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BSMV genome mediated expression of a foreign gene in dicot and monocot plant cells

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Barley stripe mosaic virus (BSMV) possesses a tripartite genome composed of RNAs α, β and γ that have been cloned into *in vitro* transcription vectors from which infectious transcripts can be obtained. The BSMV genome has been engineered here to serve as an expression vector in plant protoplasts. Open reading frame (ORF) b of RNA β, encoding a non-structural protein, was replaced by the firefly luciferase (*luc*) reporter gene to yield RNA β2–*luc*. In the presence of both RNAs α and γ, RNA β2–*luc* mediated efficient expression of the *luc* gene upon transfection into tobacco and maize protoplasts. This expression ranged from 20- to 123-fold higher than the luciferase activity obtained from transfection with a *luc* gene mRNA. Replication of RNA β and its derivatives i.e. 'minus' strand synthesis, was confirmed by Northern analysis, indicating that the high level of *luc* gene expression using RNA β2–*luc* resulted from RNA amplification. ORFa of RNA β, encoding the coat protein, was also replaced by the *luc* gene to yield RNA β1–*luc*. Although transfection of RNA β1–*luc* alone produces luciferase efficiently, neither 'minus' strand synthesis nor further increase of luciferase activity was observed in the presence of RNAs α and γ, or RNAs α, β and γ, suggesting that the deleted sequences within ORFa are *cis*-acting for replication of RNA β. Our results demonstrate that a foreign gene can be expressed by replacement of a viral non-structural protein gene that is essential for virus multiplication in plants, leading to a potential strategy for virus 'containment' with use of 'disarmed' plant viral vectors.

Key words: barley stripe mosaic virus/firefly luciferase reporter gene/gene expression in protoplasts/plant viral vectors/RNA viruses

Introduction

For expression of foreign genes in plants, there are several potential advantages in developing plant virus genome based vectors to supplement currently used methods of gene transfer (for reviews see Goodman *et al.*, 1987; Weising *et al.*, 1988; Gasser and Fraley, 1989). (i) Systemic infection of whole plants by viruses precludes the need for stable incorporation of the desired gene into the plant genome. (ii) Viruses multiply as autonomous entities to a high copy number within a plant cell and permit amplification of a foreign gene inserted into the viral genome. (iii) Gene expression via viral vectors would not encounter the 'position' effect often observed when foreign genes stably integrate into the plant chromosome. (iv) The use of viral vectors could provide flexibility in changing rapidly the genes to be expressed and (v) offers the possibility to express specific genes during a particular stage of plant growth and development.

For a number of years, cauliflower mosaic virus (CaMV; a caulimovirus) was considered as a virus of choice for the construction of plant gene expression vectors based on its more readily manipulatable double stranded (ds) DNA genome. Although expression of dihydrofolate reductase and metallothionein genes in turnip plants mediated by CaMV genome derived vectors have been obtained (Brisson *et al.*, 1984; Lefebvre *et al.*, 1987), the biology of CaMV multiplication imposes severe limitations on inserting foreign DNA into the viral genome. Moreover, since the host range of caulimoviruses is limited to only a few dicotyledonous plants, attention has focused recently on certain geminiviruses that infect a wider range of both dicots and monocots (for a review see Stanley, 1985). The genome of this latter class of viruses is composed of either one or two circular single stranded (ss) DNA molecules that replicate via a ds DNA intermediate; ds DNA clones of the viral genome are infectious and can be genetically modified. In the bipartite genome of two geminiviruses, tomato golden mosaic virus (TGMV) and cassava latent virus (CLV), the coat protein gene is not required for systemic infection. Thus, gene substitution vectors have been engineered using the TGMV (Hanley-Bowdoin *et al.*, 1988; Hayes *et al.*, 1988a) and CLV genomes (Ward *et al.*, 1988) by replacing the coat protein gene with reporter genes such as the *cat* (chloramphenicol acetyltransferase), *neo* (neomycin phosphotransferase) or *gus* (β-glucuronidase) gene. These reporter genes were shown to be expressed when the chimeric viral genomes were inoculated into tobacco plants or petunia leaf explants. The potential use of these geminivirus genome derived vectors could be limited, however, since efficient infection of plants with the viral DNA requires *Agrobacterium*-mediated inoculation (agroinfection or agroinoculation, Grimsley *et al.*, 1986, 1987; Elmer *et al.*, 1988; Hayes *et al.*, 1988b).

