How did a duplicated gene copy evolve into a restorer-of-fertility gene in a plant? The case of Oma1

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Restorer-of-fertility (Rf) is a suppressor of cytoplasmic male sterility (CMS), a mitochondrion-encoded trait that has been reported in many plant species. The occurrence of CMS is considered to be independent in each lineage; hence, the question of how Rf evolved was raised. Sugar beet Rf resembles Oma1, a gene for quality control of the mitochondrial inner membrane. Oma1 homologues comprise a small gene family in the sugar beet genome, unlike Arabidopsis and other eukaryotes. The sugar beet sequence that best matched Arabidopsis atOma1 was named bvOma1; sugar beet Rf (RF1-Oma1) was another member. During anther development, atOma1 mRNA was detected from the tetrad to the microspore stages, whereas bvOma1 mRNA was detected during the meiosis and tetrad stages. A transgenic study revealed that, whereas RF1-Oma1 can bind to a CMS-specific protein and alter the higher-order structure of the CMS-specific protein complex, neither bvOma1 nor atOma1 show such activity. We favour the hypothesis that an ancestral Oma1 gene duplicated to form a small gene family, and that one of the copies evolved and acquired a novel expression pattern and protein function as an Rf, i.e. RF1-Oma1 evolved via neofunctionalization.

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1. Introduction

In plants, hermaphrodites are converted into females by male sterility encoded by mitochondria, a phenomenon known as cytoplasmic male sterility (CMS) [1,2]. Mitochondrial genes responsible for CMS (S-orf) are composed of partial duplicates of ordinary genes (such as those coding for ATP synthase subunits) and/or origin-unknown sequences [3,4]; their primary structure varies among plant species, suggesting that each incidence of CMS has an independent origin. From the viewpoint of evolutionary genetics, the maternal inheritance of mitochondria favours the evolution of CMS because the resources for pollen production can be saved and used to increase female fitness. On the other hand, CMS causes a genetic conflict between the mitochondria and the nuclear genome (biparental inheritance), and the decrease in pollen transmission due to CMS creates pressure for the evolution of a counteracting system in the nuclear genome [2,5].

Genetic analysis of the CMS suppression system revealed a nuclear gene termed restorer-of-fertility (Rf) [6–8]. A dominant Rf allele suppresses S-orf, thereby restoring pollen fertility [9]. Differences in CMS have been defined by differences among the cognate Rf genes [10], implying genetic diversity among Rf. The proposed evolutionary mechanism for Rf has implicated a molecular arms race, analogous to the coevolution of pathogens and resistance genes [11–13]; however, the initial steps of Rf evolution are obscure at the molecular level.

Sugar beet (Beta vulgaris L.) CMS involves preSatp6 as the S-orf and Rf1 as the Rf [14,15]. preSatp6, named after a unique presequence in CMS mitochondrial atp6, is composed of a 387 amino acid sequence of unknown origin. Translation products from preSatp6 were detected in all examined organs to form a 250 kDa protein complex in the mitochondrial membrane of CMS plants [16], although this complex’s function is unknown. When the plant has a dominant Rf1 allele, preSATP6 protein in the anther is detected in a novel 200 kDa complex concomitantly with a decrease in the amount of the 250 kDa complex, whereas the total amount of preSATP6 protein is almost unchanged [16,17]. This result suggests the anther-specific alteration of a higher-order structure of the preSATP6 protein.

The Rf1 locus consists of a gene cluster that shows copy number variation (CNV) among breeding lines [15,18,19]. Genes in the cluster have homology to Oma1, a gene encoding an ATP-independent protease that participates in quality control of the mitochondrial inner membrane (yeast) and mitochondrial dynamics (mammals) [20–22]. In Arabidopsis, Oma1 is a single copy gene (hereafter atOma1); loss of atOma1 causes some disorder in oxidative phosphorylation (OXPHOS) [23].

