Biocontrol activity in a nonpathogenic REMI mutant of *Fusarium oxysporum* f. sp. *conglutinans* and characterization of its disrupted gene

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A nonpathogenic mutant (REMI10) of *Fusarium oxysporum* f. sp. *conglutinans* (*FOC*) was generated by restriction enzyme-mediated integration (REMI). REMI10 penetrated the cabbage root cortex much slower than *FOC* and never invaded the xylem through the endodermis. Previous treatment of cabbage seedlings with REMI10 reduced the disease incidence of yellows caused by *FOC*, suggesting that REMI10 has biocontrol activity. *FOC* invasion into root tissues was restricted when the seedling was treated with REMI10. The gene disrupted in REMI10 genome by plasmid insertion was identified, and designated *sap1*, which encodes a putative secreted aspartic proteinase. The *sap1* disruptants showed no reduction in virulence toward cabbage, suggesting that SAP1 is not essential for pathogenicity in *FOC*. © Pesticide Science Society of Japan

**Keywords:** cabbage yellows, *Fusarium oxysporum* f. sp. *conglutinans*, biocontrol activity, restriction enzyme-mediated integration (REMI), aspartic proteinase, nonpathogenic *Fusarium* (NPF).

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**Introduction**

*Fusarium oxysporum* Schlechtend.: Fr. causes soilborne vascular wilt diseases on various crops and causes serious economical damage.1) Yellows caused by the forma specialis *conglutinans* (Wollenweber) Snyder & Hansen in *F. oxysporum* causes destructive damage in cabbage (*Brassica oleracea* L. var. *capitata* L.) production.

Several genes encoding pathogenicity-related factors, such as cell wall-degrading enzymes, proteins in signal transduction pathways, and function-unknown proteins have been cloned in *F. oxysporum*,2–4) but the mechanisms of disease development by *F. oxysporum* have not been clarified so far. Pathogenicity mutants of *F. oxysporum* generated by restriction enzyme-mediated integration (REMI)5) have been used to identify the genes and factors responsible for pathogenicity.3,6–9) REMI has several advantages in the functional analysis of ascomycetous genes; for example, transformation frequency is relatively high and the phenotypic changes caused by plasmid insertion into a gene appeared immediately in primary transformants because most of the ascomycetous nucleus is haploid.10)

Furthermore, previous treatment of plants with nonpathogenic *Fusarium* spp. (NPF) often shows biocontrol activity against diseases caused by *F. oxysporum*.11,12) Ogawa and Komada (1986)13) suggested that NPF treatment induces systematic resistance in plants; however, the mechanisms of disease
suppression by NPF have not been clarified yet. Redman et al. (1999) obtained several nonpathogenic mutants from phytopathogenic *Colletotrichum magna* by REMI, which had activity to protect watermelon from parental (pathogenic) *C. magna* by previous treatment, and they suggested that REMI mutants were useful to investigate the mechanisms of biocontrol by nonpathogenic strains.

Recently, several studies used pathogenic *F. oxysporum* labeled with green fluorescence protein (GFP), and visualized the behavior of the fungus at the initial step of root infection. These reports showed that GFP is a useful tool for tracing the behavior of *F. oxysporum* on/in the root of host plants.

In this report, we generated 1500 transformants from *F. oxysporum* f. sp. *conglutinans* by REMI and selected a nonpathogenic strain (REMI10). REMI10 possessed biocontrol activity against cabbage yellows by previous treatment. The expression of enhanced green fluorescence protein (EGFP) in REMI10 visualized the behavior of REMI10 on/in cabbage roots. Moreover, we analyzed the genomic region truncated by the plasmid insertion in REMI10 to know if the truncated locus is involved in the pathogenicity of *F. oxysporum* f. sp. *conglutinans*.

**Materials and Methods**

1. **Fungal isolates and culture conditions**

   *F. oxysporum* f. sp. *conglutinans* isolate Cong:1-1 (FOC) was maintained on potato dextrose agar (PDA) medium (BD Biosciences, San Jose, CA, USA). Transformants carrying hygromycin B phosphotransferase gene (*hph*) were maintained on PDA containing 100 μg/ml hygromycin B (Wako Pure Chemical, Osaka, Japan).

   For genomic DNA (gDNA) extraction, *F. oxysporum* was cultured on potato dextrose broth (PDB) medium (BD Biosciences) at 28°C for 5 days without shaking and mycelia were collected by centrifugation.

