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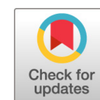

Terbinafine Resistance of *Trichophyton* Clinical Isolates Caused by Specific Point Mutations in the Squalene Epoxidase Gene

Tsuyoshi Yamada,[a,b] Mari Maeda,[a] Mohamed Mahdi Alshahni,[b] Reiko Tanaka,[c] Takashi Yaguchi,[c] Olympia Bontems,[d] Karine Salamin,[d] Marina Fratti,[d] Michel Monod[d]

Teikyo University Institute of Medical Mycology, Tokyo, Japan[a]; General Medical Education and Research Center, Teikyo University, Tokyo, Japan[b]; Medical Mycology Research Center, Chiba University, Chiba, Japan[c]; Service de Dermatologie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland[d]

ABSTRACT Terbinafine is one of the allylamine antifungal agents whose target is squalene epoxidase (SQLE). This agent has been extensively used in the therapy of dermatophyte infections. The incidence of patients with tinea pedis or unguium tolerant to terbinafine treatment prompted us to screen the terbinafine resistance of all *Trichophyton* clinical isolates from the laboratory of the Centre Hospitalier Universitaire Vaudois collected over a 3-year period and to identify their mechanism of resistance. Among 2,056 tested isolates, 17 (≈1%) showed reduced terbinafine susceptibility, and all of these were found to harbor *SQLE* gene alleles with different single point mutations, leading to single amino acid substitutions at one of four positions (Leu[393], Phe[397], Phe[415], and His[440]) of the SQLE protein. Point mutations leading to the corresponding amino acid substitutions were introduced into the endogenous *SQLE* gene of a terbinafine-sensitive *Arthroderma vanbreuseghemii* (formerly *Trichophyton mentagrophytes*) strain. All of the generated *A. vanbreuseghemii* transformants expressing mutated SQLE proteins exhibited obvious terbinafine-resistant phenotypes compared to the phenotypes of the parent strain and of transformants expressing wild-type SQLE proteins. Nearly identical phenotypes were also observed in *A. vanbreuseghemii* transformants expressing mutant forms of *Trichophyton rubrum* SQLE proteins. Considering that the genome size of dermatophytes is about 22 Mb, the frequency of terbinafine-resistant clinical isolates was strikingly high. Increased exposure to antifungal drugs could favor the generation of resistant strains.

KEYWORDS dermatophytes, squalene epoxidase, terbinafine, *Trichophyton*, antifungal resistance, reverse genetics approach

Received 20 January 2017 **Returned for modification** 8 February 2017 **Accepted** 1 April 2017

Accepted manuscript posted online 17 April 2017

Citation Yamada T, Maeda M, Alshahni MM, Tanaka R, Yaguchi T, Bontems O, Salamin K, Fratti M, Monod M. 2017. Terbinafine resistance of *Trichophyton* clinical isolates caused by specific point mutations in the squalene epoxidase gene. Antimicrob Agents Chemother 61:e00115-17. https://doi.org/10.1128/AAC.00115-17.

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Address correspondence to Michel Monod, Michel.Monod@chuv.ch.

Tinea pedis and tinea unguium are the most prevalent dermatophytoses. Both are generally caused by *Trichophyton rubrum* and *Trichophyton interdigitale*, with prevalences of approximately 80% and 20%, respectively (1). Control of tinea pedis and tinea unguium requires treatment with topical and/or oral fungicidal or fungistatic drugs (2). With some exceptions, antifungal drugs commonly used to treat dermatophytosis target the ergosterol biosynthetic pathway. Imidazoles such as econazole and triazoles such as itraconazole inhibit lanosterol 14-α-demethylase, which leads to the accumulation of sterol precursors and results in altered plasma membrane structure and function (3). In contrast, allylamines such as terbinafine are inhibitors of squalene epoxidase, an enzyme involved in the early steps of ergosterol biosynthesis (4). This inhibition results in the accumulation of squalene, which is toxic to fungi (5). Terbin-

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TABLE 1 Phenotypic and genotypic characteristics of the clinical isolates obtained from patients with dermatophyte infections

