Stable Chloroplast Transformation of the Unicellular Red Alga *Porphyridium* Species[1]

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Red algae are extremely attractive for biotechnology because they synthesize accessory photosynthetic pigments (phycobilins and carotenoids), unsaturated fatty acids, and unique cell wall sulfated polysaccharides. We report a high-efficiency chloroplast transformation system for the unicellular red microalga *Porphyridium* sp. This is the first genetic transformation system for Rhodophytes and is based on use of a mutant form of the gene encoding acetohydroxyacid synthase [AHAS(W492S)] as a dominant selectable marker. AHAS is the target enzyme of the herbicide sulfometuron methyl, which effectively inhibits growth of bacteria, fungi, plants, and algae. Biolistic transformation of synchronized *Porphyridium* sp. cells with the mutant AHAS(W492S) gene that confers herbicide resistance gave a high frequency of sulfomethuron methyl-resistant colonies. The mutant AHAS gene integrated into the chloroplast genome by homologous recombination. This system paves the way for expression of foreign genes in red algae and has important biotechnological implications.

Red algae are attractive for biotechnological purposes because they synthesize unique cell wall sulfated polysaccharides, accessory photosynthetic pigments (phycobilins and carotenoids), and unsaturated fatty acids [Arad (Malis), 1988]. However, the absence of gene transfer technology for red algae is creating a bottleneck in exploitation of their biotechnological potential. We report the development of an efficient chloroplast transformation system for the unicellular red microalga *Porphyridium* sp. This is the first gene transfer system for Rhodophytes, and is based on a mutant form of the gene encoding acetohydroxyacid synthase (AHAS; E.C. 4.1.3.18; Ray, 1984) as a dominant selectable marker.

AHAS is the target enzyme of the herbicide sulfometuron methyl (SMM), which effectively inhibits growth of bacteria, yeast, and plants. Mutant forms of the AHAS gene serve as dominant selectable markers for transformation of commercial yeast (Gysler et al., 1990) and higher plants (Li et al., 1992; Ott et al., 1996). In green algae and plants, the AHAS gene is encoded by the nucleus (Mazur et al., 1987) and nuclear transformations occur via chromosome integration at random sites (Vergunst and Hooykaas, 1999). In Rhodophytes, however, the AHAS gene is chloroplast encoded (Reith and Munholland, 1993; Lapidot et al., 1999) and therefore its use can generate stable transformants with a high probability of having the transforming DNA integrated into the chloroplast genome by homologous recombination (Boynton et al., 1988; Kindle et al., 1991; Gumpel et al., 1994).

Growth of *Porphyridium* sp. is strongly inhibited by SMM and a spontaneous resistant mutant denoted SMM-resistant (SMR) was isolated from a culture exposed to this herbicide (van Moppes et al., 1989). The mutant grows efficiently in the presence of SMM and displays SMM-resistant enzyme activity in crude extracts. We cloned the AHAS gene from wild-type *Porphyridium* sp. and from SMR. The mutant gene has a single base substitution (G $\rightarrow$ C), which leads to exchange of the conserved Trp at position 492 with a Ser residue [AHAS(W492S); Lapidot et al., 1999]. Mutations at this position are known to confer herbicide resistance in other organisms (Ibdah et al., 1996). Here, we describe biolistic delivery of the AHAS(W492S) gene to *Porphyridium* sp. cells and isolation of SMM-resistant transformants displaying AHAS activity resistant to the herbicide in crude extracts.

RESULTS AND DISCUSSION

Transformation of *Porphyridium* sp.

For microprojectile bombardment, the mutant gene was cloned with 56 upstream bp into a TA cloning vector. In our first experiments, 10^8 *Porphyridium* sp. cells per plate were bombarded with 500 μg of tungsten spheres coated with 0.8 μg of DNA. The cells were propagated in liquid selective medium (30 μM SMM) for 10 d before being plated on SMM plates (50 μM). Four transformed colonies appeared on the selective plates after 10 d. The doubling time of *Porphyridium* sp. under these experimental conditions is 17 h and we calculated a transformation frequency of about 2.5×10^{-4} μg^{-1} DNA.