A puzzling observation was seen in the clustered Oma1-like genes in the Rf1 locus. The zinc-binding motifs in their M48 peptidase domains, whether dominant or recessive, were identical to that of a mutagenized gene that lost proteolytic activity in yeast (i.e. HQVGH instead of the consensus HExxH) [15]. Therefore, we hypothesized that there might be another Oma1 homologue that functions as the authentic Oma1 in sugar beet. If so, identifying this sequence will facilitate determining how Oma1-like genes at the Rf1 locus (hereafter RF1-Oma1) evolved from Oma1. We found that another Oma1-like gene, with the consensus HExxH motif, is preserved in the sugar beet genome; however, its translation product is incapable of binding preSATP6 protein or generating the 200 kDa protein complex. Moreover, its expression pattern is different from that of RF1-Oma1. We propose that in sugar beet, Rf1 evolved via neofunctionalization.

2. Material and methods

2.1. Bioinformatic analysis

Nucleotide sequences were retrieved from the NCBI website (https://www.ncbi.nlm.nih.gov/assembly/). Database searching was conducted at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment of nucleotide and amino acid sequences was done using ClustalW (http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja) and MEGA (https://www.megasoftware.net) algorithms [24]. The alignment was visually inspected and modified manually. The microarray-based expression pattern of atOma1 was retrieved from a website (http://bar.utoronto.ca/efp2/Arabidopsis/Arabidopsis_eFPBrowser2.html) [25] using At5g51740.1 as the query.

2.2. Plant materials

The beet lines used in this study are summarized in table 1. Sugar beet (Beta vulgaris ssp. vulgaris) lines TA-33BB-O, TA-33BB-CMS, TK-81 mm-O, NK-198, NK-219 mm-O, NK-219 mm-CMS and NK-305 were
developed at the Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Japan. TA-33BB-O and TA-33BB-CMS, and NK-219 mm-O and NK-219 mm-CMS, respectively, have the same nuclear genotype but differ in their cytoplasms: suffixes ‘-O’ and ‘-CMS’ mean male fertile and male sterile cytoplasms, respectively. A wild beet (B. vulgaris ssp. maritima) accession PI 507186 was obtained from the United States Department of Agriculture. Seeds of Arabidopsis (Arabidopsis thaliana) ecotype Col-0 were a gift from Prof. Dr Satoshi Naito (Hokkaido University). All plants were grown in a greenhouse. Crosses were made as described in [26]. TA-33BB-CMS(NK-198 Rf1) is a BC6F1 derived from a cross of TA-33BB-CMS x NK-198.

2.3. Genotyping

Total cellular DNA was isolated from fresh green leaves by the CTAB method according to the procedure of [27]. PCR was performed with primers #1 and #2 (electronic supplementary material, table S1). GoTaq Green Master Mix (Promega, Madison, WI, USA) was used for genotyping. PCR cycles were: 1× 98°C 1 min; 35× (95°C 30 s, 55°C 30 s and 72°C 1 min 30 s) and 1× 72°C 3 min. PCR products were electrophoresed in 2% agarose gels.

2.4. Quantitative reverse transcription–PCR

Excised organs were frozen in liquid nitrogen and then powdered using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Total cellular RNA was isolated using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Residual genomic DNA was digested with RNase-free DNase (Promega). Procedures for cDNA synthesis and quantification were followed as described in [17]. The primers for each gene were: #3 and #4 for bvOma1, #5 and #6 for RF1-Oma1, #7 and #8 for Actin and #9 and #10 for EF1α (see electronic supplementary material, table S1). The specificity of the bvOma1 and RF1-Oma1 primers was verified by PCR with plasmids carrying the target sequences (electronic supplementary material, figure S1).

2.5. In situ hybridization

Procedures for in situ hybridization (ISH) followed the protocols outlined in the Cold Spring Harbor Arabidopsis Genetics Course (https://www.arabidopsis.org/cshl-course/5-in_situ.html) and [28]. Hybridization probes were prepared by in vitro transcription of a pBluescript SK-cloned DNA fragment using a DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany). DNA fragments for probes were generated by PCR using PrimeSTAR Max (Takara Bio, Kusatsu, Japan) as the DNA polymerase and total cellular DNA of TA-33BB-O or Col-0 as templates. The primers for each gene were: #11 and #12 for atOma1, #13 and #14 for bvOma1 and #15 and #16 for RF1-Oma1 (see electronic supplementary material, table S1).
supplementary material, table S1). Hybridized sections were observed using a BX50 light microscope equipped with a DP21 CCD camera (Olympus, Tokyo, Japan).