   For RNA extraction, *F. oxysporum* was cultured at 28°C for 4 days by shaking (120 rpm) on PDB medium, or on nitrogen-free salt medium containing 100 mM NH₄Cl and 2% (w/v) glucose (BSAF medium, 1% (w/v) bovine serum albumin (BSA), and 2% (w/v) glucose (BSA medium), or 5% (w/v) sterilized roots prepared from 2-week-old cabbage (cv. Shiki-dori) seedlings (CR medium).

2. **DNA and RNA manipulations**

   Plasmid DNA was purified with Quantum Prep Plasmid Mini Prep Kit (Bio-Rad, Hercules, CA, USA) and gDNA was extracted with RNeasy Plant Maxi Kit (QIAGEN, Hilden, Germany) following the manufacturer’s protocols. Total RNA was extracted from 6-day-cultured mycelia of *FOC* using RNeasy Plant Mini Kit and RNase-Free DNase Set (QIAGEN) as previously reported. In order to analyze fungal gene expression in host plants, total RNA was extracted from cabbage roots at 12, 24, 36, 48, 60, 72 and 84 hr after inoculation with 5 × 10⁷ bud cells/ml of *FOC*. Cabbage roots were harvested, washed thoroughly with distilled water, and total RNA was extracted from 100 mg (fresh weight) of roots using RNeasy Plant Mini Kit.

   For DNA blot hybridization, DNA fragments were separated by agarose gel electrophoresis and transferred to a Hybond N⁺ Nylon membrane (Amersham Biosciences, Piscataway, CA, USA). Preparation of the labeled probe, hybridization and detection were performed using the Alkphos Direct Labeling and Detection System with CDP-Star (Amersham Biosciences) following the instruction manual.

   PCR reaction mixture (final volume 50 μl) contained 1 × PCR Gold Buffer (Applied Biosystems, Foster City, CA, USA), 2.5 mM MgCl₂, 0.2 mM (each) dNTPs, 0.2 μM of each primer, 1.25 U AmpliTag Gold DNA polymerase (Applied Biosystems), and 50 ng of gDNA. Thermal conditions were denaturation at 95°C for 10 min; 30 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min; final extension at 72°C for 10 min.

   We applied thermal asymmetric interlaced (TAIL)-PCR to rescue the flanking regions of the transformation vector insertion site in the transformant, as described by Arie et al. (2000). Specific primers for the primary reaction were PBL11 (5′-CACACAGGAAAACAGCTATGACC-3′) or PBR12 (5′-GCTTTGTTCGCGCTGGTGATGTG-3′), and for secondary reaction, PBL13 (5′-CAGCTATGACCATGATACGCACAAG-3′) or PBR14 (5′-CCGTGCGCCGCGGGGACTGGTGG-3′) (Fig. 1A). AD primers for primary and secondary reactions were TP1 and N1. The cycling conditions followed Arie et al. (2000).

   RT-PCR was performed with total RNA (1 μg) using Ready-To-Go PCR beads (Amersham Biosciences) with gene-specific primers. Optimized RT-PCR conditions included 35 cycles of reactions with annealing at 56°C.

   All primers used in this study were synthesized by Nihon Gene Research Laboratories (Sendai, Japan).

   PCR products were cloned into pCR2.1 vector (Invitrogen, San Diego, CA, USA) following the recommendations of the supplier.

   Nucleotide sequences of DNA were determined with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) and automated fluorescent DNA sequencer ABI PRISM 377 (Applied Biosystems).

3. **Preparation of fungal protoplasts and transformation**

   *FOC* was cultured on PDB by shaking (120 rpm) at 28°C for 4 days and bud cells were collected by centrifugation at 1500 × g for 15 min at room temperature. Fresh PDB (200 ml) was inoculated with 2 × 10⁷ bud-cells and incubated at 28°C for 3 days. Mycelia in the culture were collected by centrifugation and washed twice with 1.2 M MgSO₄. The cell wall of the mycelia was digested in 10 ml of enzymatic solution containing 1% (w/v) Lysing Enzymes (Sigma, St. Louis, MO, USA), 2% (w/v) Driselase (Kyowa Hakko Kogyo, Tokyo, Japan).
4. Plants and inoculation test

Bud cells of FOC were collected as described above, and re-suspended in sterile water at a concentration of $1 \times 10^7$ bud-cells/ml.