Given that the transformation targets the chloroplast, we tried to increase the efficiency by exposing the cells to four cycles of dilution/light/dark before delivery of the DNA, a regime shown to synchronize

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cell replication (Simon-Bercovitch et al., 1999). In a preliminary experiment, cells were bombarded at 0, 4, and 8 h after the last dark cycle, grown for 10 d in liquid SMM medium (30 μM), and then seeded on 50 μM SMM- artificial seawater (ASW) plates. A very high transformation frequency was observed when the cells were transformed immediately after the dark period; cells bombarded 4 and 8 h later did not show any increase in frequency of transformation compared with asynchronous cultures. To calculate the transformation frequency, we bombarded the cells as described above and overlaid them 24 h later with 10 mL of ASW/1% (w/v) agar containing 100 μM SMM (final concentration 33 μM) without prior growth in liquid medium. This protocol of transformation gave up to 85 SMM-resistant colonies per microgram of DNA when the cells were transformed immediately after the dark period. Mock-transformed cells gave no colonies on the SMM plates. No transformation was obtained when the biolistic delivery was performed in asynchronous cultures immediately after a dark period (16 h). These results show that bombardment of synchronized cells immediately after the last dark cycle enhances DNA uptake by the chloroplasts. Studies on synchronized *Porphyridium* sp. cells indicated that it takes the cells at least 6 h of light to resume DNA synthesis; however, the amount of cell wall polysaccharide is the lowest immediately after the dark period (Simon-Bercovitch et al., 1999). The higher transformation frequency after the last dark period (0 h) in synchronized cells may stem from the decreased amount of the cell wall polysaccharide rather than from the DNA replication status.

Four transformants from four independent transformation experiments including synchronized (Syn1 and 2) as well as non-synchronized (Tsm1 and 2) cells were analyzed. In all cases, the resistant cells were grown for at least 2 months in SMM medium (100–200 μM) prior to their analysis, to increase the number of chloroplast genomes that included the introduced AHAS gene.

Analysis of Integration Events in the Chloroplast Genome of *Porphyridium* sp.

Integration via a double crossover should result in replacement of the endogenous gene by the introduced mutant AHAS(W492S) gene. Integration via a single crossover should give rise to two copies of the gene with vector sequences in between, as reported for chloroplast transformation in *Chlamydomonas reinhardtii* with a circular plasmid (Kindle et al., 1991). The mutant AHAS(W492S) gene would be expressed from the endogenous AHAS promoter, whereas the wild-type gene would be expressed from the cloned AHAS upstream sequence (Fig. 1). The W492S mutation that confers SMM resistance is close to the C terminus of the cloned gene (nucleotide 1,475), thereby reducing the probability of obtaining SMM-resistant transformants by a double crossover event on both sides of the mutation. The short region of homology downstream of the mutation increased the probability of selecting transformants that had undergone a single crossover event (Boynton et al., 1990; Kindle et al., 1991; Kindle and Sodeinde, 1994). Integration that occurred via a single crossover event was monitored by sequence analysis of PCR fragments using primers derived from the plasmid vector and the AHAS gene. Primers derived from the forward AHAS (AHAS 590–611F, primer a) and reverse vector sequences (VecR, primer b) generated a PCR fragment (1.2 kb) that included the G $\rightarrow$ C mutation at position 1,475. Primers derived from the forward vector (VecF, primer c) and reverse AHAS sequences (AHAS 1,674–1,697R, primer d) generated a PCR fragment (1.8 kb) that did not include the W492S mutation (Fig. 2). These results showed that all four transformants contained both wild-type and mutant copies of the AHAS gene, indicating that each of these SMM-resistant transformants resulted from a single crossover event. Control reactions using genomic DNA from wild type or from the spontaneous mutant SMR as templates did not yield any PCR products as expected because they did not contain the vector sequences. It is not clear whether the chloroplast genomes in transformed cells reached homoplasmicity with one copy of the wild-type gene and one copy of the mutant gene, or whether some maintained only the original wild-type AHAS gene.