The vast majority of plant viruses consists of 'plus' strand RNA viruses that are also the best studied from the point of view of the structure, translation and replication of the viral genome (for reviews see Atabekov and Morozov, 1979; Davies and Hull, 1982; Van Vloten-Doting and Neeleman, 1982; Joshi and Haenni, 1984). However, engineering gene expression vectors based on RNA viruses has awaited the successful cloning of the viral genomes into *in vitro* transcription vectors from which infectious transcripts can be obtained. Since the cloning of the cDNAs of brome mosaic

virus (BMV, a bromovirus; Ahlquist *et al.*, 1984; Ahlquist and Janda, 1984; Janda *et al.*, 1987) and tobacco mosaic virus (TMV, a tobamovirus; Dawson *et al.*, 1986; Meshi *et al.*, 1986), this major limitation is being overcome rapidly for a number of plant RNA viruses (i.e. Allison *et al.*, 1988; Vos *et al.*, 1988; Domier *et al.*, 1989; Eggen *et al.*, 1989; Heaton *et al.*, 1989; Petty *et al.*, 1988, 1989; Weiland and Dreher, 1989; Hemenway *et al.*, 1990). In the case of BMV (French *et al.*, 1986) or TMV (Takamatsu *et al.*, 1987; Dawson *et al.*, 1989), the coat protein genes have been replaced by the *cat* gene and the resulting viral vectors expressed the *cat* gene in barley protoplasts or in the inoculated leaves of tobacco plants, respectively.

In this communication, we have investigated the expression of the firefly luciferase (*luc*) gene in both mono- and dicotyledonous plant protoplasts mediated by the genome of barley stripe mosaic virus (BSMV, a hordeivirus) and compared the luciferase activity with that from transfection of protoplasts with a *luc* gene mRNA (*luc* mRNA) or a DNA plasmid containing a CaMV 35S promoter–*luc*–*nos* 3′ terminator [poly(A) region] fusion. Luciferase catalyzes the ATP-dependent oxidation of luciferin, a small organic substrate, and in the process produces light (DeLuca and McElroy, 1978). Since the luciferase assay is rapid, sensitive, inexpensive and produces quantitative results (Seliger and McElroy, 1960; deWet *et al.*, 1987), the *luc* cDNA has been used as a 'reporter' of transient and stable gene expression in plant cells (Ow *et al.*, 1986, 1987; Gallie *et al.*, 1989).

For environmental release of genetically engineered expression vectors based on viral genomes, one major hurdle to be overcome resides in controlling spread of such vectors outside the target area. To address this concern, a possible strategy permitting virus 'containment' for the use of viral vectors is discussed.

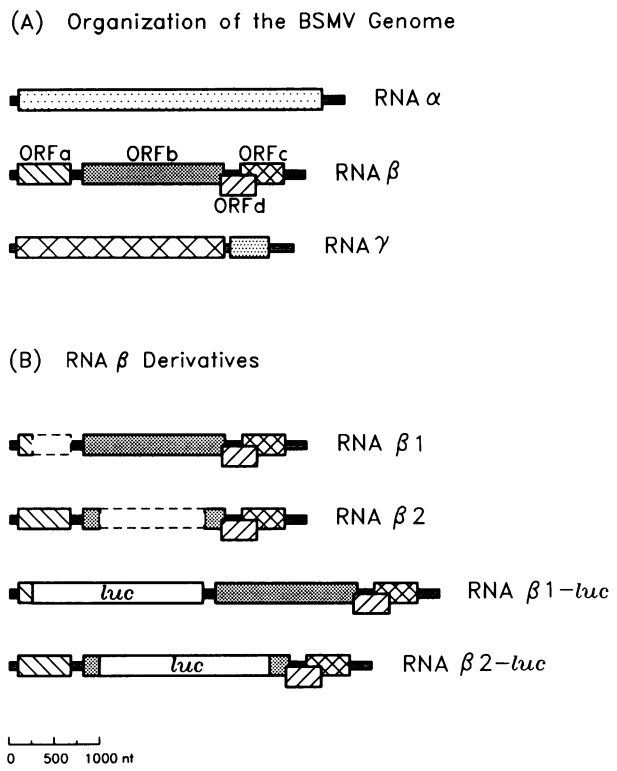

Fig. 1. Schematic representation of the genetic organization of the BSMV genome (**A**) and of RNA β derivatives (**B**). The orientation is 5′ to 3′ from left to right. The BSMV genome is composed of RNAs α, β and γ. ORFd of RNA β overlaps with ORFs b and c. In the RNA β derivatives engineered here, ORFa, encoding the coat protein of ~22.5 kd, and ORFb, encoding a non-structural protein of ~58 kd, have been deleted and replaced by the firefly luciferase (*luc*) gene leading to RNAs designated β1, β2, β1–*luc* and β2–*luc*, respectively. The extent of deleted sequences is depicted by dashed lines. Details of these constructs are described in Materials and methods.