Cabbage seeds (Brassica oleracea, cv. Shikidori), susceptible to FOC, were purchased from Takii Seed (Kyoto, Japan). Two-week-old cabbage seedlings prepared on sterilized soil (Kureha Engei Baido, Kureha, Tokyo, Japan) in a 7 cm diameter pot in a growth chamber (25°C, 80% humidity, 12 hr light (ca. 35 μE/m²s)/12 hr dark) were inoculated with FOC or each transformant by pouring 5 ml of the bud cell suspension into the pot, and maintained in the growth chamber. Disease severity of each plant was evaluated 21 days after inoculation and disease index was scored on a scale of 0 to 4, where 0=no symptom; 1=slight yellowing or swollen of lower leaves; 2=yellowing of lower leaves; 3=yellowing of lower and upper leaves; 4=yellowing and wilt of whole leaves or plant dead. Each inoculation test using more than ten seedlings was replicated three times.

5. Biocontrol activity assay

Each two-week-old cabbage seedling was removed from soil and the roots were washed in sterilized water. Each seedling was treated with the transformant by dipping the roots in the bud cell suspension or sterilized water as a control. The treated cabbage seedling was transplanted to sterilized soil in a 7 cm diameter pot and kept in the growth chamber. Three days later, the cabbage seedling was inoculated with FOC by pouring 10 ml of the bud cell suspension into the pot, and kept in the growth chamber. Disease severity of each plant was evaluated 21 days after FOC inoculation. Disease index was scored on a scale of 0 to 4, as mentioned above. Each treatment using more than ten seedlings was replicated three times.

6. Visualization of F. oxysporum on/in cabbage roots using EGFP

Cabbage seeds surface-sterilized with 70% EtOH (30 sec) and 3% sodium hypochlorite (1 min), and washed with sterilized water were germinated on an 1.5% agar plate in the growth chamber. One-week-old seedlings were removed from the plate and the roots were soaked in a bud cell suspension of strains expressing EGFP. After inoculation, these seedlings were placed on a fresh 1.5% agar plate and maintained in the growth chamber until observation. Cabbage roots harvested from the plate at different timings after inoculation were plugged in 15% agar, sliced using a microslicer (DTK-1000; D.S.K., Osaka, Japan) and mounted on a microscope slide with a drop of water. The thin image of the root (ca. 50 μm thickness) was observed under a laser scanning spectral confocal microscope (LSCM) TCS SP2 (Leica, Wetzlar, Germany).

The behavior of FOC-GFP (FOC labeled with EGFP) in cabbage previously treated with a nonpathogenic mutant was
also visualized. One-week-old cabbage seedlings were treated with bud cells of the nonpathogenic mutant as described above, then, 3 days after treatment, challenged with FOC-GFP in the same manner. Two days after FOC inoculation, the roots were harvested and subjected to observation under LSCM.

**Results**

1. **Selection of a nonpathogenic mutant of FOC and its physiological characteristics**

From 1500 hygromycin B-resistant transformants generated from FOC by REMI using pCSN43, which carries the hygromycin B resistance gene (hph) under the control of *Aspergillus nidulans trpC* promoter and terminator, digested with *Hind* III, three pathogenicity mutants were selected by bioassay with cabbage seedlings. One of the mutants, named REMI10, thoroughly lacked the ability to cause yellows on cabbage seedlings (Fig. 2). REMI10 grew on PDA medium bioassayed with cabbage seedlings. One of the mutants, named REMI10, thoroughly lacked the ability to cause yellows on cabbage seedlings (Fig. 2). REMI10 grew on PDA medium bioassayed with cabbage seedlings. One of the mutants, named REMI10, thoroughly lacked the ability to cause yellows on cabbage seedlings (Fig. 2). REMI10 grew on PDA medium bioassayed with cabbage seedlings. One of the mutants, named REMI10, thoroughly lacked the ability to cause yellows on cabbage seedlings (Fig. 2). REMI10 grew on PDA medium bioassayed with cabbage seedlings. One of the mutants, named REMI10, thoroughly lacked the ability to cause yellows on cabbage seedlings (Fig. 2). REMI10 grew on PDA medium bioassayed with cabbage seedlings. One of the mutants, named REMI10, thoroughly lacked the ability to cause yellows on cabbage seedlings (Fig. 2). REMI10 grew on PDA medium bioassayed with cabbage seedlings. One of the mutants, named REMI10, thoroughly lacked the ability to cause yellows on cabbage seedlings (Fig. 2).

