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The 3a cell-to-cell movement gene is dispensable for cell-to-cell transmission of brome mosaic virus RNA replicons in yeast but retained over 10^{45}-fold amplification

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In yeast expressing the RNA replication proteins encoded by brome mosaic virus (BMV), B3URA3, a BMV RNA3 derivative that harbours the 3a cell-to-cell movement protein gene and the yeast uracil biosynthesis gene *URA3*, was replicated and maintained in 85–95% of progeny at each cell division. Transmission of the B3URA3 RNA replicon from mother to daughter yeast did not require the 3a gene. Nevertheless, even after passaging for 165 cycles of RNA replication and yeast cell division, each of 40 independent Ura$^+$ colonies tested retained B3URA3 RNAs whose electrophoretic mobilities and accumulation levels were indistinguishable from those of the original B3URA3. These and other results suggest that unselected genes in many positive-strand RNA virus replicons can be stably retained if the presence of the gene does not confer a selective disadvantage in RNA replication.

Brome mosaic virus (BMV) is a plant virus whose genome consists of three capped, messenger-sense RNAs. RNA1 and RNA2 encode proteins 1a and 2a, respectively, which are necessary for RNA-dependent RNA replication (Ahlquist, 1992). RNA3 (Fig. 1) encodes two proteins required for systemic spread of BMV infection in plants, the 3a cell-to-cell movement protein and a coat protein (CP) (Ahlquist *et al.*, 1981; Sacher & Ahlquist, 1989; Mise & Ahlquist, 1995). The downstream CP gene is translated from a subgenomic mRNA, RNA4, which is transcribed from negative-strand RNA3 (Ahlquist, 1992). The 3a and CP open reading frames (ORFs) are dispensable for efficient replication of RNA3 or transcription of RNA4, and RNA3 derivatives with these ORFs replaced by foreign genes can be replicated and express the foreign genes (French & Ahlquist, 1987; French *et al.*, 1986; Janda & Ahlquist, 1993, 1998).

In cells of the yeast *Saccharomyces cerevisiae* expressing BMV 1a and 2a (hereafter 1a2a$^+$ yeast), BMV RNA3 derivatives are replicated and transcribed to produce subgenomic RNA4 as in plant cells (Janda & Ahlquist, 1993). Furthermore, such RNA3 derivatives can be passed from mother to daughter cells during yeast mitosis, and maintained as persistent, free RNA replicons, in the absence of any RNA3 cDNA. For example, B3URA3 (Fig. 1) is an RNA3 derivative with the CP gene replaced by the yeast *URA3* ORF, encoding a uracil biosynthesis enzyme. When B3URA3 *in vitro* transcripts were transfected into *ura3*$^-$ 1a2a$^+$ yeast and selected for growth in the absence of uracil, yeast cells were obtained that persistently maintained B3URA3 RNA and expressed *URA3* by 1a- and 2a-dependent RNA replication, allowing formation of Ura$^+$ colonies (Janda & Ahlquist, 1993).

The efficiency with which such RNA replicons are transmitted from mother to daughter cells is an important consideration for their possible use for genetic analyses. Moreover, knowing the efficiency of such transfer would provide a foundation for studies of the transmission process,

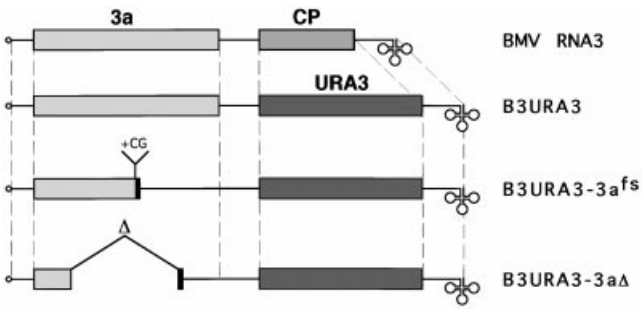

Fig. 1. Structure of BMV RNA3 and its derivatives. B3URA3 (Janda & Ahlquist, 1993) is a BMV RNA3 derivative with the 3′-proximal CP gene replaced by the coding sequence of the yeast *URA3* gene. B3URA3-3a^{fs} was constructed by modifying the B3URA3 cDNA in pB3MI8 (Ishikawa *et al.*, 1997) by cleaving the unique *Cla*I site in the 3a ORF, filling in the ends with DNA polymerase and dNTPs, and religating to create pB3MI16, resulting in a two-base frameshifting insertion between RNA3 nucleotides 603 and 604. B3URA3-3aΔ was constructed by deleting the *Afl*III–*Pfl*MI fragment (RNA3 nucleotides 306–817) in the 3a ORF of pB3MI8 to create pB3MI23.