Species and isolate no.[a]	Infection type	Growth with terbinafine[b]	Nucleotide substitution within the *SQLE* gene	Amino acid substitution	Terbinafine MIC (μg/ml)	Fold expression of *SQLE* (mean ± SD)[d]
T. interdigitale						
ATCC MYA-4439			None	None	0.00625	ND
T. rubrum						
CBS118892			None	None	ND[c]	1.08 ± 0.47
TIMM20083	Tinea pedis	+++	[1177]TTA → TTT	Leu393Phe	6.4	1.26 ± 0.48
TIMM20084	Tinea pedis	+++	[1177]TTA → TTT	Leu393Phe	3.2	0.67 ± 0.25
TIMM20093	Tinea pedis	+++	[1177]TTA → TTT	Leu393Phe	6.4	ND
TIMM20094	Tinea unguium	+++	[1177]TTA → TTT	Leu393Phe	6.4	ND
TIMM20088	Tinea unguium	+++	[1177]TTA → TCA	Leu393Ser	1.6	ND
TIMM20095	Tinea unguium	+++	[1177]TTA → TCA	Leu393Ser	ND	ND
TIMM20085	Tinea unguium	+++	[1189]TTC → TTA	Phe397Leu	3.2	ND
TIMM20086	Tinea unguium	+++	[1189]TTC → CTC	Phe397Leu	6.4	0.72 ± 0.29
TIMM20087	Tinea unguium	+++	[1189]TTC → TTA	Phe397Leu	ND	0.64 ± 0.27
TIMM20092	Tinea pedis	+++	[1189]TTC → CTC	Phe397Leu	>12.8	ND
TIMM20091	Tinea pedis	+++	[1189]TTC → ATC	Phe397Ile	0.8	ND
TIMM20097	Tinea unguium	+	[1189]TTC → GTC	Phe397Val	ND	ND
TIMM20090	Tinea unguium	+++	[1305]TTC → ATC	Phe415Ile	0.1	ND
TIMM20098	Tinea pedis	+	[1305]TTC → TCC	Phe415Ser	ND	ND
TIMM20082	Tinea unguium	+++	[1305]TTC → GTC	Phe415Val	0.4	ND
TIMM20089	Tinea unguium	+	[1380]CAT → TAT	His440Tyr	0.1	ND
T. interdigitale						
TIMM20096	Tinea unguium	+++	[1189]TTC → CTC	Phe397Leu	3.2	ND

[a]All the clinical isolates were obtained in Switzerland and deposited in the culture collection of Teikyo University Institute of Medical Mycology (TIMM).
[b]The growth ability of the clinical isolates on SDA containing 0.2 μg/ml terbinafine is indicated as weak (+) to vigorous (+++).
[c]ND, not determined.
[d]Results represent expression levels from three independent real-time PCR experiments. Expression levels of *SQLE* genes were indicated as relative fold changes compared to the CT mean of the wild-type strain CBS118892 data.

afine has been found to be (as the drug of choice) efficacious for curing both tinea pedis and tinea unguium and is now used worldwide.

Although *T. rubrum* clinical isolates resistant to terbinafine are rarely described in the literature, therapeutic failure has been recorded in Lausanne (Switzerland) in patients with tinea pedis or onychomycosis. In these cases, the infectious agent was unambiguously identified as *T. rubrum*. To date, only two cases of terbinafine resistance have been investigated at the biochemical and DNA levels in *T. rubrum* clinical isolates from onychomycoses (6–9). Resistance was shown to be due to single point mutations in the gene encoding squalene epoxidase. Both mutations introduced missense substitutions (Leu393Phe in one case and Phe397Leu in the other), resulting in a more than 100-fold higher MIC (9, 10). Antifungal resistance in many fungi is also mediated by overexpression of genes encoding multidrug transporters (11–13). However, no resistance mediated by efflux pumps of the ATP-binding cassette (ABC) transporter family or by efflux pumps of the major facilitator family has yet been documented in dermatophyte clinical isolates.

Because of the incidence of patients with tinea pedis and tinea unguium insensitive to terbinafine treatment, the aims of this work are (i) to determine the frequency of terbinafine-resistant *Trichophyton* clinical isolates in a large screening of isolates from patients for whom a mycological analysis was performed at the Centre Hospitalier Universitaire Vaudois (CHUV) and (ii) to identify the mechanisms of resistance of dermatophytes insensitive to terbinafine.