2.6. Transgene construction

Complementary DNAs were synthesized from total cellular RNAs isolated from fresh green leaves of TA-33BB-O or Col-0. The coding regions of \textit{bvOma1} and \textit{atOma1} were amplified by PCR using the primers #17 and #18 for \textit{bvOma1} and #19 and #20 for \textit{atOma1} (see electronic supplementary material, table S1). The resultant PCR fragments were cloned into pDONR/zeo via the Gateway system (Thermo Fisher Scientific, Waltham, MA, USA). A FLAG tag was added by using a PrimeSTAR Mutagenesis Basal Kit (Takara Bio) with the primers #21 and #22 for \textit{bvOma1::FLAG} and #23 and #24 for \textit{atOma1::FLAG} (see electronic supplementary material, table S1). The cloned fragments were introduced into a binary vector pMDC\textsubscript{Ω} [16] via the Gateway system. \textit{RF1-Oma1::FLAG} is the same as \textit{bvORF20::flag} that was reported in [16].

2.7. Transgenic suspension cells

The sugar beet line NK-219 mm-CMS was chosen for transformation because it has been used previously in transgene experiments [29]. Transgenic suspension cells were generated by Agrobacterum-mediated transformation [16].

2.8. Isolation of crude mitochondria

About 100–200 mg of suspension cells were ground in an extraction buffer (50 mM Tris–HCl (pH 8.0), 0.5 M mannitol, 1 mM EDTA-Na\textsubscript{2}, 0.1% (w/v) sodium ascorbate and 0.5% (w/v) Polyclar AT) with a plastic pestle, and centrifuged (5500 g at 4°C for 10 min). The supernatant was recentrifuged (6500 g at 4°C for 10 min), and the resultant supernatant was transferred to a new tube and centrifuged again (11 000 g at 4°C for 15 min). The pellet was washed in a wash buffer (50 mM Tris–HCl (pH 7.4), 0.5 M mannitol and 1 mM EDTA-Na\textsubscript{2}). After centrifugation (11 000 g at 4°C for 15 min), the pellet of crude mitochondria was suspended in the wash buffer.

2.9. Blue-native polyacrylamide gel electrophoresis

Suspended mitochondria were added to an equal volume of 2× NativePAGE Sample Buffer (Thermo Fisher Scientific) containing 2% (w/v) digitonin, and left on ice for 30 min. After centrifugation (11 500 g at 4°C for 15 min), the supernatant was applied to a NativePAGE Novex 4–16% Bis-Tris Gel (Thermo Fisher Scientific) for electrophoresis following the manufacturer’s instructions.

2.10. Co-immunoprecipitation

About 40 µg of crude mitochondria was suspended in 1× PBS containing Protein Inhibitor Cocktail for Plant Cell and Tissue Extracts (Sigma, St Louis, MO, USA) and 2.5% (w/v) digitonin, and left at 4°C for 30 min. After centrifugation (11 000 g at 4°C for 15 min), the supernatant was collected. The digitonin concentration was adjusted to 0.1% (w/v) by adding 1× PBS, and the volume of the sample was estimated. Anti-DDDDK Tag Magnetic Beads were first equilibrated in 1× PBS containing 0.1% (w/v) digitonin, then added to the sample in the ratio of approximately 4 µl bead slurry/sample as outlined in the instruction manual for the DDDDTagged Protein Magnetic Purification Kit (MBL, Nagoya Japan). Samples were end-over-end mixed overnight at 4°C. Beads were collected using a Magnetic Rack and washed twice with 1× PBS containing 0.1% (w/v) digitonin. Immunoprecipitates were eluted by boiling the beads in the SDS sample buffer (see §2.11).