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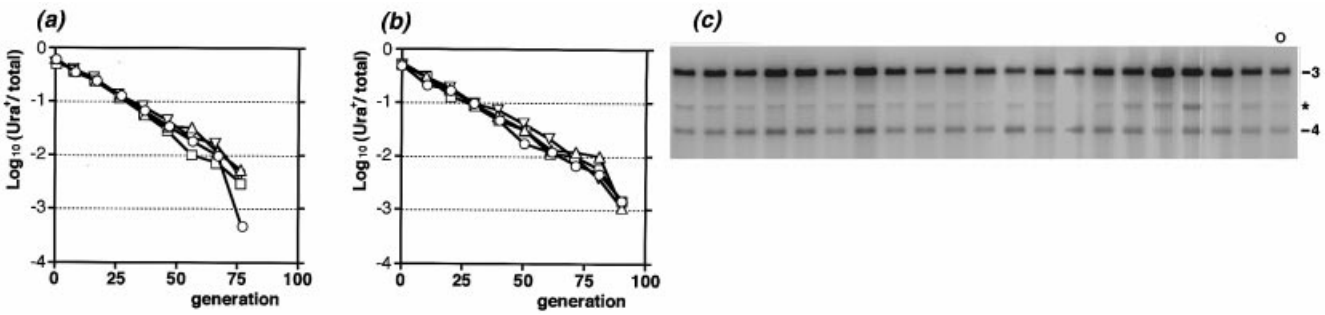

Fig. 2. B3URA3 RNA replicon is lost at a fixed frequency during culture in the presence of uracil and maintained without detectable deletions. (*a*) 1a2a$^+$ yeast cells (*ura3*$^-$ yeast strain YPH500 expressing BMV 1a and 2a proteins from plasmids pB1CT19 and pB2CT15; Janda & Ahlquist, 1993) were transfected as described (Janda & Ahlquist, 1993) with capped B3URA3 *in vitro* transcripts to obtain colonies able to grow in medium lacking uracil. Four Ura$^+$ colonies were independently cultured overnight in liquid synthetic glucose medium lacking uracil to select for the B3URA3 RNA replicon, and also lacking histidine and leucine to select for the 1a and 2a expression plasmids. All four lines were then passaged in parallel as follows. At time zero (yeast generation zero), the cells were inoculated into synthetic glucose medium containing uracil (lacking histidine and leucine). Then, at 24 h intervals, successive subcultures were established by 1000-fold dilution (each inoculum contained no less than 650 Ura$^+$ cells) into fresh medium containing uracil and lacking histidine and leucine. Every 24 h, culture samples were plated on appropriate selective media to determine the concentration of [Ura$^+$ His$^+$ Leu$^+$] and [His$^+$ Leu$^+$] cells. The plot shows $\log_{10}$([His$^+$ Leu$^+$ Ura$^+$] cells/[His$^+$ Leu$^+$] cells) vs yeast generation number for four independent subcultures (○, □, △, ▽) passaged as described. The slope of this plot represents the frequency at which the B3URA3 RNA replicon-dependent Ura$^+$ phenotype was lost. Note that the smaller the $\log_{10}$(Ura$^+$/total) value becomes (especially less than −2), the larger the experimental error becomes, because fewer colonies were used to determine the concentration of Ura$^+$ cells. (*b*) For each of the four passaged lines in (*a*), a sample containing 2500–5000 Ura$^+$ cells was re-amplified by inoculating the cells into synthetic glucose liquid medium lacking histidine, leucine and uracil, and a second, independent series of successive subcultures in the presence of uracil was established. The graph showing $\log_{10}$([His$^+$ Leu$^+$ Ura$^+$] cells/[His$^+$ Leu$^+$] cells) vs yeast generation number was created as in (*a*). (*c*) At the end of the second series of passage, 10 Ura$^+$ colonies were randomly picked from each line (total 40). These Ura$^+$ yeast cells were extracted (Janda & Ahlquist, 1993) to yield total RNA, which was denatured with glyoxal, separated by electrophoresis in 1% agarose, blotted onto a nylon membrane (Hybond-N), fixed by ultraviolet irradiation, and hybridized with a ^{32}P-labelled BMV positive-strand RNA-specific RNA probe complementary to the conserved 200 nucleotides at the 3′ end of BMV RNA. Here, we show 20 representative lanes for the colonies after passage, and that for the B3URA3-harbouring cells before passage (marked '○'). The positions of bands corresponding to B3URA3 and its subgenomic RNA4 are indicated on the right. An asterisk marks an accumulation of background RNAs swept ahead of a ribosomal RNA band.

