda and Nishiguchi 2000; Yoo *et al.* 2004; Brosnan *et al.* 2007; Kalantidis *et al.* 2008).

Currently, almost every species of cultivated vegetables, fruits, and flowers are affected by various virus diseases, which inhibit the growth of plants, reduce fruit yield and quality, or even cause the death of the whole plant. *Cucumber mosaic virus* (CMV) is the typical member of the genus *Cucumovirus*, family *Bromoviridae*, which infect a wide host range of plants (Palukaitis and Garcia-Arenal 2003). CMV is easily mechanically transmitted and most crop plants do not have resistance genes in the germplasm. Thus, CMV has been one of the primary targets for the development of transgene-mediated resistance (Harrison *et al.* 1987; Baulcombe 1996).

Although genetic engineering techniques provide an efficient way to confer virus resistance for many plant species, they are often labor-intensive and costly. Grafting can be able to maintain desired traits for scion and rootstock, and widely be used in breeding for most of fruit trees and vegetables. It has been documented that one rootstock variety can be used to graft different scion varieties. Based on this, if a viral resistance of rootstock could be improved *via* an RNAi strategy, scions grafting to this rootstock would obtain the viral resistance. And the non-GMO (genetically modified organism) scions would receive siRNAs but no other genetically modified ingredients from the rootstock. This would make positive significance for GM crops promotion.

## 2. Materials and methods

### 2.1. Plasmid construction

The CMV genome consists of three single-stranded RNAs and encodes five viral proteins, including proteins 1a encoded on RNA1, 2a and 2b encoded on RNA2, 3a and the coat protein (CP) encoded on RNA3 (Roossinck 2001). Fragments from the five genes of CMV strain Fny (Fny-CMV, Appendix A) were selected for tomato transformation experiments. These fragments were analyzed for potential off-target effects in the *Solanum lycopersicum* SGN (http://www.sgn.cornell.edu/index.pl) unigene database with the tool siRNA Scan (http://bioinfo2.noble.org/RNAiScan.htm; Xu *et al.* 2006). These fragments were amplified by polymerase chain reaction (PCR) from a vector containing the Fny-CMV gene using specific primers, 1AF/1AR, 2AF/2AR, 2BF/2BR, 3AF/3AR, and CPF/CPR (Appendix B). These primers were designed to incorporate restriction enzyme sites of *Bam*HI and *Sal*I to each amplicon. The cloning vector, pUCCRNAi (Luo *et al.* 2006) (Appendix C) contained two sets of restriction sites with the same overhang (*Xho*I/*Sal*I and *Bgl*II/*Bam*HI) and was used to insert IR sequences for the construction of ihpRNA. Constructs containing CMV-RNAi fragments were named pUCCRNAi-1A, -2A, -2B, -3A, and -CP, respectively. *Sal*I/*Pst*I restriction fragments obtained from the cloning vectors were next ligated to the plant expression vector, pCAMBIA2300-Actin1-ocs (Fang *et al.* 2008). These vectors were named as pCAMBIA2300-1A, -2A, -2B, -3A, and -CP. The generation of the plant ihpRNA expression vector is shown in Appendix A.

### 2.2. Tomato transformation and regeneration

The tomato transformation protocol (Frary and Van Eck 2005) was modified. In the study, cotyledons were employed to transformation upon emergence from seed coats and pre-cultured in medium A for 2 d. Then, the explants co-cultures with *Agrobacterium tumefaciens* suspensions for 10 min, and transferred explants to medium B for 2 d at 26°C. After that, explants were cultured on medium C for 7–10 d and the kanamycin resistance ($Kan^R$) shoots would be emergence. Then these shoots were separated and transferred to medium C1 for 1–2 weeks and then transferred to medium C2 and grown to 1–1.5 cm. For the other steps, protocol described by Frary and Van Eck (2005) was followed. All media were listed in Appendix D.

### 2.3. Molecular screening of transgenic plants

For PCR screening of transgenic plants, total DNA was isolated from leaves of $Kan^R$ plants by the hexadecyl trimethyl