INTRODUCTION

Natural hosts of *Soil-borne wheat mosaic virus* (SBWMV), the type species in the genus *Furovirus* (Torrance, 2000), are winter wheat and winter barley (Brakke, 1971). The virus is transmitted by zoospores of the plasmodiophoraceous fungus *Polymyxa graminis* (Linford & McKinney, 1954), which germinates from resting spores in the old root debris of young seedlings during the autumn in infested fields (Brakke et al., 1965; Brakke & Estes, 1967). After overwintering, infected plants start showing conspicuous mosaic symptoms on newly developing leaves, usually accompanied by stunting or rosetting of the plants when the temperature increases during the early and middle spring. Those plants that do not develop severe symptoms will recover from the disease and grow as vigorously as uninfected plants in the late spring and early summer (Brunt & Richards, 1989; Shirako & Wilson, 1999).

Early studies on fungal transmission using root washings containing zoospores or root debris containing resting spores showed that systemic infection of SBWMV occurred when the inoculated plants were grown at temperatures below 20°C (Brakke & Estes, 1967; Brakke & Rao, 1967; Rao & Brakke, 1969). In such experiments, wheat and barley plants inoculated with SBWMV by mechanical inoculation were grown in a growth cabinet below 20°C to get systemic infection (McKinney, 1948; Rao & Brakke, 1970). Thus, SBWMV appears to be adapted to host plants that grow under a cool climate from autumn to the following spring. The cause of the requirement for low temperatures for SBWMV infection, whether due to RNA replication, cell-to-cell movement or both, has not been resolved.

The genome of SBWMV consists of a 7-2 kb RNA1 and a 3-6 kb RNA2 (Shirako et al., 2000). RNA1 encodes the p152 and p211 replicase proteins, which are equivalent to the p126 and p183 proteins of *Tobacco mosaic virus*, in the 5’-terminal region and a p37 putative movement protein in the 3’-terminal region (Fig. 1A, pJS1). RNA2 encodes the capsid protein (CP) and its C-terminally extended protein formed by partial readthrough at the CP termination codon in the 5’-terminal region, and a p19 cysteine-rich protein in the 3’-terminal region (Fig. 1B, pJS2).

Here, we report on studies of SBWMV RNA replication in barley mesophyll protoplasts inoculated with infectious *in vitro* transcripts from full-length cDNA clones of RNA1 and RNA2 of a Japanese strain of SBWMV (Yamamiya & Shirako, 2000). Our results showed that the optimal temperature of SBWMV RNA1 replication is 17°C and that the temperature optimum is determined by the p152 and p211 replicase proteins and/or the 5’- and 3’-untranslated region (UTR) in RNA1.

METHODS

cDNA constructs for *in vitro* transcription of infectious RNA. Full-length cDNA clones for RNA1 and RNA2 of a Japanese strain of SBWMV (Tochigi-82), pJS1 and pJS2, have been described previously (Yamamiya & Shirako, 2000). pJS1.Rep (Fig. 1A), which contained only the p152 and p211 replicase genes between the 116...
nucleotide 5′-UTR and the 559 nucleotide 3′-UTR of RNA1, was constructed by deleting nt 5646–6666 in pJS1 as follows. The nt 3612–5645 region was amplified using primers TP48 (plus sense; nt 3612–3628 of RNA1) and TN91 (minus sense; nt 6667–6675 followed by nt 5630–5645 of RNA1) while nt 6667–7226 was amplified using TN90 (plus sense; nt 5637–5645 followed by nt 6667–6682 of RNA1) and TP25 (minus sense; annealing to the 3′-terminal 20 nts of RNA1) by PCR using pJS1 as the template. The resulting 2·0 kb and 0·6 kb PCR products were combined using TP48 and TP25 by PCR over the sequence common between the two DNA fragments, followed by digestion with BglII (nt 4529 in RNA1) and HindIII (nt 6762 in RNA1). The 1·2 kb BglII–HindIII-digested fragment was cloned into BglII/HindIII-digested vector prepared from pJS1. In pJS2.N-GFP/p19 (Fig. 1B), the CP gene and the downstream readthrough region in pJS2 were replaced with a GFP gene by a PCR-based fragment exchange and deletion method. Rightward arrows above the rectangular boxes indicate the location of a leaky UGA termination codon.