which could shed further light on virus and cell biology. To determine the efficiency with which the B3URA3 RNA replicon was transmitted from 1a2a$^+$ mother to daughter cells, we passaged such yeast in medium containing uracil, thus removing selection for *URA3* and the B3URA3 replicon. Specifically, 1a2a$^+$ yeast cells were transfected with B3URA3 *in vitro* transcripts to obtain Ura$^+$ cells, which were cultured overnight in medium lacking uracil to select for B3URA3. These cells (generation zero) then were inoculated into medium containing uracil. Every 24 h, samples were taken to determine the percentage of Ura$^+$ cells and the cells were subcultured at 1000-fold dilution in fresh, uracil-containing medium. Preliminary studies showed that, when cells reverted to a Ura$^-$ (uracil-dependent) growth phenotype under such conditions, such reversion was always associated with loss of the B3URA3 RNA replicon, while retention of the Ura$^+$ growth phenotype was linked to retention of B3URA3. Thus, B3URA3 replicon loss appears to be much more frequent than inactivating mutations of the *URA3* ORF or subgenomic mRNA promoter in B3URA3.

As shown in Fig. 2(*a*) for four independently passaged cultures, plotting $\log_{10}$(Ura$^+$ cells/viable 1a2a$^+$ cells) vs the number of yeast cell divisions yielded straight lines of reproducible slope. These results show that the Ura$^+$ phenotype was lost at a constant rate in each generation, and that the resulting Ura$^-$ cells grew at the same rate as the Ura$^+$ cells from which they were derived. From Fig. 2(*a*) and multiple independent experiments (e.g. Fig. 3*c*), the frequency of loss of Ura$^+$ phenotype was calculated to range from 5% to 15% per cell division. Thus, at each cell division, the B3URA3 RNA replicon was maintained in 85–95% of the progeny of B3URA3-containing cells.

Next, we tested whether 75 generations of B3URA3 passage in the presence of uracil had selected for strains with improved B3URA3 transmission efficiency. For each of the four passaged lines in Fig. 2(*a*), a sample (containing 2500–5000 Ura$^+$ cells) from the final passage was amplified in the absence of uracil and subjected to a second round of sequential passaging in the presence of uracil. However, as shown in Fig. 2(*b*), plotting the logarithm of Ura$^+$ frequency vs number of cell divisions over 90 yeast generations yielded straight lines with slopes similar to those of the first series of successive subcultures (Fig. 2*a*), revealing no change in the frequency of B3URA3 RNA loss.

After the second series of successive passages, we randomly picked 10 Ura$^+$ colonies from each passaged line (total of 40