2.11. SDS–polyacrylamide gel electrophoresis

Samples were suspended in SDS sample buffer (50 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 0.005% (w/v) bromophenol blue and 1% β-mercaptoethanol), and boiled for 5 min. Electrophoresis was conducted using 12% SDS–polyacrylamide gels after the method of Schägger & von Jagow [30].
2.12. Protein gel blot analysis

Separated proteins were blotted onto Hybond-P PVDF membranes (GE Healthcare, Little Chalfont, UK) with a Mini TransBlot Cell (Bio-Rad Laboratories, Hercules, CA, USA). Can Get Signal system (Toyobo, Osaka, Japan) was used for antibody/antiserum reaction. Primary antibodies/antisera included a mouse monoclonal anti-DDDDK (anti-FLAG) (MBL), and rabbit anti-preSATP6 and anti-COXI [14]. Secondary antibodies were HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA). ECL Prime (GE Healthcare) was used as the substrate for chemiluminescence. Signal bands were detected on X-ray film (GE Healthcare).

3. Results and discussion

3.1. Oma1 homologues in the sugar beet genome

Two high-quality sugar beet genome sequences were publicly available, one from the sugar beet line KWS2320 and the other from EL10 [31,32]. We searched for Oma1 homologues in these genomes using the tBLASTN algorithm with the amino acid sequence of atOma1 (At5g51740.1) as the query sequence. We identified four and eight sequences in KWS2320 and EL10, respectively (electronic supplementary material, table S2). All 12 of these sequences were predicted to be interrupted by two introns as is the case for atOma1 (figure 1).

The best matched gene to atOma1 in KWS2320 was LOC104906584 on a scaffold (NCBI reference sequence NW_017567367.1). This gene was tentatively named bvOma1 because it is the only copy having the consensus zinc-binding motif of an M48 peptidase domain (HExxH) (figure 2). Shared amino acid residues between atOma1 and bvOma1 were lost by these insertions/deletions (figure 2). The other two Oma1-homologous genes (LOC104888051 and LOC104888056) were lost by these insertions/deletions (figure 2). LOC104888051 was identical to rf1-Oma1 from a recessive rf1 allele (we use rf1-Oma1 when it is known to be CM009440.1 8.2 Mb
KWS2320 NC_025814.2 45 kb
NW_017567367.1 LOC104888056
LOC104906603 bvOma1 (LOC104906584)

NK-198 AB646135.2 41 kb
Unknown

Figure 1. Variation of Oma1 homologues in three sugar beet lines, EL10, KWS2320 and NK-198. Bold horizontal lines represent chromosomal segments and are identified by NCBI reference sequence numbers or GenBank/EMBL/DDBJ accession numbers. Brackets indicate gene loci. Boxes and wedges show exons and introns, respectively. Gene direction is coordinated with that of EL10: bvOma1, LOC104906603 and LOC104888056 are transcribed from left to right and RF1-Oma1 is from right to left. Colours of exons indicate cognate genes: blue, bvOma1; green, LOC104906603; red, RF1-Oma1 (or rf1-Oma1); and yellow, LOC104888056. LOC104888056 of NK-198 is apparently a pseudogene due to the presence of a frame shift mutation (indicated by ψ). Length of intervals is shown by double-headed arrows. Note that line lengths are not proportional. The nucleotide sequence of bvOma1 in NK-198 is unknown (but see electronic supplementary material, figure S3).
LOC104888056 had the least homology to rf1-Oma1. The rf1-Oma1 and LOC104888056 were 45 kb apart. Both the NW_017567367.1 and NC_025814.2 scaffolds were assigned to chromosome 3, but their physical locations on the chromosome are unknown.