RESULTS

Isolation of dermatophyte strains showing reduced susceptibility to terbinafine. A total of 1,644 *T. rubrum* and 412 *T. interdigitale* clinical isolates, which were mainly collected from patients with tinea pedis or tinea unguium, were tested for growth ability on Sabouraud dextrose agar (SDA) containing 0.2 μg/ml terbinafine. Only 16 *T. rubrum* and one *T. interdigitale* isolate grew on the medium (Table 1). To

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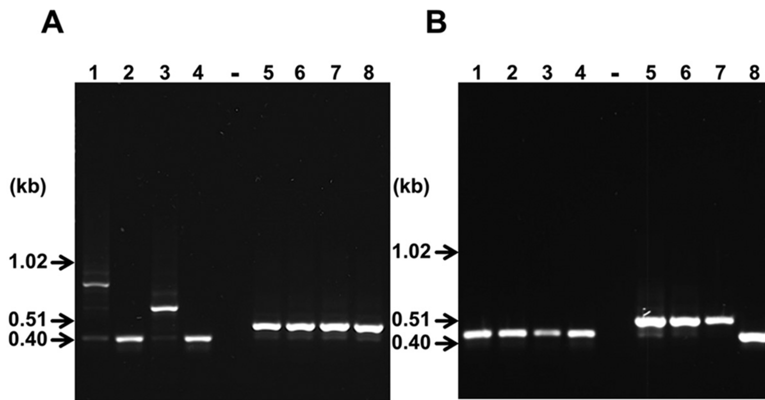

FIG 1 Agarose gel electrophoresis of the TRS-1 (lanes 1 to 4) and TRS-2 (lanes 5 to 8) regions from terbinafine-resistant *T. rubrum* clinical isolates with the same point mutation in the *SQLE* gene. (A) Strains with the replacement of Leu[393] with Phe in SQLE. Lanes 1 and 5, TIMM20083; lanes 2 and 6, TIMM20084; lanes 3 and 7, TIMM20093; lanes 4 and 8, TIMM20094. (B) Strains with the replacement of Phe[397] with Leu in the SQLE. Lanes 1 and 5, TIMM20085; lanes 2 and 6, TIMM20086; lanes 3 and 7, TIMM20087; lanes 4 and 8, TIMM20092. Leu[397] is encoded by TTA in TIMM20085 and TIMM20087 and by CTC in TIMM20086 and TIMM20092.

investigate further the reduced terbinafine susceptibility of such strains, their squalene epoxidase (*SQLE*) genes were amplified by PCR and sequenced. As shown in Table 1, the *SQLE* genes of all the strains were revealed to contain point mutations leading to amino acid substitutions at one of four amino acid positions (Leu[393], Phe[397], Phe[415], and His[440]) within the SQLE protein, strongly suggesting that a structural change in the SQLE protein might be involved in the reduced susceptibility of these strains to terbinafine.

We used the length polymorphism of the tandem repetitive subelement 1 (TRS-1) and TRS-2 amplicons tentatively to differentiate *T. rubrum* resistant isolates carrying identical point mutations. The length polymorphism of TRS-1 allowed the differentiation of TIMM20083 and TIMM20093 from TIMM20084 and TIMM20094 (Fig. 1A). These four strains carried identical A-to-T substitutions at position 1179 of the open reading frame (ORF) of the *SQLE* gene, leading to the replacement of Leu[393] by Phe in the SQLE protein. The length polymorphism of TRS-2 allowed the differentiation of TIMM20086 from TIMM20092 (Fig. 1B). These two strains carried identical T-to-C substitutions in position 1189 of the open reading frame of the *SQLE* gene, leading to the replacement of Phe[397] by Leu in the SQLE protein. TIMM20085 and TIMM20087, which carried identical C-to-A substitutions at position 1191 of the open reading frame of the *SQLE* gene, could not be differentiated. Similar to TIMM20086 and TIMM20092, Phe[397] was replaced by Leu in the SQLE protein.

The MIC values, measured by the broth microdilution method, varied from one isolate to another and appeared to depend on the detected mutation (Table 1). While the MIC values were almost the same for all Leu393Phe mutants, substantial differences were observed between Phe397Leu mutants. In particular, TIMM20092 showed a higher terbinafine tolerance than the other two Phe397Leu mutants (TIMM20085 and TIMM20086). The MIC values of TIMM20095 (Leu393Ser), TIMM20087 (Phe397Leu), TIMM20097 (Phe397Val), and TIMM20098 (Phe415Ser) were not estimated because these isolates dramatically decreased their conidial productivity.