Further analysis of integration events was performed on Southern blots of Tsm1 by Southern analysis of the Tsm1 transformant that was grown in SMM for 4 months. Genomic DNA of wild-type and Tsm1 cells was probed with vector and AHAS sequences. The diagram in Figure 3 shows how integration of pSMR1000 into the chloroplast genome at a single site would generate four AHAS bands in an *Eco*RI digest. The AHAS probe hybridized to two fragments (1.0 and 1.5 kb) in both wild-type and Tsm1 DNA. Tsm1 DNA revealed two additional fragments of 0.8 and 4 kb. The 0.8-kb band extends from the AHAS *Eco*RI site (E/974) of the gene copy shown at the left to the introduced vector *Eco*RI site (E/MCS). The 4-kb fragment extends from the latter (E/MCS) to the *Eco*RI site of the other AHAS gene. The 4-kb band also hybridized with the vector DNA (Fig. 3). Therefore, our results show that both AHAS genes, the mutant and the wild type, are present in the transformed cells as well as the vector. Hybridization with uncut wild-type and Tsm1 DNA using vector DNA probe revealed a single high-M_r band that comigrated with the uncut DNA; we did not detect any fragment that could correspond to an extrachromosomal supercoiled plasmid (Fig. 2). These data, combined with the PCR results, excluded the possibility that the mutant AHAS gene was re-