Results and discussion

Engineering BSMV genome to serve as an expression vector

BSMV contains a tripartite genome composed of RNAs α, β and γ. The sequence of the entire viral genome has been determined (Gustafson and Armour, 1986; Gustafson *et al.*, 1987, 1989) and all three genomic RNAs have been cloned into transcription vectors that allow generation *in vitro* of infectious transcripts by T7 RNA polymerase (Petty *et al.*, 1988, 1989). The genetic organization of the BSMV genome is shown in Figure 1A. Each RNA carries a 'cap' structure at the 5′ terminus (Agranovsky *et al.*, 1979) and a tRNA-like structure at the 3′ end (Kozlov *et al.*, 1984; reviewed by Joshi and Haenni, 1990). RNA α and the 5′ proximal ORF in RNA γ encode large polypeptides that are presumably part of the viral replicase (RNA-dependent RNA polymerase). Aside from the coat protein encoded by ORFa in RNA β, the functions of the other non-structural proteins encoded by ORFb, ORFc and ORFd in RNA β and the 3′ proximal ORF in RNA γ are not yet elucidated. The latter three ORFs of RNA β, however, are required for systemic infection of plants (I.T.D.Petty and A.O.Jackson, personal communication). Synthesis of subgenomic RNAs appears to be the general mechanism for translation of all these 'internal' ORFs in infected cells (reviewed by Jackson and Lane, 1981; Carroll, 1986; Atabekov and Dolja, 1986; Jackson *et al.*, 1989).

In the present work, RNA β derivatives have been constructed to determine the capacity of RNA β to express foreign proteins (Figure 1B). In-frame deletions have been introduced into ORFa and ORFb to yield RNAs β1 and β2, respectively. Approximately 80% of the coding sequences of ORFa and 74% of ORFb were removed. An 'intronless' *luc* gene was subsequently fused in-frame into the deleted ORFa and ORFb to give rise, respectively, to RNAs β1–*luc* and β2–*luc*. Capped transcripts of RNA β, its derivatives, and RNAs α and γ were obtained by *in vitro* transcription and were then used to transfect plant protoplasts. To normalize the level of expression obtained, a capped *luc* mRNA was used as a reference, synthesized *in vitro* from a T7 transcription vector that had been modified to provide a poly(A) tail of 30 (A) residues at the 3′ end of the message (details of the above constructs are described in Materials and methods).

Luciferase gene expression in tobacco protoplasts

Expression of the *luc* gene in tobacco leaf mesophyll protoplasts mediated by BSMV RNAs β2–*luc* and β1–*luc* was investigated. Figure 2 shows that in protoplasts transfected with RNA β2–*luc* alone, only a small amount of luciferase activity over the background level was detected

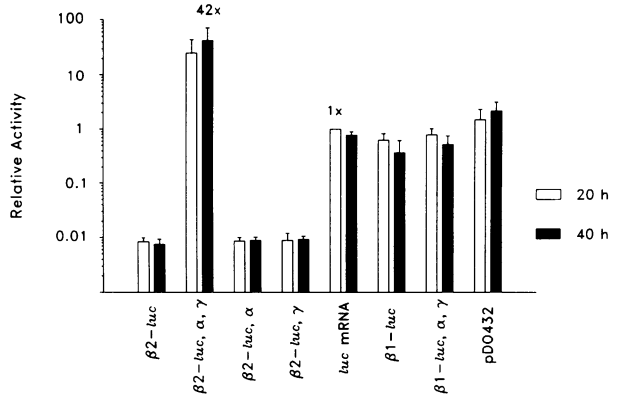

Fig. 2. BSMV RNAs $\beta 2-luc$ and $\beta 1-luc$ mediated synthesis of luciferase in tobacco protoplasts. Protoplasts were transfected with ~5 μg each of BSMV specific RNAs, ~5 μg of *luc* mRNA or ~5 μg of pDO432. Luciferase activity found in a tenth of the soluble extract from ~5 × 10^5 protoplasts (20 h and 40 h post-transfection) was determined. The value obtained using *luc* mRNA (~20 000 light units) at 20 h post-transfection, after subtracting the background activity (from a mock transfection without input of substrate), was normalized to 1 to determine relative values. The mean value of two to three independent experiments is presented with error bars to indicate population standard deviations.

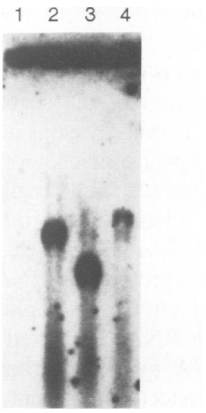

Fig. 3. Autoradiogram of Northern blot analysis to detect minus strand synthesis corresponding to BSMV RNAs β, β2 and $\beta 2-luc$ in tobacco protoplasts. Protoplasts (~5 × 10^5) were transfected with RNAs β, β2 or $\beta 2-luc$ in the presence of RNAs α and γ (lanes 2, 3, and 4, respectively) or with a combination of RNAs β, β2 and $\beta 2-luc$ but without RNAs α and γ (lane 1). Transfections were performed using ~5 μg each of BSMV specific RNAs and protoplasts were harvested 20 h after transfection. Total RNA from transfected protoplasts was extracted, electrophoresed on a 1% formaldehyde−agarose gel, blotted onto nitrocellulose membrane and hybridized to ^{32}P-labeled probe (*in vitro* transcribed plus strand RNAs β, β2 and $\beta 2-luc$) to detect minus strand synthesis. The filter was exposed to X-ray film as described in Materials and methods.

at either 20 or 40 h post-transfection. This result was anticipated since ORFb in RNA β, where the *luc* gene was inserted, is an 'internal' gene and its expression would be expected to require *de novo* synthesis of the corresponding subgenomic RNA. Subgenomic RNA synthesis is an integral part of viral replication and would require replicase-associated proteins from RNAs α and γ. Indeed, RNA $\beta 2-luc$ was proficient in mediating high level expression of the *luc* gene when cotransfected along with RNAs α and γ. Compared with transfection with RNA $\beta 2-luc$ alone, levels of expression greater than three orders of magnitude were obtained when RNA $\beta 2-luc$ was co-introduced with RNA α and γ. Consistent with the requirement for both RNAs α and γ (I.T.D.Petty and A.O.Jackson, personal communication), *luc* gene expression was not enhanced when either RNA α or γ was omitted.