Point mutations detected in the *SQLE* gene of resistant strains confer resistance to terbinafine. We examined whether the single amino acid substitutions in the SQLE protein of the 17 *Trichophyton* isolates were involved in the terbinafine resistance phenotypes. Point mutations corresponding to the amino acid substitutions in these isolates were introduced into the endogenous *SQLE* gene of a terbinafine-sensitive dermatophyte strain by genetic manipulations. To enhance the generation of such mutations, *Arthroderma vanbreuseghemii* was used as a recipient organism, for which a variety of more efficient genetic manipulation tools have been developed than for *T. rubrum* and *T. interdigitale* (14). *A. vanbreuseghemii*, *T. rubrum*, and *T. interdigitale SQLE*

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TABLE 2 Plasmids used in this study

Plasmid	Description[a]	Source or reference
pAg1	Streamlined version of the binary vector pBIN19 containing sequences necessary for replication in *E. coli* and *A. tumefaciens* (*oriV* and *trfA*), *E. coli* neomycin phosphotransferase (*nptII*), and the transferable DNA (T-DNA) region, with a multiple cloning site within the T-DNA region	37
pAg1-*AbKu70*/T2	*AbKu70a* fragment (the 5′ UTR of *AbKu70* gene; 2.49 kb), 5′ FRT sequence, P_{trpC} (GenBank accession no. X02390), *nptII*, T_{cgrA} (AFUA_8G02750), P_{ctr4} (TERG_01401), *Pcflp*, T_{trp1} (M74901), *AbKu70b* fragment (the 3′ UTR of *AbKu70* gene; 2.19 kb)	This study
pAg1-*AvSQLE*/T	*SQLEa* fragment (5′-UTR and ORF of the *AvSQLE* gene; about 3.88 kb), 5′ FRT sequence, P_{trpC}, *nptII*, T_{cgrA}, P_{ctr4}, *Pcflp*, T_{trp1}, 3′ FRT sequence, *SQLEb* fragment (the 3′-UTR of the *AvSQLE* gene; about 2.31 kb)	This study
pAg1-*mAvSQLE*/T series	*SQLEa* fragment (the 5′-UTR of *AvSQLE* gene and each mutated *AvSQLE* ORF leading to the Leu393Phe, Leu393Ser, Phe397Leu, Phe397Ile, Phe397Val, Phe415Ser, Phe415Val or His440Tyr substitution in AvSQLE) (3.88kb), 5′-FRT sequence, P_{trpC}, *nptII*, T_{cgrA}, P_{ctr4}, *Pcflp*, T_{trp1}, 3′-FRT sequence, *SQLEb* fragment (the 3′-UTR of *AvSQLE* gene) (about 2.31kb)	This study
pAg1-*sSQLE*/T	*SQLEa* fragment (5′ UTR of the *AvSQLE* gene and the *sSQLE* ORF encoding wild-type TrSQLE; 3.88kb), 5′ FRT sequence, P_{trpC}, *nptII*, T_{cgrA}, P_{ctr4}, *Pcflp*, T_{trp1}, 3′ FRT sequence, *SQLEb* fragment (the 3′ UTR of the *AvSQLE* gene; 2.31 kb)	This study
pAg1-*msSQLE*/T series	*SQLEa* fragment (5′ UTR of *AvSQLE* gene and each mutated *sSQLE* ORF leading to the Leu393Phe, Leu393Ser, Phe397Leu, Phe397Ile, Phe397Val, Phe415Ser, Phe415Val, or His440Tyr substitution in TrSQLE; 3.88kb), 5′ FRT sequence, P_{trpC}, *nptII*, T_{cgrA}, P_{ctr4}, *Pcflp*, T_{trp1}, 3′ FRT sequence, *SQLEb* fragment (the 3′ UTR of the *AvSQLE* gene; about 2.31 kb)	This study

[a]ORF, open reading frame; *sSQLE*, synthetic *SQLE*.