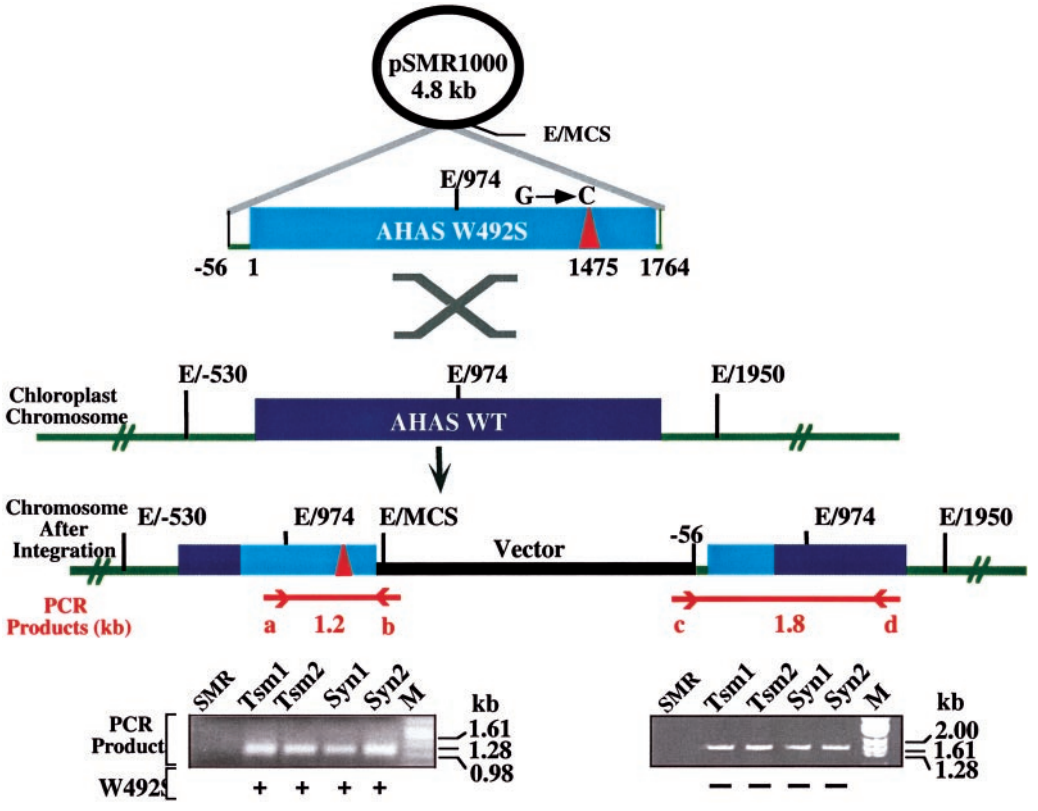

Figure 1. Analysis of the AHAS locus of transformed cells. Top, The diagram shows pSMR1000 (4.8 kb), the plasmid used for transformation, that included the complete coding region of the AHAS W492S gene (1,764 bp), with 56 upstream bp. The G → C mutation at position 1,475 (W492S) is marked with a red triangle and vector sequences are marked with a thick black line. The chloroplast chromosome is represented by a green line. *Eco*RI sites are marked as E, followed with their position. The plasmid-derived *Eco*RI site is marked E/MCS. The mutant and wild-type genes are shown by light- and dark-blue rectangles, respectively. The location of the integration event is arbitrary. The predicted PCR fragments are marked with red lines below the chromosome that has the endogenous and the integrated mutant genes and the binding sites of the primers are marked with arrowheads. The AHAS fragment with the W492S mutation was amplified with primers a (AHAS 590–611F) and b (VecR). The wild-type AHAS fragment was amplified with primers c (VecF) and d (AHAS 1,674–1,697R). The resulting PCR products obtained from the spontaneous mutant SMR and the transformants of *Porphyridium* sp. Tsm1, Tsm2, Syn1, and Syn2 are shown below the integration scheme and the presence (+) or absence (−) of the W492S mutation is marked.

tained as an episome, as observed occasionally in green algae (Suzuki et al., 1997).

Integration of the plasmid DNA into the chloroplast genome via single crossover events paves the way for introducing foreign genes into the chloroplast genome along with the AHAS(W492S)-selectable marker. Under selective conditions, the mutant AHAS(W492S) gene and the vector sequences were maintained for a year after the transformation.

SMM Activity of Transformed Algae

Growth of the wild-type algae was indistinguishable from that of the transformants in the absence of the herbicide, indicating that integration of the mutant AHAS gene did not interfere with cell growth. However, in the presence of the herbicide, wild-type cells did not grow at all, whereas growth of the transformants was indistinguishable from that of growth in the absence of SMM (Fig. 3A).

AHAS activity was examined in crude extracts of wild-type cells of the spontaneous mutant SMR, and of Tsm1 and Syn1 transformants in the presence of increasing concentrations of SMM. Enzyme activity in crude extracts of the SMM-resistant mutant (SMR) was not affected by 100 μM SMM, whereas activity of the wild-type extract was reduced to 50% by 3 μM SMM. Extracts of the transformants (Syn1 and Tsm1) showed an intermediate level of activity with 50% inhibition at 30 and 70 μM SMM, respectively (Fig. 3B).

The original herbicide-resistant strain SMR, which was isolated as a spontaneous mutant after exposure to SMM (van Moppes et al., 1989), has a single AHAS gene per chloroplast genome and is homoplasmic for the AHAS(W492S) mutation. In the transformed cells, the activity assay indicated that both copies of the gene, the mutant and the wild type, were expressed. Likewise, the PCR analysis (detailed above) detected both the mutant and wild-type copy of the