Compared with luciferase activity in protoplasts 20 h after transfection with *luc* mRNA, a 42-fold higher level of expression, on average, was reached using RNA $\beta 2-luc$ in conjunction with RNAs α and γ. Interestingly, protoplasts transfected with the combination of RNAs α, γ and $\beta 2-luc$ produced more luciferase activity over time whereas the converse is true with protoplasts transfected with *luc* mRNA. This is consistent with the expectation that a viral genome could be amplified via RNA replication, whereas a mRNA introduced into protoplasts would be subjected to only degradation without *de novo* synthesis. To insure that the higher level of expression is not due to the use of a poorly translated or an unstable *luc* mRNA substrate, plasmid pDO432, which carries a CaMV 35S promoter−*luc*−*nos* 3′ terminator fusion served as an additional reference. As shown in Figure 2, RNA $\beta 2-luc$ mediated luciferase synthesis was also considerably greater than that from this plasmid substrate.

Initially, RNA $\beta 1-luc$ was constructed for use in an attempt to compare the relative efficiency of foreign gene amplification and expression between the two ORFs. However, contrary to RNA $\beta 2-luc$, RNA $\beta 1-luc$ alone synthesized luciferase in tranfected protoplasts to a level that is comparable with that obtained from the *luc* mRNA. Since the *luc* gene in RNA $\beta 1-luc$ was fused in-frame into the deleted ORFa, direct translation as a 5′ proximal gene would be expected. Luciferase synthesis from RNA $\beta 1-luc$, however, showed no significant increase upon addition of RNAs α and γ (Figure 2) or in the presence of RNAs α, γ plus either RNA β or RNA β2 (deleted ORFb) that could provide the intact coat protein (data not shown). These results implied that RNA $\beta 1-luc$ might be defective in replication, and that the deletion generated within ORFa covers a *cis*-acting signal involved in RNA β replication.

RNA replication concomitant with luciferase gene expression

Replication of RNA β and its derivatives was further examined by Northern blot analysis. Since replication of the viral genome in plus strand (messenger-sense) RNA viruses occurs initially via synthesis of complementary minus strand RNA by the viral replicase, we checked for the presence of minus strand RNA in protoplasts transfected with RNA β or its derivatives together with RNAs α and γ. Total RNA from transfected protoplasts was extracted and subjected to Northern blot analysis using as a probe a combination of plus strands from RNA β and its derivatives, which were labeled with ^{32}P during transcription *in vitro*. As shown in Figure 3, hybridizing bands indicating minus strand RNA synthesis corresponding to RNAs β, β2, and $\beta 2-luc$ were detected (lanes 2, 3 and 4, respectively), confirming that these RNAs had indeed replicated in transfected protoplasts. Replication was strictly dependent on the presence of RNAs α and γ, as minus strand RNA was not detectable when protoplasts were transfected with a combination of three substrates, RNAs β, β2 and $\beta 2-luc$, but in the absence of RNAs α and γ (lane 1). It is interesting to note that RNA

α and γ-dependent synthesis of the RNA $\beta2-luc$ minus strand is consistently lower than that found with RNAs β or $\beta2$. Whether this is due to the larger size of the $\beta2-luc$ molecule, a disruption of the secondary structure of the altered RNA β, or to other reasons has not been determined. Nevertheless, the lower efficiency of minus strand synthesis in RNA $\beta2-luc$ was sufficient to allow for greater production of luciferase relative to *luc* mRNA and pDO432.

In contrast to RNAs $\beta2$ and $\beta2-luc$ that retained the ability to replicate in protoplasts, RNAs $\beta1$ and $\beta1-luc$ were found to be defective for replication. In protoplasts cotransfected with RNAs α, γ and either $\beta1$ or $\beta1-luc$, minus strand RNA synthesis from either RNAs $\beta1$ or $\beta1-luc$ was not detected (data not shown). This result is consistent with the tentative conclusion that sequences within ORFa are important *in cis* for replication of RNA β. It should be pointed out, however, that several deletions within ORFa of RNA β have recently been found to affect infectivity in *Chenopodium amaranticolor* but not in barley plants (I.T.D.Petty and A.O.Jackson, personal communication). Whether the deleted sequences within RNA $\beta1$ affect replication in a host cell specific manner remains to be tested.