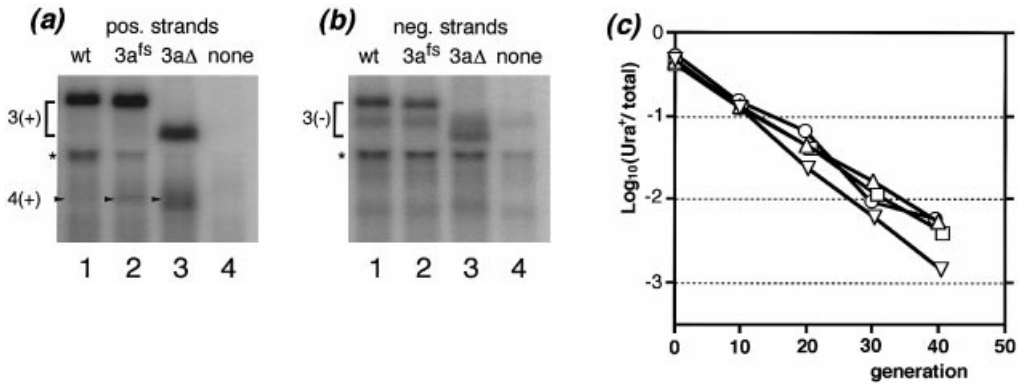

Fig. 3. Accumulation and maintenance of the B3URA3 RNA replicon and 3a-disrupted derivatives in yeast. (*a*, *b*) As an alternative to inefficient transfection of yeast with *in vitro* transcripts, Ura$^+$ 1a2a$^+$ yeast strains bearing B3URA3 (wt, lane 1), B3URA3-3a^{fs} (3a^{fs}, lane 2) or B3URA3-3aΔ (3aΔ, lane 3) as free RNA replicons were obtained by induction from DNA plasmids followed by plasmid curing, as follows: 1a2a$^+$ yeast cells were transformed with a DNA plasmid (see Fig. 1) expressing the desired B3URA3 derivative from the galactose-inducible, glucose-repressible *GAL1* promoter, and then were cultured in synthetic medium lacking histidine, leucine and tryptophan to select for the 1a and 2a expression plasmids and B3URA3 plasmid, respectively, and containing galactose to induce transcription of the B3URA3 derivative. Ura$^+$ cells with *URA3* expressed from the BMV RNA replicon were selected by plating these yeast on synthetic medium lacking uracil, histidine, leucine and tryptophan and containing glucose to repress plasmid transcription of the B3URA3 derivative (Ishikawa *et al.*, 1997). These [Ura$^+$ His$^+$ Leu$^+$ Trp$^+$] colonies were then cultured in synthetic glucose medium lacking uracil, histidine and leucine but containing tryptophan to remove selection for the B3URA3 derivative plasmid. [Ura$^+$ His$^+$ Leu$^+$ Trp$^-$] cells were isolated from these cultures and Southern blot analysis confirmed that the DNA plasmids encoding B3URA3 derivatives were lost and that no B3URA3 DNA sequences were present in any other form. These [Ura$^+$ His$^+$ Leu$^+$ Trp$^-$] yeast cells were extracted (Janda & Ahlquist, 1993) to yield total RNA, which was denatured with glyoxal, separated by electrophoresis in 1% agarose, blotted onto a nylon membrane (Hybond-N), fixed by ultraviolet irradiation and hybridized with BMV RNA-specific probes. The probe to detect BMV-related positive-strand RNAs in (*a*) was ^{32}P-labelled RNA complementary to the conserved 200 nucleotides at the 3′ end of BMV RNA. The probe to detect BMV-related negative-strand RNAs in (*b*) was ^{32}P-labelled RNA having the sequence of the conserved 200 nucleotides at the 3′ end of BMV RNA. Lanes 4 (marked 'none') contain RNA from 1a2a$^+$ yeast without B3URA3 RNA. The positions of positive- and negative-strand RNA3 bands are shown by brackets. In lane 3 for B3URA3-3aΔ in (*a*), the band just below subgenomic RNA4 is believed to represent a degradation product of B3URA3-3aΔ RNA. An asterisk marks an accumulation of background RNAs swept ahead of a ribosomal RNA band. (*c*) The specific yeast strains analysed in (*a*) and (*b*), containing B3URA3 (□), B3URA3-3a^{fs} (△) or B3URA3-3aΔ (▽) RNA replicons, were successively subcultured in medium containing uracil and analysed as in Fig. 2. Circles represent data for parallel subcultures of one of the B3URA3-containing cell lines of Fig. 2 (obtained by direct transfection of 1a2a$^+$ yeast with B3URA3 *in vitro* transcripts). The difference in slope between Fig. 2 and Fig. 3 may represent experimental variation derived from slight differences in culture conditions. Equivalent results were obtained in another experiment using independent isolates of 1a2a$^+$ yeast bearing B3URA3-3a^{fs} or B3URA3-3aΔ.

colonies), amplified them in medium lacking uracil, extracted RNA and analysed it by Northern blot hybridization with a B3URA3 probe. The accumulation level and electrophoretic pattern of B3URA3-related RNAs in these 40 colonies paralleled those in the original B3URA3 RNA-harbouring 1a2a$^+$ cells (Fig. 2*c*). While these results do not rule out point mutations in the 3a gene, they show that the 3a ORF on B3URA3 RNA was maintained without detectable deletion during continuous replication and mitotic transmission for over 165 yeast generations.

Such long term retention of 3a sequences appeared surprising since the 3a gene is dispensable for RNA3 replication and subgenomic mRNA synthesis (French & Ahlquist, 1987; Janda & Ahlquist, 1998). By contrast, during expression of foreign genes from vectors engineered from many positive-strand RNA viruses, genes not necessary for virus multiplication or spread tend to be quickly deleted, in whole or in part, by recombination (e.g. see Dawson *et al.*, 1989 for tobacco mosaic virus; Mueller & Wimmer, 1998 for polio virus). Similar results have also been found for BMV gene expression vectors (M. Janda & P. Ahlquist, unpublished results). Even for natural viral sequences, a beet necrotic yellow vein virus genomic RNA segment required for fungal transmission but not virus multiplication is quickly shortened by internal deletion when infectious transcripts are mechanically inoculated on a host plant, bypassing selection for fungal transmission (Bouzoubaa *et al.*, 1991). For BMV, it has been reported that RNA3 derivatives with deletions in the 3a gene accumulate after prolonged incubation of infected barley plants, implying the dispensability and possible negative effect of the 3a gene for the accumulation of BMV in old barley plants (Damayanti *et al.*, 1999).

