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mRNA amplification system by viral replicase in transgenic plants

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We have constructed transgenic tobacco plants (M1×2-FCP2IFN plants) expressing viral RNA replication genes of brome mosaic virus (BMV) and BMV RNA3 derivative (FCP2IFN) carrying the human gamma interferon (IFN-γ) gene. In M1×2-FCP2IFN plants the RNA3 derivative expressed from the integrated cDNA was replicated and subgenomic RNA (i.e. mRNA of IFN-γ) was produced by BMV replicase. The accumulation level of the mRNA of IFN-γ was approximately 5-fold higher than that by the cauliflower mosaic virus (CaMV) 35S RNA promoter. In addition IFN-γ accumulated in M1×2-FCP2IFN plants.

Transgenic plant; Viral replicase; Brome mosaic virus

1. INTRODUCTION

Recently transgenic plants have been used for producing desired proteins such as nuropeptides [1], blood factors [2] and antibody [3]. However, yields of those protein products in transgenic plants are often disappointingly low. This may be due to lack of a strong promoter suitable for high expression of foreign genes in plant cells. It seems that one of the key determinants for the success in agricultural production of a valuable foreign protein is how to level up the expression of foreign genes in transgenic plants.

To develop a more efficient system for high-level synthesis of foreign proteins in plants, an RNA virus replication system based on RNA-dependent RNA synthesis is very attractive since the replication system at RNA level can apply to amplification of mRNA for a foreign gene in transgenic plant cells and may surmount several impediments encountered in the production of foreign protein in the DNA-directed transcription system. In this paper we present a new system (an in vivo mRNA amplification system) in which DNA-directed mRNA can be amplified in vivo by viral replicase expressed from the integrated cDNAs in transgenic plants.

We used brome mosaic virus (BMV), a positive sense single-stranded RNA plant virus. The genome of BMV is divided among three 5′-capped separate RNAs (designated RNAs 1, 2 and 3) [4]. RNA 1 and RNA 2 encode the helicase-like 1a protein and the polymerase-like 2a protein, respectively, which are required for viral RNA replication [5–8]. BMV RNA3 encodes the 3a protein, which is thought to be involved in cell-to-cell movement of viruses in plants [9,10], and the coat protein gene. The coat protein is translated from a subgenomic mRNA, RNA4, which is synthesized from the negative strand of RNA3 [11].

Recently, we have demonstrated that transgenic tobacco plants (M1×2 plants) expressing BMV RNA replication genes 1a and 2a under control of the CaMV 35S RNA promoter support RNA-dependent replication of RNA 3 in transfected protoplasts [12]. In this paper we introduced a cDNA of BMV RNA 3 derivative (FCP2IFN) [13] carrying the human gamma interferon (IFN-γ) gene linked to the 35S promoter into the chromosomal DNA of M1×2 plants. We show the replication of FCP2IFN and production of subgenomic mRNA of IFN-γ in M1×2-FCP2IFN plants.

2. MATERIALS AND METHODS

2.1. *Plasmid construction*

A plasmid pARK22 (kindly provided from Dr. H. Anzai) containing a *bar* gene was digested with *Pst*I, made blunt with T4 DNA polymerase and self-ligated to remove the *Pst*I site, creating pARK22(−Pst). The pARK22(−Pst) was cut with *Eco*RI and made blunt with T4 DNA polymerase, and then ligated with *Pst*I linkers, creating pARK22(E-P). The binary vector pBICP35 [14] containing the 35S promoter and termination signal was partially cut with *Pst*I and digested with *Hin*dIII. The resulting plasmid was ligated with the *Pst*I–*Hin*dIII-cut fragment of pARK22(E-P), creating pBICBP35. The fragment of *Sna*BI–*Eco*RI-cut pBB3 [14] (i.e. full-length cDNA of RNA 3) was inserted into *Stu*I–*Eco*RI-cut pBICBP35, creating pBICBPBR3. The fragment of *Xba*I–*Eco*RI-cut fragment of pBTCP2IFN was inserted into *Xba*I–*Eco*RI-cut pBICBPBR3, creating pBICBPCP2IFN.

2.2. *Plant transformation*

pBICBPCP2IFN was mobilized into *Agrobacterium tumefaciens* (strain LBA4404) using *E. coli* HB101 harboring pRK2013, as described [15]. Transformation of *N. tabacum* cv. Petit Habana (SR1) was carried out using the leaf disc transformation method [16]. Transformed shoots and roots were selected by propagation on medium containing both kanamycin and bialaphos and eventually transferred to a glasshouse for production of mature plants.

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2.3. *RNA isolation and Northern blot analysis*

RNA was isolated from tobacco plants as described [13]. The RNA was denatured in formaldehyde/formamide and fractionated on a 1.5% agarose gel containing 1.8% formaldehyde and transferred to a BIODYNE membrane. Detection of positive-sense viral RNA was by a ^{32}P-labeled SP6 transcript from a subclone containing the 200-base *Hin*dIII (nt 1914)–*Eco*RI 3′-terminal fragment of BMV RNA 3 cDNA which is conserved among all BMV RNAs. To detect negative-sense viral RNA we used ^{32}P-labeled positive-sense RNA of the 200-base 3′-terminal fragment.