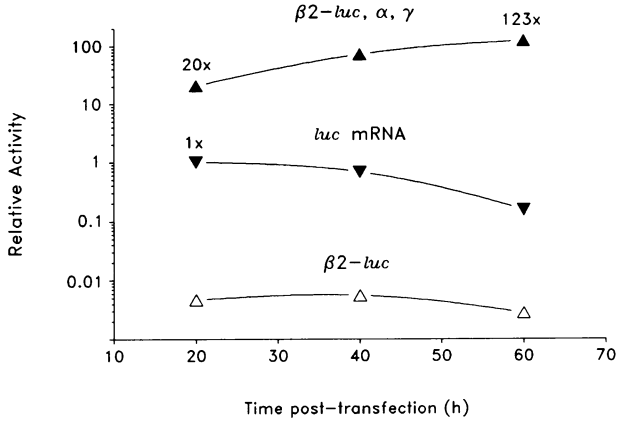

Fig. 4. BSMV RNA $\beta2-luc$ mediated *luc* gene expression in protoplasts derived from maize suspension culture. Protoplasts were transfected with ~5 μg each of BSMV specific RNAs, ~5 μg of *luc* mRNA or ~5 μg of pDO432 (not shown). The luciferase activity in a tenth of the soluble extract from ~5 × 10^5 protoplasts (~7500 light units) obtained using *luc* mRNA at 20 h after transfection was normalized to 1 to calculate relative values; the background value (from a mock transfection) had been subtracted. The mean value of two independent experiments is presented.

Luciferase gene expression in maize protoplasts

As a model system to examine whether BSMV RNA $\beta2-luc$ could mediate expression of the *luc* gene in monocotyledonous plant cells, we used protoplasts derived from a Black Mexican Sweet maize suspension culture line, BMSI, since viable protoplasts could be readily isolated in high yield for use in transformation (Fromm *et al.*, 1985, 1986). As observed previously using tobacco protoplasts, transfection into BMSI protoplasts with RNA $\beta2-luc$ alone did not produce a significant amount of luciferase (Figure 4). In the presence of RNAs α and γ however, RNA $\beta2-luc$ was able to express the *luc* gene at a high level. As observed in tobacco protoplasts (Figure 2), a decline in luciferase activity was found over time using *luc* mRNA as the transfecting substrate. On the other hand, the level of luciferase activity from RNA $\beta2-luc$ (in the presence of RNAs α and γ) increased with time on average from 20-fold higher at 20 h post transfection to 123-fold at 60 h post transfection when compared with the level obtained from *luc* mRNA 20 h after transfection. This strongly implicates gene amplification as a result of viral RNA replication. With pDO432, low luciferase activity similar to the level of RNA $\beta2-luc$ alone was obtained under those conditions (data not shown). It is possible, as observed by Fromm *et al.* (1987), that the transcriptional activity in BMSI protoplasts can be relatively low if the cells are not actively dividing at the time of protoplast preparation. Viral RNA mediated gene expression, however, may exhibit less reliance on the host transcriptional machinery since RNA amplification, presumably the key to enhanced expression of a foreign gene, is performed by an RNA-dependent RNA polymerase directly translated from the viral genome.

Search for a strategy permitting virus 'containment'

Tremendous progress has been made in recent years in the development of gene transfer systems for higher plants. Besides *Agrobacterium*-mediated transformation, direct gene transfer technologies using calcium phosphate coprecipitation, PEG treatment, microinjection, electroporation and 'particle gun' bombardment are being developed to transform plants that are refractory to infection by *Agrobacterium*. In view of these efforts, it appears that all major dicotyledonous and monocotyledonous crop species will be amenable to improvement by genetic engineering within the next few years. Significant achievements for crop improvement have already been made by engineering herbicide tolerance, insect resistance and viral disease resistance into crop plants (for reviews see Goodman *et al.*, 1987; Weising *et al.*, 1988; Gasser and Fraley, 1989).

As an alternative to stable gene transfer for crop improvement, there are certain potential advantages in developing plant viral genome based vectors to express foreign genes (for reviews see Hull and Davies, 1983; Siegel, 1985; Van Vloten-Doting *et al.*, 1985). One major concern for field release of genetically engineered viral vectors, however, is in the control of their spread outside of the target area. Hence, it would be important to engineer conditionally 'disarmed' viral vectors that could multiply only within the target host. Towards this aim, both the viral genome and the host plant would need to be genetically modified to form an obligate association. To envision such an approach, a disarmed viral vector could be engineered by deleting a gene essential for virus multiplication in plants and replacing it with a foreign gene to be expressed. The corresponding 'helper' plant could be transformed with the viral gene and engineered to supply the complementing function *in planta*. Thus, in this ideal scenario, the vector would become host-dependent and would be obliged to multiply and express the foreign gene only in the transgenic helper plants.