2. **Behavior of REMI10 and FOC on/in cabbage roots**

REMI10 and FOC were transformed with pNEO-EGFP and pHYG-EGFP, respectively. pHYG-EGFP carrying *egfp* under the control of *A. nidulans gpd* promoter and terminator was provided by Dr. T. Motoyama (RIKEN, Wako, Saitama, Japan), and pNEO-EGFP was constructed by replacing *hph* in pHYG-EGFP in a gene-resistant gene (*NPTII*) from pII199, which was provided by Dr. T. Tsuge and Dr. I. Imazaki (Nagoya University, Nagoya, Japan). Among more than 50 transformants for each, we selected transformants expressing EGFP and named them REMI10-GFP and FOC-GFP, respectively. Mycelia, conidia, and chlamydospores of REMI10-GFP and FOC-GFP were observed constitutively with green fluorescence under 488 nm excitation (U-MIWB/ GFP filter unit, Olympus, Tokyo, Japan), and their growth on PDA medium and pathogenicity to cabbage were equilibrated with those of parental REMI10 and FOC, respectively.

Sequential observation of cabbage roots inoculated with REMI10-GFP or FOC-GFP revealed that REMI10-GFP invaded the root cortex through the cuticle much slower than FOC-GFP (Fig. 3A). Moreover, in contrast with the invasion of FOC-GFP into the vascular bundle through the endodermis in 48–72 hr, REMI10-GFP never intruded into the vascular bundle of cabbage roots during the period (Fig. 3A) and even 20 days after inoculation (data not shown). Three replicates of three seedlings each per treatment were undertaken. All experiments exhibited similar results and the figures represent the data from those experiments.

3. **Biocontrol of cabbage yellows by previous treatment with REMI10**

Treatment of cabbage roots with REMI10 at 3 days prior to FOC inoculation (REMI10+FOC in Fig. 2) significantly reduced the severity of yellows (FOC in Fig. 2). Development of symptoms, such as yellowing of leaves and browning of vascular bundles, was suppressed in cabbage plants treated with REMI10. The behavior of FOC-GFP in/on cabbage roots treated previously with REMI10 was imaged in Fig. 3B. We could find few mycelia of FOC-GFP in root tissues.

4. **Rescue of the gene disrupted in REMI10 by insertion of the transformation vector**

DNA blot hybridization showed insertion of a single copy of the transformation vector pCSN43 in the REMI10 genome. DNA fragments around the vector-insertion site were amplified by TAIL-PCR and their nucleotide sequences were partly determined. The sequence data suggested that a gene in the FOC genome was truncated by insertion of the vector pCSN43 at the *Hind* III restriction site (Fig. 1A, B). A fragment (494 bp., probe 1 in Fig. 1B) amplified by PCR with the specific primers fap11 (5′-CCAGTGCCATCGCGTATCT-3′, nt. 1508–1489) and fap14 (5′-GATGGACCAAATCACCACAAAC-3′, nt. 1015–1034) from genomic DNA of FOC was labeled and used to select clones carrying this gene from a cosmid genomic library of FOC (Yoshida, unpublished data). One positive cosmid clone was selected. Two *Hind* III-restricted fragments and one *EcoR* V-restricted fragment from

![Chart](chart.png)

**Fig. 2.** Nonpathogenic mutant (REMI10) derived from *Fusarium oxysporum* f. sp. *conglutinans* Cong:1-1 (FOC) and its biocontrol activity against cabbage yellows. Two-week-old cabbage (cv. Shikidori) seedlings were treated with REMI10 by dipping the roots in the bud cell suspension (1×10⁷ cells/ml) or sterilized water as a control. Three days after REMI10 treatment, the treated seedlings were inoculated with FOC by adding 10 ml of the bud-cell solution (1×10⁷ cells/ml), and maintained in a growth chamber at 25°C, 80% humidity, and 12 hr light (ca. 35 μE/m²/s) dark. Disease severity of each plant was determined 21 days after inoculation. Disease index was scored on a scale of 0 to 4, where 0=no symptoms; 1=slight yellowing or swollen of lower leaves; 2=yellowing of lower leaves; 3=yellowing of lower and upper leaves; 4=yellowing and wilt of whole leaves or plant death. The mean values obtained from three replicates with more than ten plants were presented. Treatments followed by a common letter were not significantly different according to Tukey’s test (**P**=0.05). The experiments were repeated three times with similar results.
the cosmid which hybridized with probe 1 were sub-cloned (Fig. 1B), and the nucleotide sequences of the sub-cloned fragments were partially determined. We combined the nucleotide sequences (determined by TAIL-PCR and the sub-cloned fragments), and deposited the 2468 bp. sequence in the DBBJ/EMBL/GenBank databases under accession number AB028020 (Fig. 1B). In this paper we described the nucleotide position according to this accession.