2.4. *Protein analysis*

Total protein was extracted from transgenic plants with Laemmli buffer [17], and separated on a 15% polyacrylamide gel containing 0.1% SDS. Western blot analysis was carried out as described [13]. Electrophoresed proteins were transferred to an Immobilon-P transfer membrane (Millipore) and probed with antibody against IFN-γ.

3. RESULTS

3.1. *Production of transgenic tobacco plants expressing both BMV replicase and FCP2IFN*

Transgenic tobacco plants (M1×2 plants) [12] expressing BMV replication gene 1a and 2a proteins were transformed with an *Agrobacterium* binary vector (designated pBICBPCP2IFN) which contains full-length cDNA of FCP2IFN [13] linked to the CaMV 35S RNA promoter and a *bar* gene as a selection marker that confers resistance to bialaphos (Fig. 1). FCP2IFN is a BMV RNA 3 derivative having the IFN-γ gene replacing the truncated coat protein (CP2) gene [18] in RNA3. FCP2IFN is able to replicate and produce subgenomic mRNA of IFN-γ in tobacco protoplasts when coinoculated with BMV RNA1 and RNA2 transcripts [13].

Expression of BMV replicase and FCP2IFN was tested as described below. Transgenic tobacco plants resistant to both bialaphos and kanamycin were obtained. Integration of cDNAs was confirmed by Southern blot analysis. Transgenic tobacco plants expressing both BMV replicase and FCP2IFN were designated M1×2-FCP2IFN plants. F_1 progenies with a different pattern of gene expression were obtained by self-pollination of primary transformants, since both BMV replication genes and cDNA of FCP2IFN in R_0 plants are not homozygous. In this study we used three kinds of F_1 progenies expressing replicase alone (M1×2 plants), FCP2IFN alone (designated FCP2IFN plants) and both BMV replicase and FCP2IFN (M1×2-FCP2IFN plants).

3.2. *Replication of FCP2IFN and production of subgenomic mRNA in transgenic tobacco plants expressing both BMV replicase and FCP2IFN*

The expression and replication of FCP2IFN and the production of subgenomic mRNA in M1×2-FCP2IFN plants were analyzed by Northern blotting with strand-specific probes for the conserved 3′-terminal non-coding sequence of the wild-type BMV RNAs (Note that these 3′-terminal sequences are not present in viral replicase transcripts expressed in M1×2 plants). The use of a positive strand-specific probe did not give any detectable bands in M1×2 plants (Fig. 2A, lane 2). However, a band with mobility slower than that of FCP2IFN observed in FCP2IFN-infected tobacco protoplasts was detected in both M1×2-FCP2IFN and FCP2IFN plants (Fig. 2A, lanes 3 and 4). These bands must be the 35S promoter-driven transcripts with non-viral and polyadenylated sequences at the 3′ end. In addition to the 35S promoter-driven transcripts, in M1×2-FCP2IFN plants two more bands with the same mobility as those of FCP2IFN and its subgenomic RNA were observed in FCP2IFN-infected tobacco protoplasts (Fig. 2A, lane 4). The replication of FCP2IFN and transcription of subgenomic RNA from FCP2IFN by viral replicase in M1×2-FCP2IFN plants was confirmed by the presence of the negative-strand RNA which comigrated with the negative-strand FCP2IFN in FCP2IFN-infected tobacco protoplasts (Fig. 2B). These results indicate that in M1×2-FCP2IFN plants BMV replicase initiated negative strand synthesis at a correct site and eliminates the extra nucleotides at their 3′ end and leads to replication of authentic FCP2IFN and transcription of subgenomic mRNA from the negative-strand FCP2IFN [14,19]. The accumulation level of the subgenomic RNA was approximately 5-fold larger than that of the 35S-driven FCP2IFN and was approximately 0.05% of the total extracted RNA (data not shown).

3.3. *Accumulation of human gamma interferon in M1×2-FCP2IFN plants*

Immunological analysis using antibody against

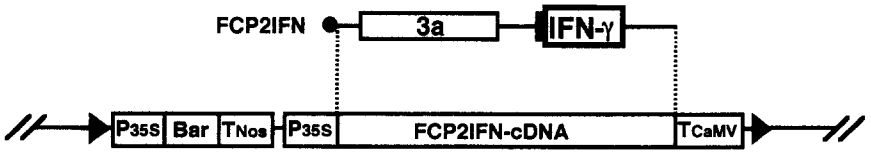

Fig. 1. Schematic representation of the binary vector for RNA 3 derivative carrying gamma interferon gene (FCP2IFN). ●, cap structure; 3a, 3a gene; ▶, T-DNA border sequence; P_{35s}, cauliflower mosaic virus 35S promoter; T_{CaMV}, 35S terminator; Bar, bar gene; T_{Nos}, nopaline synthase terminator. The shadowed box indicates 24 nucleotides coding for the N-terminal amino acids of a full-length coat protein.