In this respect, the coat protein gene would not be appropriate to serve as the complementating gene since transgenic plants that synthesize the coat protein are protected against viral infections. This was first demonstrated with TMV (Powell Abel *et al.*, 1986) and subsequently observed in the case of many other plant RNA viruses (Tumer *et al.*, 1987; Loesch-Fries *et al.*, 1987; Van Dun *et al.*, 1987; Hemenway *et al.*, 1988; Cuozzo *et al.*, 1988; Van Dun and Bol, 1988; Stark and Beachy, 1989; Lawson *et al.*, 1990).

Moreover, even if the coat protein were not involved in the systemic spread of certain plant viruses, it needs to be retained in the viral genome in order to obtain an inoculum of packaged virions. In the BMV (French *et al.*, 1986) and TMV (Takamatsu *et al.*, 1987; Dawson *et al.*, 1989) genome based vectors engineered to date, it is precisely the coat protein gene that was replaced by the foreign *cat* gene. Insertion of the *cat* gene into the complete TMV genome, while retaining the coat protein gene, has also been reported. Unfortunately, however, the chimeric virus in this instance was unable to retain the foreign *cat* gene during viral replication (Dawson *et al.*, 1989).

In the BSMV genome based expression vector engineered here, ORFb in RNA β was replaced by the *luc* gene. We have shown that the resulting RNA $\beta 2-luc$ can mediate efficient expression of the *luc* gene in both mono- and dicotyledonous plant protoplasts upon replication in the presence of RNAs α and γ. Interestingly, ORFb encodes a non-structural protein that is essential for virus multiplication in whole plants (I.T.D.Petty and A.O.Jackson, personal communication). Consistent with that finding, luciferase activity was not detected in extracts of whole plants inoculated with RNAs α, γ and $\beta 2-luc$ (not shown). Thus, it would be tempting to engineer the expression of ORFb into host plants and test whether propagation of a BSMV genome derived vector and expression of the foreign gene it carries would be restricted solely to transgenic helper plants. Towards this aim, it has been found that BSMV can systemically infect *Nicotiana benthamiana* (Petty *et al.*, 1989), leaving open the possibility of gene transfer into a model BSMV host plant using conventional gene transfer methodology. Precedence for transgenic plants that complement a mutation of the viral genome already exists. Indeed, Deom *et al.* (1987) have established that transgenic plants expressing the TMV 30 kd protein gene, which is involved in the cell-to-cell spread of the virus, can potentiate the systemic spread at the non-permissive temperature of a TMV mutant (Lsl) that is temperature sensitive for cell-to-cell movement.

With respect to the issue of viral containment, the eventuality of RNA recombination must be addressed. Recombination occurs naturally in plant RNA viruses and moreover, Bujarski and Kaesberg (1986) and Allison *et al.* (1990) have demonstrated experimentally that this phenomenon can indeed occur *in planta*. However, the molecular mechanisms of RNA viral recombination are not yet entirely elucidated. A mechanism involving template switching by the viral replicase first from a replicative intermediate of a viral mutant RNA to a complementing mRNA present in the helper host plant and then back to the viral mutant replicative intermediate would seem unlikely. Thus, it may be that the occurrence of viral RNA recombination will not prove to be an important factor. In support of this conclusion, it should be noted that in transgenic plants complementing the TMV movement function, no appearance of wild type TMV has been reported upon inoculation with the TMV mutant (Lsl) (Deom *et al.*, 1987). Another consideration is that virus 'escape' might occur via RNA pseudorecombination if the complementing mRNA were packaged. However, since the mRNA would not bear viral replicase recognition signals at the 5′ and 3′ ends, it would not be able to replicate and infection of any non-helper plant would be aborted.

In conclusion, although BSMV is not among the best studied RNA viruses, it was chosen as a model for this investigation for a variety of reasons. Aside from the availability of nucleotide sequence information and cDNA clones, a major consideration was its wide experimental host range (Jackson *et al.*, 1989). BSMV can infect cereals of agronomic importance including barley, oat, wheat and corn, as well as dicots (e.g. *Nicotiana benthamiana*) that can be used as model hosts for investigation. Moreover, BSMV genomic RNAs are assembled individually into rod-shaped virions. With respect to obtaining viral inocula for field infections, it is an important consideration that rod-shaped viruses generally impose less constraints in packaging RNAs of varying sizes. All these characteristics suggest that development of a helper plant-dependent BSMV vector system should present considerable interest to crop improvement.

Materials and methods

Enzymes and chemicals

Enzymes used for cloning, T7 RNA polymerase and RNA cap structure analog (m^7GpppG) were from Bio-Labs. ATP, GTP, UTP, CTP, RNasin, RQ$_1^{TM}$ DNase and RNase-free bovine serum albumin (BSA) were purchased from Promega. Cellulase 'Onozuka R-10' and macerase (Macerozyme R-10) were from Serva, hemicellulase from Sigma and pectinolyase (Pectolyase Y-23) from Seishin Pharmaceutical Co., Ltd. Polyethylene glycol (PEG) 1540 was purchased from Polysciences and luciferin from Analytical Luminescence Laboratory (San Diego, California).

