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Transient expression of siRNA targeted against the TYLCV AV1, AC1 and AC3 genes for high resistance in tomato

Sheng Sun[1], Xiu-Ping Kang[1], Xiao-Juan Xing, Zhi-Feng Chen, Shao-Wen Zheng, Guo-Ming Xing*

College of Horticulture, Shanxi Agricultural University, Taigu, Shanxi 030801, China

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ABSTRACT

Tomato yellow leaf curl virus (TYLCV) is a globally devastating disease that affects the production of many crops, particularly tomato, leading to considerable economic losses. RNA interference, which allows sequence-specific gene silencing at the post-transcriptional level, is an effective method to obtain virus-resistant lines of crops. *Agrobacterium*-mediated transient expression assay has emerged as a rapid and useful approach to evaluate gene functions in plants without the need to produce transgenic lines. In this study, we transformed the plant expression vector pBIN438-AV1–AC1–AC3(i/r), which consists of the inverted repeat of ΔAV1, ΔAC1, and ΔAC3 fusion fragments into *Agrobacterium tumefaciens* EHA105 to generate tomato lines with high viral resistance. The viral resistance of the fusion gene was evaluated by transient expression. The test plants did not show disease symptoms at 35 d postinoculation with TYLCV. This study provided an important method for plant antiviral breeding and safe tomato production.

1. Introduction

Tomato yellow leaf curl virus (TYLCV), which belongs to the *Begomovirus* Genus of Geminiviridae, is a globally devastating viral disease of cultivated tomato (Yang et al., 2004). Symptoms of tomato infection include leaf curling, crimple, yellow, plant dwarf, and yield loss ranging from 20% to 100% depending on the stage of plant growth at the time of infection, leading to severe economic losses in tomato production areas (Pan et al., 2012). In mid-2006, TYLCV was first detected in symptomatic tomato plants in Shanghai (Wang et al., 2007). Since then, TYLCV has moved toward other provinces (such as Zhejiang, Jiangsu, Guangdong, Fujian, and Shandong) with unprecedented speed, making it the most virulent begomovirus in tomato crops (Zhang et al., 2009). Therefore, feasible and effective approaches for antiviral breeding need to be developed.

Classical breeding has introduced resistance genes from wild origin into the cultivated tomato by cross breeding. Some TYLCV resistance genes, such as TY-1, TY-2, TY-3, TY-4, and TY-5, are effective but do not provide complete resistance (Brunetti et al., 1997). Recent studies have introduced and expressed foreign genes that affect the infection cycle to endow plant resistance (Brunetti et al., 1997). Truncated, entire, or mutated genes of TYLCV (Bendahmane and Gronenborn, 1997; Kunik et al., 1994; Yang et al., 2004) or other foreign genes have been targeted for the viral host (Akad et al., 2007; Edelbaum et al., 2009). This approach enhances resistance to TYLCV at some extent. RNA interference (RNAi), a natural mechanism that directs specific gene silencing, is an invaluable research tool for evaluating gene functions and controlling plant viruses (Hamilton and Baulcombe, 1999). RNAi-induced antiviral defense in plants was established in the late 1990s; in this mechanism, the plant-expressing part of the gene fragment of the virus genome shows enhanced resistance against virus infection (Agius et al., 2012; Sharma et al., 2013). Many studies demonstrated that siRNA expression is an effective strategy against TYLCV (Ben Tamarzizt et al., 2009; Fuentes et al., 2006; Peretz et al., 2011; Ramesh et al., 2007), especially in tomato (Sahu et al., 2010, 2012).

With the evolution of TYLCV, these engineered plants have shown temporary resistance to the virus and delayed symptoms. Thus, we employed targeted multigene silencing to generate highly resistant tomato. Previous research showed that the genome of TYLCV has two open reading frames on the virion-sense strand (AV1 and AV2) and four on the complementary-sense strand (AC1, AC2,

Abbreviations: TYLCV, tomato yellow leaf curl virus; RNAi, RNA interference; NCBI, National Center for Biotechnology Information; siRNA, small interference RNA; ORF, open reading frames; PDS, phytoene desaturase.

* Corresponding author. Tel.: +86 451 6946299; fax: +86 0451 6946622.
E-mail address: sunsheng77@aliyun.com (G.-M. Xing).

[1] These authors contributed equally to this work.

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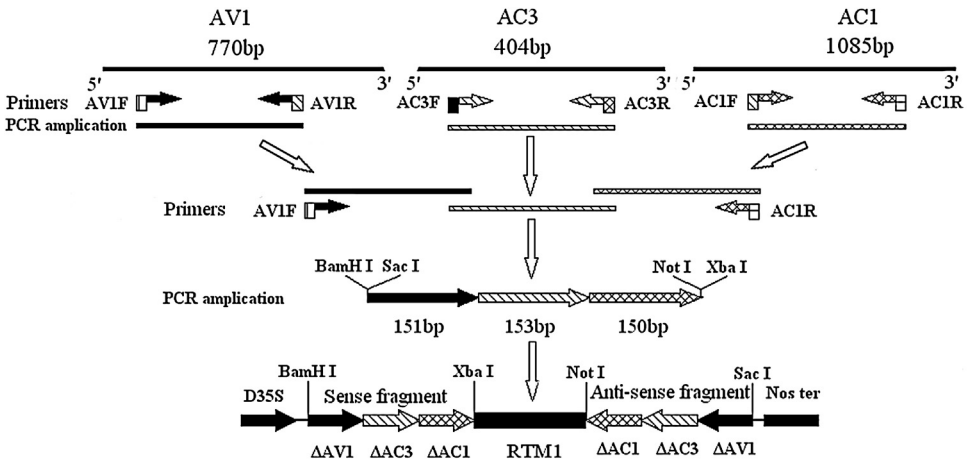

Fig. 1. Strategy for fusion gene construction in the T-DNA region of pBIN438-AV1–AC3–AC1(i/r). Target genes (AV1, AC3, and AC1) were amplified by specific primers, containing 10 bp in length to fusion gene, and ligated into one gene by overlap PCR amplification. Two copies of fusion gene were ligated with RTM1 intron in an inverted repeat manner. The recombinant fragments were then inserted into the binary vector pBIN438 under the control of double 35S promoter.