genes each comprise a 1,532-bp ORF encoding 489 amino acids. The amino acid sequence of *A. vanbreuseghemii* SQLE (AvSQLE) is completely identical to that of *T. interdigitale* (GenBank accession number EZF33561). However, it differs in six amino acids at distinct positions (Val^{24}, Thr^{52}, Glu^{174}, Asp^{352}, Ala^{392}, and Ile^{483}) from that of *T. rubrum* SQLE (TrSQLE) (GenBank accession number EGD89476), with some potential impacts on the structure of mutated AvSQLE proteins expressed in *A. vanbreuseghemii*. Based on this possibility, expression of wild-type and mutated TrSQLE proteins in *A. vanbreuseghemii* was also investigated. The synthetic *SQLE* gene (designated *sSQLE*), which encodes the wild-type TrSQLE protein, and the *SQLE* gene alleles harboring point mutations leading to single amino acid substitutions in SQLE proteins were generated from the wild-type *AvSQLE* gene by overlap extension PCR. Four types of *AvSQLE*-targeting vectors harboring those alleles (Table 2) were constructed and introduced into the terbinafine-sensitive *A. vanbreuseghemii* strain 1062Av1401, according to the gene replacement strategy shown in Fig. 2A and B. Our initial attempt at transformation was unsuccessful, except for the production of transformants (named Av-FRT-1-3 and Tr-FRT-52-9, where FRT is FLP recombinase target) harboring the *AvSQLE* or *sSQLE* gene without any point mutation. However, subsequent attempts at transformation using these two clones as the recipient strains led to the successful production of clones harboring the mutated *SQLE* gene alleles, leading to the Leu393Phe, Leu393Ser, Phe397Ile, Phe397Leu, Phe397Val, Phe415Val, or His440Tyr substitution in AvSQLE and TrSQLE proteins (Fig. 2C). However, mutant strains harboring a Phe415Ser substitution could not be generated.

The growth properties of the obtained *A. vanbreuseghemii* transformants harboring the mutated *AvSQLE* or *sSQLE* gene on solid medium containing terbinafine were compared with growth of the parent strain 1062Av1401. The two clones, i.e., Av-FRT-1-3 and Tr-FRT-52-9, which harbor the wild-type *AvSQLE* or *sSQLE* gene, were used as the control strains. As shown in Fig. 3, 1062Av1401, Av-FRT-1-3, and Tr-FRT-52-9 were unable to grow on SDA containing 0.005 μg/ml terbinafine, while all the clones harboring the mutated *SQLE* genes maintained growth activity. The MICs of terbinafine and itraconazole against these resistant clones were measured using the CLSI broth microdilution method (15). As shown in Table 3, clones that harbor the mutated *AvSQLE* and *sSQLE* genes were 8- to 512-fold less susceptible to terbinafine, respectively, than their respective control strains, demonstrating that the terbinafine tolerance of the clinical *Trichophyton* strains was conferred by single amino acid substitutions in the

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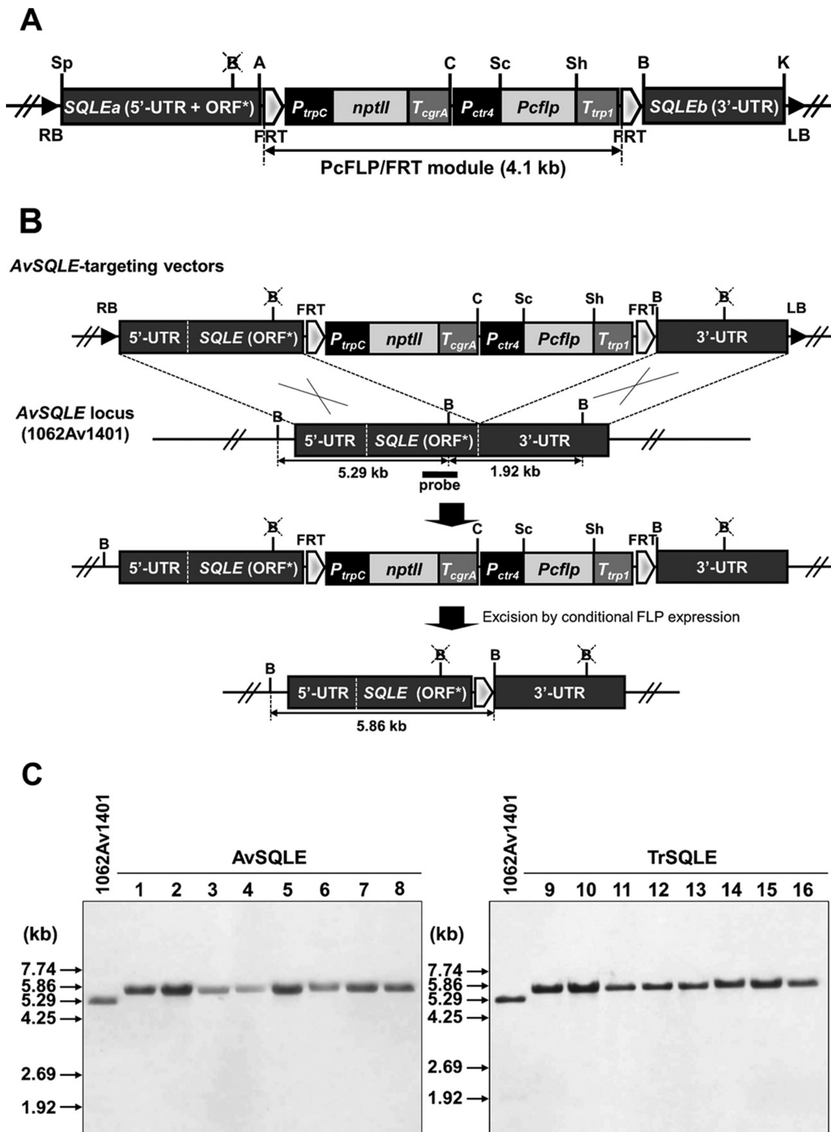