Plasmid constructions

All DNA and *Escherichia coli* (strains HB101 or DH5α) manipulations were performed essentially as described by Maniatis *et al.* (1982). Plasmids pTα10, pTβ2GAN2 and pTγ16, from which infectious RNAs α, β and γ, respectively, of the type strain of BSMV could be obtained by linearization of the plasmids with *Mlu*I followed by *in vitro* transcription using T7 RNA polymerase (Petty *et al.*, 1988, 1989), were kindly provided by Drs I.T.D.Petty and A.O.Jackson (University of California, Berkeley).

To introduce an in-frame deletion into ORFb in RNA β, pTβ2GAN2 was linearized with *Hin*cII and *Eco*RI and the plasmid backbone re-circularized in the presence of a synthetic linker-adaptor (5′-GACGGATCCG-3′/5′-AATTCGGATCCGTC-3′) which encoded a unique *Bam*HI site to yield plasmid pVRJ21. The new ORFb in RNA β2, from pVRJ21, encoded a peptide of 140 amino acids. To fuse the *luc* cDNA in-frame into the deleted ORFb, pVRJ21 was linearized at the *Eco*RI site, the staggered *Eco*RI ends were filled in with PolIk (*E.coli* polymerase I Klenow fragment), and the plasmid further cleaved with *Bam*HI. A firefly *luc* cDNA fragment was prepared by cleavage of pKW112-72 (Wood, 1989) with *Xho*I, followed by filling in the staggered ends with PolIk, and subsequent cleavage by *Bam*HI. The 5′-*Bam*HI-*luc* cDNA blunt end 3′ fragment was joined to the 5′-*Bam*HI to blunt end 3′ pVRJ21 backbone to yield pVRJ23. This plasmid resulted in fusion of the N-terminal 61 amino acids encoded by ORFb to a GGA codon followed by the *luc* coding region, beginning with its N-terminal second codon.

To introduce an in-frame deletion into ORFa in RNA β, pTβ2GAN2 was cleaved with *Xba*I and *Nde*I and the plasmid backbone reclosed in the presence of a synthetic linker-adaptor (5′-CTAGACCTCGAGTGACC-TGCTGTCGAAGCGGTAAAAGGATGTACA-3′/5′-TATGTACATCC-TTTTACCGCTTCGACAGCAGGTCACTCGAGGT-3′). Addition of the linker adaptor (which also encoded an *Xho*I site) restored the deleted termination codon of ORFa, and the remainder of the non-coding intergenic region between ORFa and ORFb to yield pVRJ20. The new ORFa encoded a peptide of 46 amino acids. To place the *luc* cDNA in-frame into the deleted ORFa, pVRJ20 was cleaved at the *Xba*I site, followed by filling in the staggered *Xba*I ends with PolIk, and subsequently cleaved with *Xho*I. The plasmid backbone obtained was then ligated to a *luc* cDNA fragment from pKW112-72, which was prepared from cleavage with *Bam*HI, followed by filling in the *Bam*HI staggered ends, and subsequent cleavage with *Xho*I. This gave rise to plasmid pVRJ22, which resulted in fusion of the first 43 amino acid codons of ORFa to a GGA codon followed by the *luc* coding region, beginning with its N-terminal second codon. Upon transcription *in vitro* with T7 RNA polymerase, *Mlu*I linearized plasmids pVRJ21,

pVRJ23, pVRJ20 and pVRJ22 produced RNA β derivatives designated RNAs $\beta 2$, $\beta 2-luc$, $\beta 1$ and $\beta 1-luc$, respectively.

To obtain a *luc* mRNA to use as reference, the *in vitro* transcription vector pT7E19(+) described by Petty (1988) was first modified by cleavage at the *Xba*I and *Pst*I sites and reclosed in the presence of a synthetic linker-adaptor [5′-CTAGAGCTC(A_{30})CTGCA-3′/5′-G(T_{30})GAGCT-3′] to yield pRJ18 which provided a poly(A) tail at the 3′ end of the transcript. The *luc* cDNA excised by *Bam*HI digestion of pD0432 (Ow *et al.*, 1986) was then inserted into *Bam*HI linearized pRJ18 to yield pRJ19. The proper orientation of insertion was determined by restriction analysis by *Cla*I. The *luc* mRNA transcript was obtained from *in vitro* transcription by T7 RNA polymerase upon linearization of pRJ19 with *Hin*dIII.