**Inoculation of protoplasts with in vitro transcripts.** One ml of protoplast suspension (containing 3 × 10⁵ cells) was centrifuged at 100 g for 5 min and the supernatant was removed. The pelleted cells were suspended in 20 μl 0·65 M mannitol and 10 μl of in vitro transcripts at a concentration of about 200 ng μl⁻¹ were added, followed by immediate mixing with 100 μl PEG solution (40 % polyethylene glycol, average M, 1450 from Sigma/Aldrich, 30 mM CaCl₂, pH 5·7) (Samac et al., 1983). The mixture was placed on ice for 1 min, followed by addition of 1 ml 0·65 M mannitol and incubation on ice for 15 min. Inoculated protoplasts were washed twice in 0·65 M mannitol by centrifugation and suspended in 1 ml of medium consisting of 0·2 mM KH₂PO₄, 1 mM KNO₃, 1 mM MgSO₄, 1 μM KI, 0·1 μM CuSO₄, 10 mM CaCl₂, 0·65 M mannitol, pH 6·5 (Takebe et al., 1968). The cell suspension was transferred to a 24-well plate (Corning) and incubated at 17°C, or to microfuge tubes and incubated in heat blocks with Peltier elements (ALB 301, IWKAI) at appropriate temperatures for up to 48 h in the dark.

**Expression of CP as the GST fusion protein in Escherichia coli cells and preparation of anti-GST–CP antisera.** The SBWMV CP gene was fused in-frame to the 3′ terminus of the glutathione S-transferase (GST) gene in plasmid pGEX-6P-1 (Amersham Pharmacia). The GST–CP fusion protein was expressed in transformed E. coli strain MC1061 cells by induction with 1 mM IPTG. Insoluble GST–CP inclusion body was isolated from E. coli cells lysed with bacterial protein extraction reagent (Pierce) and denatured in 1 × SDS-PAGE sample buffer (1 × SB), followed by 7·5 % SDS-PAGE. The 66 kDa GST–CP fusion protein was eluted from gel

---

Preparation of barley mesophyll protoplasts. Barley mesophyll protoplasts were prepared by the methods of Okuno et al. (1977) and Loesch-Fries & Hall (1980) with minor modifications. Seedlings of barley (Hordeum vulgare L. cv Miniorinumig) were grown on 1 : 1 peat moss and vermiculite at 25°C with 16 h of light per day. Plants were watered with half-strength Hoagland’s solution (Sigma/Aldrich) every day. Two grams of leaves were cut at about 5 cm from the ground surface and the epidermis was peeled off carefully by hand. Exposed mesophyll tissues were immediately placed in an enzyme solution consisting of 0·65 M mannitol, 2 % Cellulase Onozuka R-10 (Yakult), 0·1 % Macerozyme R-10 (Yakult) and 0·1 % BSA (Fraction V; Sigma/Aldrich), pH 5·7, and incubated without stirring at 30°C for 3 h in the dark. The enzyme solution containing released protoplasts was collected and centrifuged at 100 g for 3 min. The pellet was suspended in 5 ml 0·65 M mannitol, which was layered on 20 % sucrose in a 15 ml tube, followed by centrifugation at 100 g for 8 min. Protoplasts at the interface were collected, suspended and washed twice in 0·65 M mannitol by centrifugation at 100 g for 5 min. The final pellet was suspended in 0·65 M mannitol at a concentration of 3 × 10⁵ cells ml⁻¹.

Fig. 1. (A) RNA1 constructs. pJS1 (WT) contains the full-length cDNA clone of SBWMV RNA1. In pJS1Rep, the region between the termination codons for the p211 and p37 genes was deleted from pJS1 by PCR-based deletion mutagenesis. (B) RNA2 constructs. pJS2 (WT) contains the full-length cDNA clone of SBWMV RNA2. In pJS2.N-GFP/p19, the CP gene and the downstream readthrough region in pJS2 were replaced with a GFP gene by a PCR-based fragment exchange and deletion method. Rightward arrows above the rectangular boxes indicate the location of a leaky UGA termination codon.
slices by the method of Hager & Burgess (1980) and 2 mg of the purified fusion protein was used to immunize a rabbit at Sawady Technology, Tokyo, Japan.

**Western blot analysis.** Protoplasts from one well of a 24-well plate were collected by centrifugation and suspended in 20 μl 1× SB, followed by heating at 95°C for 3 min. Ten μl of sample containing proteins from 1-5 x 10^6 cells was loaded per lane in a 12.5% SDS-polyacrylamide gel for electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membrane (S&S) and subjected to detection of SBWMV CP using anti-GST–CP antiserum and goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma/Aldrich) using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrate, as described (Shirako & Ehara, 1986).