AC3 and AC4) (Navot et al., 1991). AV1 encodes coat protein (CP) and is likely involved in virus nucleo-cytoplasmic shuttling; its siRNA targeted against CP can confer resistance to TYLCV in transgenic tomato plants (Gorovits et al., 2013; Zrachya et al., 2007b). AV2 participates in virus infection and suppresses RNA silencing (Zrachya et al., 2007a). AC1 initiates the rolling circle replication, and AC1 silencing impedes the growth and spread of TYLCY (Fuentes et al., 2006; Ramesh et al., 2007). AC2 encodes a host-range factor, which also suppresses RNA silencing (Dong et al., 2003; Glick et al., 2008; Zhang et al., 2012). AC3 encodes a replication accessory factor that enhances viral DNA accumulation; its siRNA affects the accumulation of TYLCY in plants (Sabourrezk et al., 2006). AC4 is a small RNA regulator and RNAi suppressor (Chellappan et al., 2005). In our study, AV1, AC1, and AC3 are target sequence candidates for the production of virus-resistant plants. Tomato was infiltrated with *Agrobacterium* harboring the intron-hairpin RNA construct that simultaneously targets AV1, AC1, and AC3. This approach of expressing the gene of interest can induce RNAi with 100% efficiency. We showed that tomato plants can produce siRNAs using *Agrobacterium*-mediated transient expression system, which was developed as a rapid and useful method to enable RNA silencing in plants (Johansen and Carrington, 2001). These tomato plants, inoculated with TYLCV at 20 d postagroinfiltration, showed no disease symptoms at over 35 d postinoculation.

2. Materials and methods

2.1. Selection of target sequences

The reference sequences of TYLCV AV1, AC1, and AC3 gene (GenBank accession number AM050555.1) were obtained from the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) and compared with those of the TYLCV sequence found in other regions in China by nucleotide (nt) BLAST. The other accession numbers of TYLCV are JQ768339, JX239482, KC312665, KC312662, JQ768340, GU348995.1, GU951436.1, JF817218.1, and JN990922.1. After nucleotide sequence alignment, conserved regions of TYLCV AV1, AC1, and AC3 were selected as candidate sequences. All fragments were further analyzed by siRNA design algorithm DEQOR to make sure that they are capable of producing potent siRNAs (Henschel et al., 2004).

2.2. Plasmid construction

The basal binary vector pBIN438 for all constructs contained enhanced 35S promoter from cauliflower mosaic virus, TEV 5′-non-translated sequence, and NOS terminator, with little modification.

The pBIN438–AV1–AC1–AC3(i/r) targeted against the AV1, AC1, and AC3 genes of TYLCV was constructed using strategy shown in Fig. 1. First, the truncated TYLCV AV1, AC1, and AC3 gene fragments were amplified by PCR from TYLCY-infected tomato plants from Shanxi Province. Specific primers was designed and showed in Table 1. Primers AV1R, AC3F, AC3R, and AC3R possess arms to further construct fusion gene while AV1F and AC1R to further construct gene in binary vector. Second, the three PCR products were mixed fully and used as template for second PCR with AV1F and AC1R as primers. Conditions of PCR were one cycle at 94 °C (denaturation) for 1 min, and then 25 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and then 72 °C for 10 min. The resulting PCR product was cloned in pGEM-T-EASY vector (Promega, Madison, WI, USA) and confirmed by sequencing. Last, fusion gene was then sub-cloned into intron side from *RTM1* gene of Arabidopsis (Chisholm et al., 2000) in pBluescript II KS+ (Stratagene, La Jolla, CA). Then, a 1 kb BamH I–Sac I fragment, containing the two oppositely oriented arms, was cloned into the same sites in the binary vector pBIN438. The resulting plasmid was designated pBIN438-AV1–AC1–AC3(i/r).

The pBIN438-AV1(i/r): 151 bp truncated AV1 was amplified by using specific primers AV1F and AV1r. The resulting PCR product was digested by restriction enzyme to generate sense (BamHI & Xba I) and antisense (Not I & Sac I) fragments and then sub-cloned into *RTM1* intron side in pBluescript II KS+. Clones were verified by sequencing and the correct fragment was cloned into the same sites in the binary vector pBIN438.

The pBIN438-AC1(i/r): 150 bp truncated AC1 was amplified by specific primers AC1f and AC1r. The resulting PCR product was digested by restriction enzyme to generate sense (BamHI & Xba I) and antisense (Not I & Sac I) fragments and then sub-cloned into *RTM1* intron side in pBluescript II KS+. Clones were verified by sequencing and the correct fragment was cloned into the same sites in the binary vector pBIN438.

The pBIN438-AC3(i/r): 153 bp truncated AC3 was amplified by specific primers AC3f and AC3R. The resulting PCR product was digested by restriction enzyme to generate sense (BamHI & Xba I) and antisense (Not I & Sac I) fragments and then sub-cloned