**Figure 2.** Alignment of amino acid sequences deduced from atOma1, bvOma1 (KWS2320), LOC104906603 (KWS2320), rf1-Oma1 (KWS2320) and LOC104888056 (KWS2320). Residues are numbered from the first methionine. Dashes are incorporated for maximum matching. Zinc-binding motifs are enclosed in a box, and positions of introns are shown by triangles and dashed lines.
In EL10, all Oma1 homologues were located on a contig from chromosome 3 (GenBank accession number CM009440.1) (figure 1). Whereas bvOma1 was identified, LOC104906603 was not present in the EL10 genome, suggesting polymorphism in the presence/absence of LOC104906603 among sugar beet lines. This supposition was confirmed by the analysis of our beet collections (electronic supplementary material, figure S3). We found the Rf1 locus of EL10 consisted of six copies of RF1-Oma1. LOC104888056 of EL10 was identified in a region 10 kb apart from the Rf1 locus. Copies of RF1-Oma1 were on the opposite strand of other Oma1 homologues.

A 383 kb genomic region containing the Rf1 locus of sugar beet line NK-198 was previously described [15]. We found the NK-198 counterpart of LOC104888056, but it had an 8 b insertion in the first exon that led to a frame shift mutation (electronic supplementary material, figure S4).

Our previous reports indicated that no sugar beet line examined so far has lost rf1-Oma1 [18,26], and in this study, we found that bvOma1 and RF1-Oma1 are ubiquitous among all examined lines. Additional Oma1-like duplicates occur, but they are not always conserved among sugar beet lines; hence, we focused our analysis on bvOma1 and RF1-Oma1.

3.2. Differences in the spatial and temporal expression patterns between Oma1 homologues

The expression patterns of bvOma1, RF1-Oma1 and atOma1 were compared. We considered atOma1 to be an outgroup Oma1 for the beet Oma1-like genes, because atOma1 is the only Oma1 homologue in Arabidopsis, and it can complement the deficiency of yeast Oma1 [23]. Web-retrieved microarray-based expression data of atOma1 indicated the detection of atOma1 mRNA from almost all organ/tissues, whereas increased expression was seen in young stamens, mature pollen, immature seeds and dry seeds (expression levels 2–9 times that of root) (electronic supplementary material, figure S5).

We analysed bvOma1 expression in the sugar beet line TA-33BB-O, in which no LOC104906603 was seen (electronic supplementary material, figure S3). Quantitative reverse transcription–PCR (qRT–PCR) detected bvOma1 mRNA from roots, green leaves, peduncles without flowers, immature anthers and other floral organs (electronic supplementary material, table S3). Highest expression was measured in immature anthers (three times that of root). We also quantified the mRNA of rf1-Oma1 in TA-33BB-O, which is identical to the KWS2320 rfl-Oma1. Although rfl-Oma1 is unable to restore pollen fertility, its open reading frame (ORF) is uninterrupted and is highly conserved among sugar beet lines [18]. The transcripts were relatively abundant in immature anthers compared to other organs (473–487 times that of root).

We focused on the expression patterns of atOma1, bvOma1 and RF1-Oma1 in anthers. We used the Arabidopsis accession Col-0 for ISH of atOma1. No signal was seen at the meiosis stage (figure 3a). Sections with tetrads gave signals within the tapetal tissues, the inner-most cell layer of anther locules and tetrads (figure 3b). Sections with microspores produced signals within both tapetal tissues and microspores (figure 3c). A sense-probe yielded signals within microspores.

We conducted ISH using sugar beet line TA-33BB-CMS(NK-198 Rf1), which has S mitochondria but is male fertile due to a dominant Rf1 introduced from NK-198 by recurrent backcrossing, from which we expected a stronger signal of RF1-Oma1 than that obtained in TA-33BB-O. Expression signals of bvOma1 were obtained from tapetal tissues and microspores in sections of the microspore stage but hardly seen in sections at the meiotic and tetrad stages (figure 3b). On the other hand, RF1-Oma1 mRNA was detected from tapetal tissues in sections at both the meiotic and tetrad stages (figure 3c). Faint signals were also seen in microspore mother cells, whereas the signals in tetrads were uneven, and we therefore think their significance was questionable (figure 3c). No signal was observed in sections at the microspore stage.