5. Estimation of ORF and function of sap1

We revealed the putative coding region consisting of an open reading frame (ORF) of 1680 bp. (nt. 482–1160; 1207–1330; 1380–2259) interrupted by two introns (46 and 49 bp.) both of which carried the conserved splicing motifs (GTAAG and CAG) in filamentous fungi. We designated this sap1. Blastp searches against the NCBI database (National Center for Biotechnology Information, Bethesda, MD, USA) showed high similarity of the deduced SAP1 to aspartic proteinases of ascomycetes, such as Glomerella cingulata GC SAP (identity 44%, #U43775), Neosartorya fischeri ASP (identity 46%, #XP_001259355), Aspergillus fumigatus PEP1 (identity 46%, #XP_753324), Fusarium oxysporum ASP (identity 45%, #AAL69900), Trichoderma asperellum PAPA (identity 44%, #ATT09023), Podospora anserina PAPA (identity 44%, #O13340), Cryphonectria parasitica EAPA (identity 44%, #P11838). Blastp searches against the Fusarium group genome database (Broad Institute, Cambridge, MA, USA) also showed high similarity of SAP1 to predicted aspartic proteinases of Fusarium spp. such as F. oxysporum (identity 87%, #FOXG_02464.2), F. verticillioides (identity 81%, #FVEG_05650.3), F. graminearum (identity 59%, #FGSG_06501.3). We selected several sequences among them, and aligned these sequences with FOL SAP1 by Clustal X 1.83 and arranged by hand (Fig. 4).

In SAP1, a set of putative active-center motifs (DTG) with aspartyl residue (D), which characterizes an aspartic proteinase, was determined (aa. 262–264 and 447–449; bold characters in Fig. 4). It was also predicted that SAP1 have a signal peptide (17 aa. in the N-terminal of proteins) by SignalP 3.0, suggesting that SAP1 is secreted.

DNA blot hybridization for gDNA of FOC digested with four different restriction enzymes (Hind III, EcoR I, EcoR V and Sal I) with probe 1 (Fig. 1B) revealed that sap1 presents in the FOC genome as a single copy (data not shown).

6. Expression of sap1

Secretion of aspartic proteases in filamentous fungi is generally induced by the addition of exogenous protein, for example, BSA, and repressed by ammonium salts in the medium. RT-PCR with sap1-specific primers fap14 and fap11 (Fig. 1B) was performed using cDNAs synthesized from mRNA extracted from FOC mycelia grown on CR, BSA, BSAF, and PDB media as templates. sap1 was expressed in mycelia grown on CR, BSA, and PDB media (Fig. 5A). No expression of sap1 was observed on BSAF medium (Fig. 5A).
RT-PCR using total RNAs from cabbage roots as templates showed that sap1 transcription in cabbage roots inoculated with FOC could be detected at 48 hr after inoculation (Fig. 5B).

7. sap1-disruptants of FOC and their pathogenicity to cabbage

To delete sap1 in the genome of FOC by gene replacement, plasmid pFDAP-2 was constructed as follows (Fig. 1B–D).

About 2.0 kb of the 5'-flank and 1.5 kb of the 3'-flank of the sap1 coding region, which were amplified by PCR with plasmid pCR 2.1 vector (Fig. 1D). The ca. 2.0 kb fragment of the 5'-flank of sap1 was released by KpnI/SalI digestion, and ligated to the KpnI/SalI digest of pCR2.1 carrying the ca. 1.5 kb fragment of the 3'-flank of sap1 (Fig. 1D). The hph cassette with trpC promoter and terminator was released from pCSN43 by digestion with SalI and ligated into the SalI site between the above-mentioned 5'- and 3'-flanks of sap1 in pCR2.1 (pFDAP-2, Fig. 1C, D). The ca. 6 kb linearized fragment was released by digestion of pFDAP-2 with KpnI/NotI (Fig. 1C), and FOC was transformed with this fragment by replacing ca. 4.6 kb of the sap1 coding region.