FIG 2 Introduction of point mutations into the endogenous *SQLE* gene of *A. vanbreuseghemii* by gene replacement strategy. (A) Schematic representation of a series of binary *AvSQLE*-targeting vectors. DNA fragments (SQLEa and SQLEb) containing the 5′ UTR of the *AvSQLE* gene and the open reading frames encoding wild-type and mutated *A. vanbreuseghemii* or *T. rubrum* SQLE proteins (ORF*) as well as the 3′ UTR of the *AvSQLE* gene were subcloned into the pAg1-*AbKu70*/T2 upstream (SpeI/ApaI) and downstream (BamHI/KpnI) of the PcFLP/FRT module, respectively (Table 2 and Fig. S1). The *nptII* cassette is composed of *Aspergillus nidulans trpC* promoter (P_{trpC}), *E. coli* neomycin phosphotransferase gene (*nptII*), and the *A. fumigatus cgrA* terminator (T_{cgrA}). P_{ctr4}, *T. rubrum ctr4* promoter (34); *Pcflp*, the synthetic *flp* gene with *Penicillium chrysogenum*-optimized codon usage (35); T_{trp1}, *Cryptococcus neoformans trp1* terminator (36); FRT, FLP recombinase target sequence; LB and RB, left and right borders, respectively; A, ApaI; B, BamHI; C, ClaI; K, KpnI; Sc, SacI; Sh, SphI; Sp, SpeI. (B) Schematic representation of the *AvSQLE* locus before and after homologous recombination and excision of the PcFLP/FRT module. Site-specific recombination between the flanking FRT sequences was induced by conditional expression of *Pcflp* after transformation. All the internal BamHI sites contained in the amplified fragments were inactivated by overlap extension PCR. (C) Southern blotting. Aliquots of approximately 10 μg of total DNA from each mutant strain were digested with BamHI and separated by electrophoresis on 0.8% (wt/vol) agarose gels. 1062Av1401 indicates the parent strain. Lane 1, Av-FRT-1-3 (AvSQLE's control); lane 2, Av-S38A (Leu393Phe); lane 3, Av-1-3 (Leu393Ser); lane 4, Av-S714J6 (Phe397Leu); lane 5, Av-7-5 (Phe397Ile); lane 6, Av-4-1 (Phe397Val); lane 7, Av-S28M (Phe415Val); lane 8, Av-2-4 (His440Tyr); lane 9, Tr-FRT-52-9 (TrSQLE's control); lane 10, Tr-T31C (Leu393Phe); lane 11, Tr-2-3 (Leu393Ser); lane 12, Tr-T719J (Phe397Leu); lane 13, Tr-4-1 (Phe397Ile); lane 14, Tr-75-6 (Phe397Val); lane 15, Tr-T4B (Phe415Val); lane 16, Tr-2-1 (His440Tyr). A 566-bp fragment of the *AvSQLE* gene was amplified by PCR with the primer pair AvSQLE-F23 and AvSQLE-R21 (Table 4) and used as a hybridization probe. DNA standard fragment sizes are shown on the left.