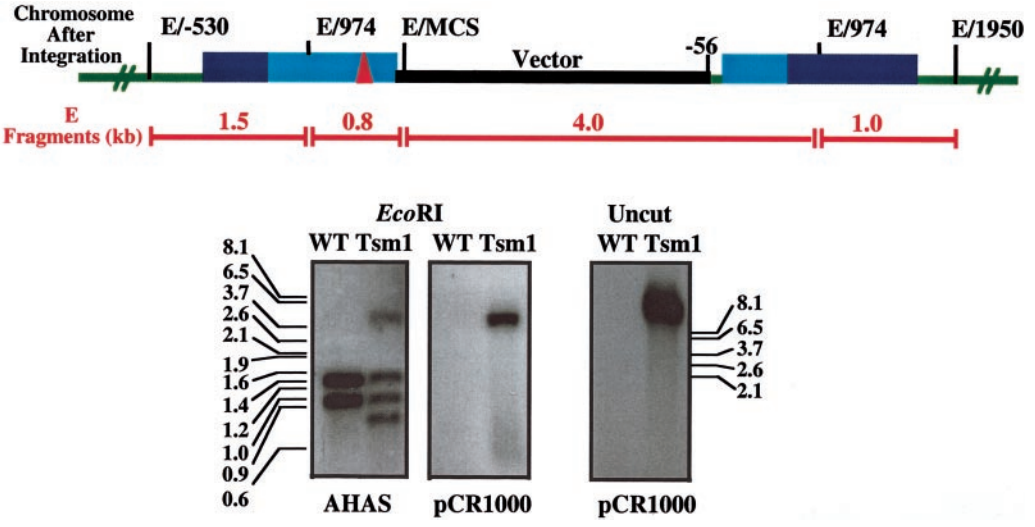

Figure 2. Southern analysis of transformed *Porphyridium* sp. Total DNA of wild-type (WT) and transformed (Tsm1) cells was digested with *Eco*RI and hybridized sequentially with the AHAS and vector probes (left double panel). Uncut DNA was hybridized with the vector probe (right). The predicted *Eco*RI bands observed in the Southern analysis are marked below the recombined chromosome as a red ruler. The 1.0- and 1.5-kb bands in the *Eco*RI digest correspond to the endogenous AHAS gene and the 0.8- and 4-kb bands are specific to the transformants, only the latter hybridizes with the vector probe. Hybridization of vector sequences with the uncut Tsm1 DNA comigrates with ethidium bromide staining (not shown) of the uncut DNA.

AHAS gene in the transformants. A similar intermediate level of AHAS resistance to SMM was found in crude extracts of somatic hybrids produced by fusing protoplasts of a DCMU-resistant mutant of *Porphyridium* sp. with those of the SMM-resistant mutant (Sivan and Arad Malis, 1998).

The AHAS enzyme is a heterotetramer that consists of two large catalytic subunits and two small regulatory subunits (Chipman et al., 1998). Recent studies using mutants of this enzyme show that wild-type and mutant catalytic subunits can assemble into a hybrid enzyme (Bar-Ilan et al., 2001). Thus, the intermediate resistance to SMM in crude extracts of the transformed algae (Fig. 3B) could result from such hybrid enzyme molecules, suggesting that both wild-type and mutant enzyme subunits are expressed in the chloroplast of transformed cells.

The establishment of an efficient stable chloroplast transformation system for the unicellular red alga *Porphyridium* sp. will facilitate future biotechnological developments. Use of the *Porphyridium* AHAS gene as a dominant selectable marker conferring herbicide resistance should be applicable to all Rhodophytes, including seaweeds of commercial impact (i.e. various species of *Porphyra* and *Gracilaria*).

MATERIALS AND METHODS

Cells

Porphyridium sp. UTEX 637 (Starr and Zeikus, 1987) was grown in ASW (Jones et al., 1963) at 25°C on a rotary shaker at 130 rpm under continuous illumination of cold white

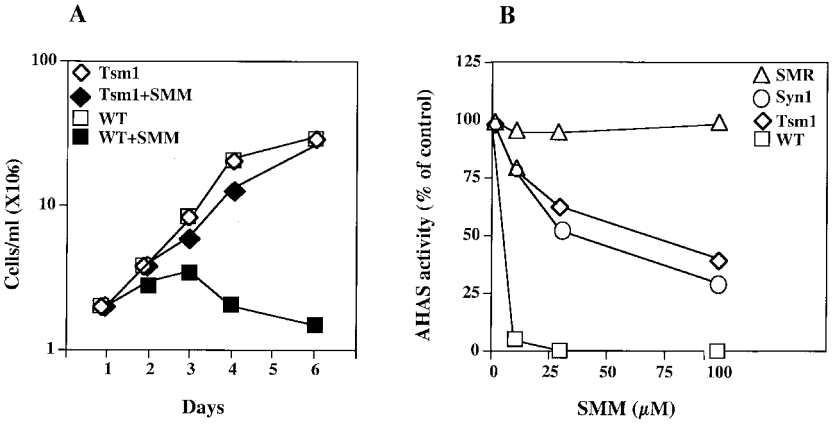

Figure 3. SMM resistance of transformed cells. A, Effect of SMM on growth of the transformant Tsm1 (diamonds) and wild-type (squares) *Porphyridium* sp. with (black) or without (white) 100 μM SMM. Cells were counted daily. B, AHAS activity of Tsm1 (diamonds), Syn1 (circles), SMR (triangles), and wild-type (squares) *Porphyridium* sp. in the presence of SMM. AHAS activity in each extract is calculated as percent of activity without SMM.