Fig. 4. Alignment of fungal aspartic proteinases. FOCsap1, AB028020, deduced amino acid sequences from sap1 of Fusarium oxysporum f. sp. conglutinas (this study); F. oxysporum, FOXG_02464,2, hypothetical protein similar to aspartic proteinase of F. oxysporum; F. verticillioides, FVEG_05650,3, hypothetical protein similar to aspartic proteinase of F. verticillioides; F. graminearum, FGSG_06501,3, hypothetical protein similar to aspartic proteinase of F. graminearum; G. cingulata, U43775, secreted aspartic proteinase GCSAP precursor of Glomerella cingulata; N. fischeri, XP_001259355, aspartic endopeptidase ASP of Neosartorya fischeri; A. fumigatus, XP_753324, aspartic endopeptidase Pep1/aspergilipolipase F of Aspergillus fumigatus; F. venenatum, AAL69900, aspartic proteinase ASP of Fusarium venenatum; T. asperellum, AAT09023, aspartyl proteinase PAP A of Trichoderma asperellum; P. anserina, O13340, podosporapepsin PAP A precursor of Podospora anserina; C. parasitica, P11838, endotheiapsin EAP A precursor/aspartate protease of Cryphonectria parasitica. The active site motifs (DTG) in N and C-terminal lobes which characterize aspartic proteinases are indicated by outlined letters on a black background. The putative signal peptide is indicated by a shaded box and putative KEX2 processing site16) is indicated by underlining.

RT-PCR using total RNAs from cabbage roots as templates showed that sap1 transcription in cabbage roots inoculated with FOC could be detected at 48 hr after inoculation (Fig. 5B).

7. sap1-disruptants of FOC and their pathogenicity to cabbage

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1.8 kb including \( \text{sap1} \) with \( \text{hph} \) by two times-homologous recombination (Fig. 1B, C).

\( \text{sap1} \) disruptants were selected by genomic DNA blot hybridization using \( \text{probe 2} \) (Fig. 1B) prepared from the \( \text{Hin} \) III-restricted sub-cloned fragment of the cosmid (Fig. 1A). We obtained five mutants (d-1 to d-5) which lack \( \text{sap1} \) (Fig. 6A). We could not detect \( \text{sap1} \) transcription in these mutants under \( \text{sap1} \) expression-inducible conditions (data not shown). These \( \text{sap1} \) disruptants showed no phenotypic differences, such as growth rate, pigmentation on PDA, and formation and size of conidia in parental \( \text{FOC} \). The inoculation test with cabbage cv. Shikidori showed that d-1 to d-5 still possessed pathogenicity to cabbage, which was equivalent to that of \( \text{FOC} \).

**Discussion**

In this study, we generated a nonpathogenic mutant, REMI10, from the cabbage yellows pathogen, \( F. \text{oxysporum} \) f. sp. \( \text{conglutinans} \) (\( \text{FOC} \)), by REMI. Previous treatment of cabbage roots with REMI10 suppressed the disease caused by \( \text{FOC} \), showing that REMI10 carries biocontrol ability against cabbage yellows (Fig. 2).

Observation under LSCM revealed that REMI10-GFP, a transformant of REMI10 expressing EGFP, could colonize on the surface of cabbage roots and could penetrate through the cuticle and invade the cortex but much slower than \( \text{FOC} \) (Fig. 3A). We did not observe that REMI10-GFP went through the endodermis to reach the vascular bundles. These results suggested that the endodermis in roots is a critical point to establish yellows and REMI10 could not overcome this. Figure 3B clearly showed that cabbages treated with REMI10 three days before inoculation prevented the invasion of \( \text{FOC} \)-GFP into the root cortex.

These results suggested that although REMI10 lost the ability to cause yellows on cabbage, it possessed not only the ability to localize on/in cabbage roots but also biocontrol activity against yellows disease.