**Northern blot analysis.** Protoplasts from one well of a 24-well plate were collected by centrifugation and suspended in 0.1 M glycine, pH 9.5, 0.1 M NaCl, 10 mM EDTA, 2% Triton-X 100. After centrifugation at 10,000 g for 3 min to remove large organelles, SDS was added to the supernatant to a final concentration of 2% and RNA was extracted with phenol/chloroform and precipitated in ethanol. The final pellet was suspended in water. Extracted RNA was denatured in 40 mM MOPS, pH 7.5, 10 mM Na acetate, 1 mM EDTA, 1 M formaldehyde at 65°C for 5 min, followed by immediate chilling on ice. Denatured RNA was run on a 0.8% agarose gel prepared in MOPS/formaldehyde buffer, blotted onto a nylon membrane (Hybond-N+; Amersham Pharmacia) and fixed on the membrane with 50 mM NaOH for 20 min. The membrane was rinsed in 2× SSC (0.3 M NaCl, 30 mM Na citrate) for 1 min and incubated at 68°C in 50% formamide, 0.02% SDS, 0.1% lauryl sarcosine, 2% block stock solution (Roche) in 5× SSC (hybridization buffer) for 1 h. The membrane was further incubated at 68°C for 6 h in the hybridization buffer containing digoxigenin (DIG; Roche)-labelled RNA probes. RNA probes annealing to nt 3124–3731 and nt 5092–6218 of RNA1 and nt 2658–3182 of RNA2 were prepared by *in vitro* transcription of pGEM-T plasmids containing cDNA inserts prepared by PCR using pJS1 or pJS2 as the templates in the presence of DIG–UTP according to the manufacturer’s protocol. After washing twice for 5 min in 2× SSC containing 0.1% SDS and twice for 15 min in 0.1× SSC containing 0.1% SDS, the membrane was incubated with anti-DIG alkaline phosphatase-conjugated goat Fab fragment (Roche) at a 1:10,000 dilution for 30 min. Probe-annealing bands on the membrane were detected using CDP-Star (New England Biolabs) as the substrate in a chemiluminescence detector (LAS1000, Fuji Film). In all Northern blots, the amount of RNA sample loaded was adjusted based on ribosomal RNAs stained with ethidium bromide.

### RESULTS AND DISCUSSION

**Establishing infection of barley mesophyll protoplasts with infectious *in vitro* transcripts from pJS1 and pJS2,N-GFP/p19**

Prior to this study, there was no report of an SBWMV or SBWMV RNA infectivity assay using barley or wheat protoplasts. Therefore, we examined the efficiency of infection of infectious *in vitro* transcripts in barley mesophyll protoplasts using GFP fluorescence as a marker. *In vitro* transcripts from pJS1 and pJS2,N-GFP/p19 (Fig. 1B) were co-transfected into barley protoplasts and the protoplasts were incubated for 24 h at 17°C, which is the optimal temperature for systemic infection of wheat plants with SBWMV. As shown in Fig. 2, about 50% of the protoplasts fluoresced strongly 24 h after transfection using approximately 2 μg each of RNA1 and RNA2 *in vitro* transcripts for 3 x 10^5 cells in 1 ml of the cell suspension buffer. Trials to increase transfection efficiency to more than 50% using increased amounts of *in vitro* transcripts were not successful. Since both RNA1 and RNA2 are required for expression of the GFP gene, we assumed that more than 70% of protoplasts received at least one species of RNA.

**Detection of CP from barley mesophyll protoplasts incubated at different temperatures after transfection with *in vitro* transcripts from pJS1 and pJS2**

Barley mesophyll protoplasts transfected with *in vitro* transcripts from pJS1 and pJS2 were incubated at 15, 17, 20, 22 and 25°C for 48 h. Protoplasts were collected by centrifugation and total proteins were subjected to Western blot analysis using anti-GST–CP antiserum. The 19 kDa CP was detected from protoplasts incubated at 17°C (Fig. 3, lane 3). A weak band was detected at 15 and 20°C (Fig. 3, lanes 2 and 4, respectively). A faint band was also detected at 22°C but no band was detected at 25°C (Fig. 3, lanes 5 and 6, respectively). At 17°C, the 83 kDa CP-RT protein was also clearly detected, indicating that translational readthrough at the UGA termination codon occurred efficiently at 17°C within 48 h after transfection. On the contrary, the 24 kDa N-CP protein, which contains a 40 amino acid extension to the N terminus of the CP due to translational initiation at a CUG codon 120 nucleotides upstream from the AUG initiation codon for CP (Shirako, 1998), was not detectable within 48 h after RNA transfection.