3.3. Examination of molecular interactions with preSatp6

RF1-Oma1, but not rfl-Oma1, can give rise to a 200 kDa protein complex containing preSATP6 when expressed in suspension cells of CMS sugar beet [16,17,19]. We examined whether atOma1 and bvOma1 had the same activity. FLAG tag-fused atOma1 and bvOma1 cDNAs were constructed using a virus-derived promoter for constitutive expression. The two transgenes were introduced into suspension cells of CMS sugar beet via Agrobacterium (electronic supplementary material, figure S6). Results of immunodetection of preSATP6 from protein samples resolved by Blue-native polyacrylamide gel electrophoresis (PAGE) are shown in figure 4a. No alteration was seen on the blot of the transgenic suspension cells expressing atOma1 or bvOma1, whereas RF1-Oma1 from NK-198 produced an immunodetected 200 kDa band (figure 4a).
Figure 3. Detection of atOma1, bvOma1 and RF1-Oma1 mRNAs in anther tissues by ISH. Images of antisense and sense probes are shown. MMC, microspore mother cell; T, tapetal cell; Tds, tetrads; MSp, microspore. Scale bars are 50 µm. (a) atOma1 expression in Arabidopsis Col-0 anthers. Meiosis, tetrad and microspore stages correspond to developmental stages 6, 7 and 9 of [33]. (b,c) bvOma1 and RF1-Oma1 expression in sugar beet anthers, respectively. Meiosis, tetrad and microspore stages correspond to the developmental stages of Meiosis, Tetrad and Microspore (Sb-1) described by Arakawa et al. [17].
It was possible that alteration occurred but was masked by other signal bands. Because alteration in the molecular mass of preSATP6-containing complexes is tightly associated with protein–protein binding between preSatp6 and RF1-Oma1, we tested the protein–protein interaction between preSatp6 and atOma1 or bvOma1 by co-immunoprecipitation. Total mitochondrial proteins from the transgenic suspension cells were precipitated with antibody against FLAG. The precipitates, however, did not react with preSATP6 antiserum (figure 4b), indicating that protein–protein binding between preSatp6 and atOma1 or bvOma1 was below the limit of detection by this assay.

The Oma1-like genes form a small gene family in beets, unlike in the model plants Arabidopsis and rice [15,23]. Gene duplication is the most likely mechanism to explain the generation of this gene family. Two members of this family, bvOma1 and RF1-Oma1 (including rf1-Oma1), are ubiquitous among sugar beet lines. Whereas bvOma1 occurs as a single copy gene in all lines examined so far, RF1-Oma1 shows CNV among beet lines, as was reported previously [18,19,26]. In our previous study, an organizational comparison of beet Rf1 alleles suggested that intra- and/or intergenic recombination played an important role in this variation [17]. We should point out that analogous variations occur in other...
plant Rf loci encoding pentatricopeptide repeat proteins (PPRs) that participate in post-transcriptional regulation of genes responsible for CMS [12,34,35]. It is possible that beet RfI and the PPR-type Rfs share evolutionary mechanisms.

The protein product of RfI-Oma1 binds the preSATP6 protein and forms a 200 kDa protein complex, and suppression of CMS is tightly associated with this activity [16,17]. In this study, we found that such activity is absent from the protein products of bvOma1 and atOma1, suggesting that Oma1 was originally incapable of binding the preSATP6 protein. RfI-Oma1 also differs from bvOma1 and atOma1 in its spatial expression pattern. We hypothesize that RfI-Oma1 evolved through neofunctionalization of a duplicated Oma1 gene. Furthermore, the differences in the spatial expression patterns of bvOma1 and atOma1 suggest the possibility that the evolution of RfI-Oma1 might involve additional mechanisms, such as complementarily degenerative mutations [36] in RfI-Oma1 and other Oma1-like gene(s). However, these are only speculations, and further detailed studies are necessary to clarify the evolution of sugar beet RfI.

Data accessibility. Our data are provided as electronic supplementary material.

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References