In vitro transcriptions

Capped RNA transcripts using T7 RNA polymerase were prepared essentially as described by Nielson and Shapiro (1986). Briefly, the incubation reaction (100 μl) containing 40 mM Tris−HCl pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 100 μg/ml RNase-free BSA, 10 mM DTT, 500 μM each of ATP, CTP and UTP, 50 μM GTP, 500 μM m^7GpppG, 100 U RNasin, 10 μg of linearized CsCl purified plasmid DNA template and 100 U of T7 RNA polymerase were incubated for 30 min at 37°C; 50 μM GTP was added and incubation was continued for 30 min. Template DNA was removed by subsequent digestion by 5 U of RQ_1^{TM} DNase for 30 min at 37°C. The incubation mixture was extracted with phenol followed by chloroform. The RNAs were precipitated by adding 25 μl of 4 M ammonium acetate and 350 μl of ethanol. The dried pellets were dissolved in 5 μl of TE (10 mM Tris−HCl pH 7.8, 1 mM EDTA) and stored at −70°C. Under these conditions, ~1 μg RNA was made per μg of template DNA transcribed as estimated by analyzing an aliquot on a 1% agarose gel followed by ethidium bromide staining. Synthesis of ^{32}P-labeled RNA transcripts was performed as above except that m^7GpppG was omitted and the reaction mixture (20 μl) contained 500 μM each of ATP, GTP and UTP, 12 μM CTP, 50 μCi [α-^{32}P]CTP (400 Ci/mmol; New England Nuclear), 0.5 μg each of linearized pTβ2GAN2, pVRJ21 and pVRJ23 (or pTβ2GAN2, pVRJ20 and pVRJ22) and 20 U T7 RNA polymerase. Incubation was for 60 min at 37°C; template DNA was digested with RQ_1^{TM} DNase (2 U) and the reaction mixture was treated as above. Under those conditions, ~60% incorporation of [α-^{32}P]CTP was obtained. The dried pellets were dissolved in 50 μl TE and used for hybridization in Northern blot analysis.

Protoplast isolation

Leaf mesophyll protoplasts of *Nicotiana tabacum* cv. Wisconin 38 were isolated from aseptically grown shoot cultures and purified in K3 medium containing 0.4 M sucrose as described by Nagy and Maliga (1976). Protoplasts from the *Zea mays* cv. Black Mexican Sweet suspension culture line, BMSI, were isolated according to Fromm *et al.* (1985) except that a final purification step, floating the protoplasts in K3 medium containing 0.4 M sucrose, was added.

Protoplast transfections and culture

Protoplasts were transfected with various RNAs and DNA by a PEG−$CaCl_2$ procedure similar to that described by Dawson *et al.* (1978) and Loesch-Fries *et al.* (1985). Briefly, protoplasts purified by floating in sucrose were diluted in 0.4 M mannitol containing 3 mM $CaCl_2$ and 5×10^5 to 1×10^6 protoplasts were collected by centrifugation and placed on ice. RNAs or DNA (in 15 μl) were directly added to the protoplast pellet just before their resuspension in 400 μl of 40% PEG-1540 containing 3 mM $CaCl_2$ pH 5.5. After mixing for 30 s, the PEG−protoplast suspension was diluted slowly with 4 ml of cold mannitol−$CaCl_2$ solution and left on ice for 20 min with occasional gentle shaking. The protoplasts were collected, washed twice with mannitol−$CaCl_2$ solution and resuspended in 1 ml of culture medium containing 20 μg of gentamicin sulfate. Tobacco protoplasts were cultured under constant light in K3 medium containing 0.4 M mannitol and maize protoplasts were incubated in the dark in MS medium containing 0.4 M mannitol essentially as described by Fromm *et al.* (1987). At appropriate times, aliquots were removed and protoplast pellets were frozen at −70°C until further analyzed.

Luciferase assays

Protoplast pellets were resuspended in 100 μl of extraction buffer (100 mM potassium phosphate pH 7.5, 1 mM DTT). Cell extracts were prepared by three cycles of freeze−thawing followed by sonication (3 × 1 s pulses). The extracts were clarified by centrifugation for 5 min at 8000 r.p.m. at 4°C. Luciferase activity was determined by mixing 10 μl of the supernatant with 100 μl of luciferase assay buffer (50 mM HEPES pH 7.8, 20 mM $MgCl_2$, 10 mM ATP, 1 mg/ml BSA) and the reaction was initiated by injection of 100 μl of 0.5 mM luciferin into the mixture. Light intensity measured in a luminometer was integrated over a 30 s period (Monolight 2001, Analytical Luminescence Laboratory, San Diego, California).

RNA extraction and Northern blot analysis

Total RNA from protoplasts (~5×10^5) was isolated by an acid guanidium thiocyanate−phenol−chloroform extraction procedure as described by Chomczynski and Sacchi (1987). Briefly, the protoplast pellets were resuspended in 100 μl of denaturing solution and 100 μl of phenol and extracted for 10 min at 0°C. The aqueous phase was separated by centrifugation upon addition of 20 μl of chloroform and the RNA precipitated with ethanol. The material was further processed and electrophoresed on 1% formaldehyde−agarose gels according to Rave *et al.* (1979). Blotting onto nitrocellulose membranes and hybridization with ^{32}P-labeled RNA transcripts were performed as described by Maniatis *et al.* (1982). Filters were hybridized and washed at 68°C. Following hybridization, filters were treated with RNase A according to Wahl *et al.* (1987) to remove any non-specific binding of the probe. Blots were exposed to X-ray film with an intensifying screen for 2−4 days at −70°C.

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