Observation of pathogenic fungi with GFP is a good tool to elucidate the process of vascular infection of pathogenic \( F. \text{oxysporum} \) and the interaction between pathogenic and non-pathogenic \( F. \text{oxysporum} \) strains in soil. In this study, GFP-tagged strains (\( \text{FOC-GFP} \) and REMI10-GFP) were useful to study the interactions between \( \text{FOC} \) and REMI10. Previously, it was reported that there was no exclusion of pathogenic \( F. \text{oxysporum} \).
oxysporum by nonpathogenic isolates in the mode of action of biocontrol.\textsuperscript{25} In this study, in contrast, we could find few FOC in the root cortex when the plant had been treated with REMI10 previously, suggesting that the pathogenic strain was excluded from the root by treatment with the nonpathogenic strain. These suggested that the mode of action is different depending on the strain. We assume that colonization on the root surface and slow intruding to the root cortex by nonpathogenic REMI10 gave cabbage a chance to induce disease resistance and, moreover, that the endodermis may play an important role in the recognition of microbe invasion.

In the nonpathogenic mutant REMI10, sap1 encoding a putative secreted aspartic proteinase was truncated by insertion of the transformation vector (Fig. 1A). Aspartic proteinases have implicated as pathogenicity factors in several plant pathogens, such as Botrytis cinerea, Glomerella cingulata, and Sclerotinia sclerotiorum.\textsuperscript{18,26,27} and in animal pathogens, such as Candida albicans and Rhizopus spp.\textsuperscript{28–30} In B. cinerea, aspartic proteinase is necessary to extend mycelia in the host tissues,\textsuperscript{11} and in C. albicans, aspartic proteinase is essential for attachment and infection to the host.\textsuperscript{32–35} In the deduced amino acid sequence of SAP1, we could find a putative signal peptide and active-center motifs, which are popular in fungal aspartic proteinases (Fig. 4).\textsuperscript{18} Transcript of sap1 was detected when FOC was cultured on medium containing cabbage roots or BSA. On the other hand, no sap1 transcript was detected when FOC was grown on medium containing NH\textsubscript{4}Cl as sole nitrogen (Fig. 5A). This did not contradict the reported producible conditions of aspartic proteinase by fungi.\textsuperscript{10} Alignment of SAP1 and hypothetical proteins homologous to SAP1 in the genome database of the Fusarium group (FOXG_024646, FVEG_05650, FGSG_06501) showed variations around the first active-center motif (aa. 262–264 and aa. 447–449 in FOC; outlined letters on a black background in Fig. 4). In F. oxysporum and F. graminearum, one of the active-center motifs, which are typical in aspartic proteinases, is missing. It is possible that annotation of the genes in the database contains errors.

RT-PCR detected the expression of sap1 in cabbage roots from 48 hr after inoculation (Fig. 5B). This is different from previous reports showing that B. cinerea produced aspartic proteinase in the initial stage of infection\textsuperscript{26} and S. sclerotiorum expressed asparyl proteinase from the beginning of infection in sunflower.\textsuperscript{25} In this experiment, we extracted RNA from cabbage roots after washing the root surface to exclude fungi on the surface. We suppose that FOC establishes the invasion and colonization of cabbage roots 36 hr after inoculation.

In order to investigate the relevance of SAP1 to pathogenicity, we generated sap1 disruptants from FOC. Contrary to our expectations, all disruptants were fully virulent to cabbage as the parental FOC (Fig. 6B), and we verified that sap1 is not essential for pathogenicity in FOC. This suggested that unexpected mutation occurred in the genome of REMI10 during the transformation procedure. As sap1 disruptants showed no great phenotypic differences between Cong:1-1, the role of SAP1 in FOC has not been determined so far.

We consider that REMI is an effective means to produce an array of mutants in fungus, and REMI mutants will provide good understanding not only of the mechanisms of infection by phytopathogenic fungus but also interactions between the host plant and pathogenic and/or nonpathogenic fungus; however, like REMI10, REMI transformants unexpectedly acquire genetic mutations at more than one site. REMI10 should have an additional mutation at an unidentified pathogenicity-related genomic region and we are interested in its analysis. Proteomic analyses have revealed the protein-expression profiles under various conditions in fungi. Proteomic analyses can be applied to FOC and REMI10 to understand the mode of pathogenicity and biocontrol.

We concluded that an artificially generated nonpathogenic mutant of F. oxysporum could be a good tool to investigate not only the mechanisms of pathogenicity but also the mode of action of biocontrol by nonpathogenic F. oxysporum. Moreover, visualization using GFP would help to understand interactions between the nonpathogenic mutant and the pathogen in/on plant tissues.

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