Since the 3a gene does not contribute to RNA replication but was maintained in B3URA3 replicons during long term replication and mitotic transmission, these results suggested that 3a function might be necessary for efficient cell-to-cell transmission of BMV RNA replicons from mother to daughter yeast cells. Several independent results appeared consistent

with this possibility. In the natural plant hosts of BMV, 3a protein localizes to plasmodesmatal connections between adjacent cells, and is necessary for cell-to-cell movement of infection (Fujita *et al.*, 1998; Mise & Ahlquist, 1995). The ability of 3a protein to cooperatively bind nucleic acids in a sequence-nonspecific manner is thought to be involved in delivering viral RNA from one plant cell to the next (Jansen *et al.*, 1998; Fujita *et al.*, 1998), and so might mediate or enhance transmission of B3URA3 RNA from mother to daughter yeast cells.

To test whether the 3a gene contributed to B3URA3 transmission from mother to daughter yeast, we constructed two B3URA3 derivatives with 3a gene disruptions: B3URA3-3a^{fs}, with a two-base frameshifting insertion between RNA3 nucleotides 603 and 604, and B3URA3-3aΔ, with a deletion of RNA3 nucleotides 306–817 (Fig. 1; Ahlquist *et al.*, 1981). As with B3URA3, introduction of either of these derivatives into 1a2a^{+} yeast generated Ura^{+} strains that replicated the B3URA3 derivative and readily formed colonies on media lacking uracil. Northern blot analysis (Fig. 3*a*, *b*) confirmed that these cells contained the expected B3URA3-related RNA replicons and their negative-strand replication intermediates (Janda & Ahlquist, 1993). When these strains were passaged in the presence of uracil, the frequency of loss of these two 3a-deficient B3URA3 RNA derivatives was similar to or, for B3URA3-3aΔ, only slightly higher than that of the original B3URA3 RNA (i.e. 10–15% per cell division; Fig. 3*c*). Thus, the 3a coding region is not required for B3URA3 RNA transmission from mother to daughter yeast and makes little if any contribution to the efficiency of this transmission.

Given the absence of demonstrable selection pressure for the 3a gene in yeast, it is noteworthy that this gene was not deleted from B3URA3 during over 165 cycles of RNA replication and yeast cell division, corresponding to the equivalent of 10^{45}-fold amplification of B3URA3 by RNA-dependent RNA replication. Similarly, though eventually deleted, some unselected foreign gene insertions in positive-strand RNA virus expression vectors have proven more genetically stable than others (Donson *et al.*, 1991; Varnavski & Khromykh, 1999). The detectable appearance of deletion variants in a virus population depends on the frequency of recombination events generating such deletions (e.g. Dawson *et al.*, 1989; Donson *et al.*, 1991) and on the replicative fitness of such deletions relative to the starting genome. In other words, even after being generated, a new deletion derivative would remain a rare variant in the population unless it has a replicative advantage over the starting genome. Many foreign gene insertions reduce the replication and/or stability of viral RNAs, giving their deletion derivatives a significant replicative advantage. By contrast, in B3URA3, the wild-type BMV 3a gene may be sufficiently adapted to replication in its RNA3 surroundings that 3a deletions have little or no replicative advantage, as appears to be the case for B3URA3-3aΔ (Fig. 3*a*). Thus, retention of the 3a gene in B3URA3 may be due to the absence of a selective disadvantage, rather than the presence of a selective advantage.

While the BMV 3a gene did not contribute significantly to B3URA3 cell-to-cell transmission in yeast, it remains possible that BMV RNA replicons are actively partitioned to yeast daughter cells by 3a-independent processes. The BMV RNA replication complex is associated with the endoplasmic reticulum (ER), predominantly the perinuclear ER, in both plant cells and in yeast (Restrepo-Hartwig & Ahlquist, 1996, 1999). Thus, the ordered segregation of the nuclear envelope (which in yeast remains intact through the cell cycle) and peripheral ER during yeast cell division (Warren & Wickner, 1996) may transport functional BMV RNA replication complexes and their templates to daughter cells.

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