IFN-γ revealed that IFN-γ with M_rs of approximately 23 kDa and 18 kDa was detected in total protein extract from M1×2-FCP2IFN plants but not in protein from either M1×2 or FCP2IFN plants (Fig. 3). These 23 kDa and 18 kDa proteins may be glycosylated and non-glycosylated IFN-γ, respectively [13].

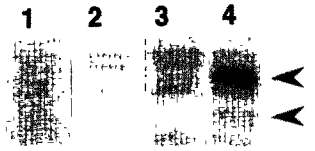

Fig. 3. Immunological analysis of gamma interferon produced in transgenic tobacco plants. Total proteins were extracted from non-transgenic tobacco plants (lane 1), M1×2 plants (lane 2), FCP2IFN plants (lane 3) and M1×2-FCP2IFN plants (lane 4), and separated on 15% SDS-PAGE. IFN-γ was identified by immunoblotting. The position of IFN-γ is indicated in the margin.

4. DISCUSSION

The results presented here show that DNA-directed RNA transcripts were amplified and subgenomic mRNAs from the transcripts were produced by viral replicase expressed in transgenic plants. This mRNA amplification system holds potentiality to overcome low production of mRNA by the DNA-directed transcription system. Furthermore, this system is more suitable for mass production of foreign proteins in plants than a direct viral system in which foreign proteins are produced by inoculating plants with a genetically engineered virus carrying foreign genes. These mutant viruses having foreign genes by gene replacement or insertion in viral genome have poor or no ability to infect a whole plant [8,20–22]. Even though the recominant virus replicates in a whole plant [23–25], the infection procedure will be an obstacle to scale up this system to a farm level.

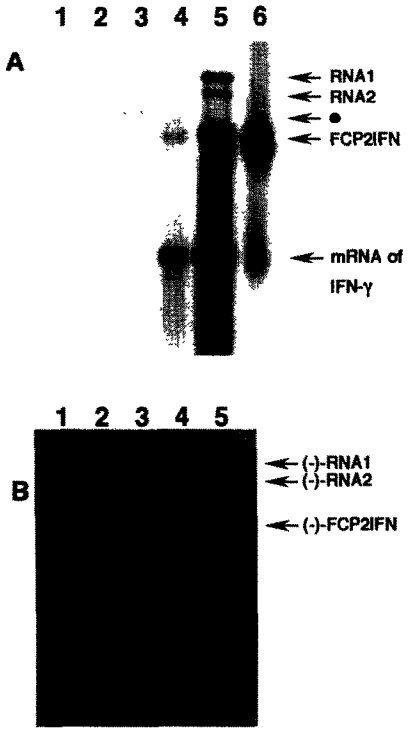

Fig. 2. Northern blot analysis of replication of FCP2IFN in transgenic tobacco plants expressing BMV replicase. Total RNAs were extracted from non-transgenic tobacco plants (lane 1), M1×2 plants (lane 2), FCP2IFN plants (lane 3), M1×2-FCP2IFN plants (lane 4) and tobacco protoplasts inoculated with FCP2IFN together with RNA 1 and RNA 2 (lane 5). Lane 6 contains 500 pg of in vitro synthesized FCP2IFN and its subgenomic mRNA of IFN-γ. The RNA (5 μg in lane 1–4A, 1.25 μg in lane 5A, 10 μg in lane 1–4B, 2.5 μg in lane 5B) was separated on a 1.5% agarose gel containing formaldehyde, and transferred to a nylon membrane. (A) Positive-sense RNA was detected by a ^{32}P-labeled in vitro transcript complementary to the conserved 3′ terminal 200 bases of FCP2IFN and wild-type BMV genomic RNAs.(B) Negative-sense RNA was detected by a ^{32}P-labeled positive sense in vitro transcript of the conserved 3′ 200 base of FCP2IFN and wild-type BMV genomic RNAs. The positions of viral RNAs are indicated in the margin. ● indicates the 35S promoter-driven transcripts of FCP2IFN.

The expression level of mRNA in our in vivo mRNA amplification system is approximately fivefold greater than that of the CaMV 35S promoter-driven transcripts, however this system has many potentialities for further improvement. In this research tobacco plants (non-host plant of BMV) were used for the easiness in obtaining transgenic plants. The use of host plants of BMV in the system will improve the efficiency of mRNA amplification, since the replication level of BMV in host plants such as barley or *Nicotiana benthamiana* is much higher than that in non-host tobacco plants [26].

The use of a cDNA cassette containing self-cleavage sequence at the 3′ end of FCP2IFN will improve the template activity of foreign gene transcripts to viral replicase compared with the CaMV 35S driven-FCP2IFN which has heterologous sequences at the 3′ end [19].

The amplification of mRNA can be controlled at the transcription level in either the foreign gene or viral replication genes if these genes are joined to various promoters instead of the constitutive CaMV 35S promoter. Inducible and organ-specific promoters will open the way to produce the desired proteins at the desired time and desired tissues.

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