fluorescent lamps at 90 μmol photons m^{-2} s^{-1} [Sivan and Arad (Malis), 1998].

Plasmid Construction and Primers

The mutant AHAS gene with 56 upstream and 6 downstream bp was amplified by PCR using forward (CCAGGTATTATTCTAGACTGAACTG) and reverse (GAATAATTCTCTTAATTATTCTT) primers. The resulting 1,826-bp PCR product was ligated into a TA cloning vector (pCR1000, Invitrogen, Carlsbad, CA) to give pSMR1000.

The chloroplast genomes of wild-type and transformed cells were analyzed with primer pairs derived from the vector and AHAS gene sequences. These were: AHAS forward (primer a, AHAS 590–611F; GCGATCTAGTTGCTCCCAGCTC) and vector reverse (primer b, VecR; CGGCCAGTGAATTCGCGGCCGCGGT) for the mutant, and vector forward (primer c, VecF; CGAGCTCACTAGTTTAATTAAAAG C) and AHAS reverse (primer d, AHAS 1674–1697R; CCAGGAGCTACCATAGGATAACAG) for the wild-type gene. The G $\rightarrow$ C substitution in the mutant AHAS allele is at position 1,475.

Transformation of *Porphyridium* sp.

Logarithmic (24 h) and synchronized cells were collected by centrifugation and washed once with water (pH 4.0) at room temperature to remove excess cell wall polysaccharide. Algal cells (10^8) were plated on ASW/1% (w/v) agar 1 h prior to bombardment with a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories, Hercules, CA). Tungsten particles (0.7 μm) were coated with circular supercoiled pSMR1000 DNA according to Bio-Rad protocols. Each bombardment delivered 500 μg of tungsten spheres coated with 0.8 μg of DNA at 1,300 psi from a distance of 9 cm. After 24 h, the cells were transferred to liquid ASW containing 30 μM SMM for 10 d, replated onto ASW/1% (w/v) agar plates with 50 μM SMM, and incubated for an additional 10 d. Colonies that appeared under these selective conditions were propagated in liquid ASW with 100 to 200 μM SMM for 2 months before Southern analysis.

For calculating the transformation efficiency, we synchronized cells with four cycles of dilution/12 h of light/12 h of dark (Simon-Bercovitch et al., 1999) and bombarded them 0, 4, and 8 h after the last dark period. The plates were overlain 4 d later with solid ASW/1% (w/v) agar medium with 33 μM SMM (final concentration). Colonies were counted 7 to 10 d later and propagated in 100 to 200 μM SMM.

AHAS Activity

Wild-type cells were grown in ASW; SMR, Tsm1, and Syn1 were grown in ASW with 100 μM SMM. Log phase cells were washed twice with water at pH 4.0 at room temperature and resuspended at a concentration of 10^9 cells mL^{-1} in extraction buffer (0.1 M KPO_4, pH 7.6; 10 mM EDTA; 1 mM dithiothreitol; and 0.1 mM FAD). The cells were broken by four cycles of freeze/thaw in liquid N_2. Enzyme activity was assayed in the crude extracts by the standard colorimetric method at 37°C in a buffer containing 0.1 M KPO_4, pH 7.6, with 100 mM pyruvate and increasing concentrations of SMM (Weinstock et al., 1992).

Southern Analysis

Aliquots of 10 μg of genomic DNA were digested with restriction enzymes, blotted, and hybridized with the 1.8-kb AHAS gene or the 3-kb vector sequence (pCR1000) probe.

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