**SBWMV RNA replication is most efficient at 17°C**

Barley protoplasts transfected with *in vitro* transcripts from pJS1 and pJS2 were incubated for 24 h at 15, 17, 20, 22 and 25°C. Intracellular RNA was extracted, denatured with formaldehyde and run on an agarose gel. Fig. 4(A)
shows accumulation of RNA1 in barley protoplasts as indicated by an arrow on the right. Using a probe annealing to nt 5692–6218 of the p37 gene, RNA1 was detected most abundantly at 17˚C (lane 2). The intensity of the RNA1 band decreased in the order 20, 15 and 22˚C (Fig. 4A, lanes 3, 1 and 4, respectively). At 25˚C, RNA1 was detected only as a very faint band (Fig. 4A, lane 5). There was another band detected on this blot at the position of ~1.5 kb (Fig. 4A, asterisk), most intensely at 17˚C and faintly at 20˚C. This band was not detected using a probe annealing to nt 3123–3731 in the p152 gene (Fig. 4A, lane 6). Based on the migration rate and specificity to probes in Northern blots, the 1.5 kb RNA is likely to be the subgenomic RNA for the p37 putative movement protein encoded in the 3' terminal region. Similarly, RNA2 was detected most abundantly at 17˚C (Fig. 4B, lane 2) using a probe annealing to nt 2658–3182 in the p19 gene on RNA2. RNA2 was also detectable, although less abundantly, at 15, 20 and 22˚C, but was barely detected at 25˚C (lanes 1, 3, 4 and 5, respectively). A 0.9 kb band (Fig. 4B, asterisk) was clearly detected at 17, 20 and 22˚C (Fig. 4B, lanes 2, 3 and 4, respectively). This RNA could be the subgenomic RNA for the p19 cysteine-rich protein encoded in the 3' terminal region.

**The p152 and p211 replicase proteins and/or the 5' and 3' terminal UTR of RNA1 determine adaptation of SBWMV RNA replication at 17˚C**

When barley mesophyll protoplasts were transfected with *in vitro* transcripts from pJS1 alone, the full-length RNA1 and the possible subgenomic RNA for the p37 protein were detected most abundantly at 17˚C using a probe annealing to nt 5692–6218 (Fig. 5A, lane 2). These two RNAs were detected in reduced amounts at 15, 20 and 22˚C, but were barely detectable at 25˚C (Fig. 5A, lanes 1, 3, 4 and 5).

---

**Fig. 3.** Western blot analysis of the SBWMV CP from barley mesophyll protoplasts transfected with *in vitro* transcripts from pJS1 and pJS2. Samples were collected 48 h after RNA transfection. Protoplasts transfected with *in vitro* transcripts were incubated at 15, 17, 20, 22 and 25˚C. The antiserum was prepared against the GST–CP fusion protein expressed in *E. coli* cells using pGEX-6P-1.

**Fig. 4.** Northern blot analysis of RNA1 (A) and RNA2 (B) replicated in barley mesophyll protoplasts after transfection with *in vitro* transcripts from pJS1 and pJS2. After RNA transfection, protoplasts were incubated at 15, 17, 20, 22 and 25˚C (lanes 1, 2, 3, 4 and 5, respectively) for 24 h. (A) Blotted membrane was probed with DIG-labelled antisense transcripts annealing to nt 5692–6218 in RNA1 to detect both genomic and subgenomic RNAs (lanes 1–5). Lane 6, RNA was extracted from protoplasts incubated at 17˚C for 24 h and the blotted membrane was probed with DIG-labelled probe annealing to nt 3123–3731 to detect the genomic RNA but not the subgenomic RNA. (B) Blotted membrane was probed with DIG-labelled antisense transcripts annealing to nt 2658–3182 in RNA2 to detect both genomic and subgenomic RNAs. Positions of the genomic RNAs are shown by arrows, whereas bands possibly corresponding to subgenomic RNAs are indicated by asterisks on the right. Sizes of marker RNAs are indicated.
This result indicates that RNA1 itself can replicate in the absence of RNA2 and its gene products, and that the requirement for low temperatures is determined by RNA1. We further analysed replication of a mutant RNA1 from pJS1.Rep in which only the p152 and p211 genes are present between the 5'- and 3'-UTRs (Fig. 1A). Using a probe annealing to nt 3124–3731, the 6.2-kb RNA1.Rep band was detected at 17°C but not at 25°C (Fig. 5B, lanes 5 and 6, respectively).

The above results clearly indicate that the requirement for lower temperatures for SBWMV infection in plants are primarily determined at the level of viral RNA replication. Since RNA1.Rep showed the same characteristics as wild-type RNA1 in the RNA replication profile, involvement of the p37 putative movement protein and the p19 cysteine-rich protein with unknown function is excluded. The determinant for temperature sensitivity may reside not only in the p152 or p211 replicase proteins but also in the cis elements in the 5'- and 3'-UTRs as in the case of Red clover necrotic mosaic virus, in which the 3'-UTR of RNA1 was shown to contain the determinant for temperature sensitivity (Mizumoto et al., 2002). Whether the p37 putative movement protein also requires low temperatures for its activity in planta remains to be determined.

ACKNOWLEDGEMENTS

We are grateful to Myron Brakke for valuable comments on this manuscript. This work was supported by a grant from the Japan Society for the Promotion of Science.

REFERENCES