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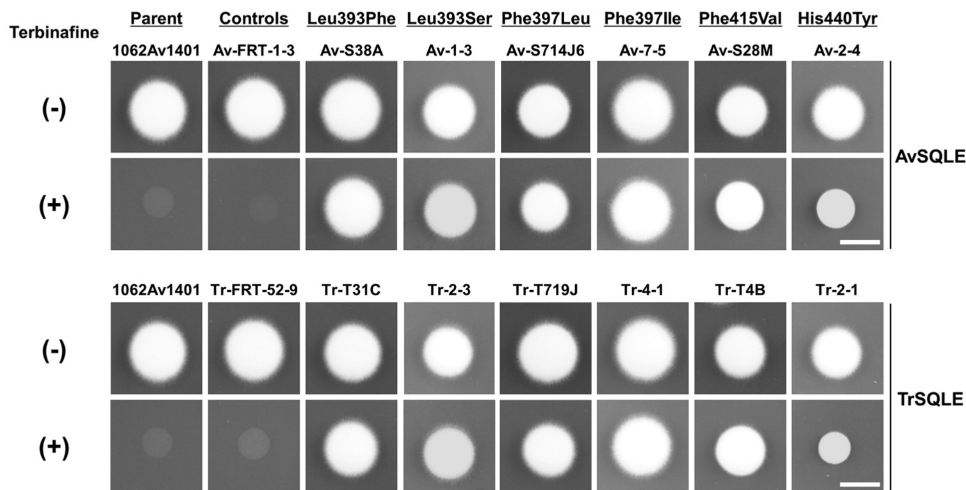

FIG 3 The growth properties of *A. vanbreuseghemii* transformants expressing mutated AvSQLE and TrSQLE proteins on solid terbinafine-containing medium. Aliquots of 10 μl of conidial suspensions containing 1×10^5 cells were spotted onto SDA with (+) or without (−) 0.005 μg/ml terbinafine and incubated at 28°C for 3 days. Bar, 1.0 cm.

SQLE protein. Noteworthy clones with the Phe397Val substitution showed reduced growth rates (Fig. 4A). This finding has correlates with the observation that the clinical isolate TIMM20097 harboring the Phe397Val substitution grew slowly compared to growth of the wild-type *T. rubrum* and the isolate TIMM20085 harboring the Phe397Leu substitution (Fig. 4B). Similarly, the isolate TIMM20098 harboring the Phe415Ser substitution showed much more retarded growth than the wild-type *T. rubrum* and the isolate TIMM20082 harboring the Phe415Val substitution.

No apparent difference was observed in the susceptibilities to itraconazole between transformants harboring the mutated *SQLE* genes and control strains (Table 3).

TABLE 3 Susceptibilities to terbinafine and itraconazole of *A. vanbreuseghemii* transformants expressing mutated forms of the *SQLE* gene and corresponding expression levels

Species and strain	Amino acid substitution	MIC (μg/ml)		Fold expression of *SQLE* (mean ± SD)[a]
		Terbinafine	Itraconazole	
T. interdigitale				
ATCC MYA-4439	None	0.00625	0.12	ND
A. vanbreuseghemii				
1062Av1401	None	0.0125	0.06	1.02 ± 0.24
Av-FRT-1-3	None	0.00625	0.12	1.28 ± 0.06
Av-S38A	Leu393Phe	1.6	0.12	1.08 ± 0.11
Av-1-3	Leu393Ser	0.4	0.12	ND
Av-S714J6	Phe397Leu	3.2	0.12	1.37 ± 0.11
Av-7-5	Phe397Ile	1.6	0.06	ND
Av-S28 M	Phe415Val	0.4	0.12	ND
Av-2-4	His440Tyr	0.2	0.12	ND
T. rubrum				
Tr-FRT-52-9	None	0.00625	0.06	1.27 ± 0.14
Tr-T31C	Leu393Phe	3.2	0.12	1.63 ± 0.18
Tr-2-3	Leu393Ser	0.4	0.06	ND
Tr-T719J	Phe397Leu	3.2	0.06	1.35 ± 0.16
Tr-4-1	Phe397Ile	1.6	0.06	ND
Tr-T4B	Phe415Val	0.4	0.12	ND
Tr-2-1	His440Tyr	0.05	0.25	ND

[a]Results are from three independent real-time PCR experiments. ND, not determined. Expression levels of *SQLE* genes are indicated as relative fold changes compared to the CT mean of the parent strain 1062Av1401 data.

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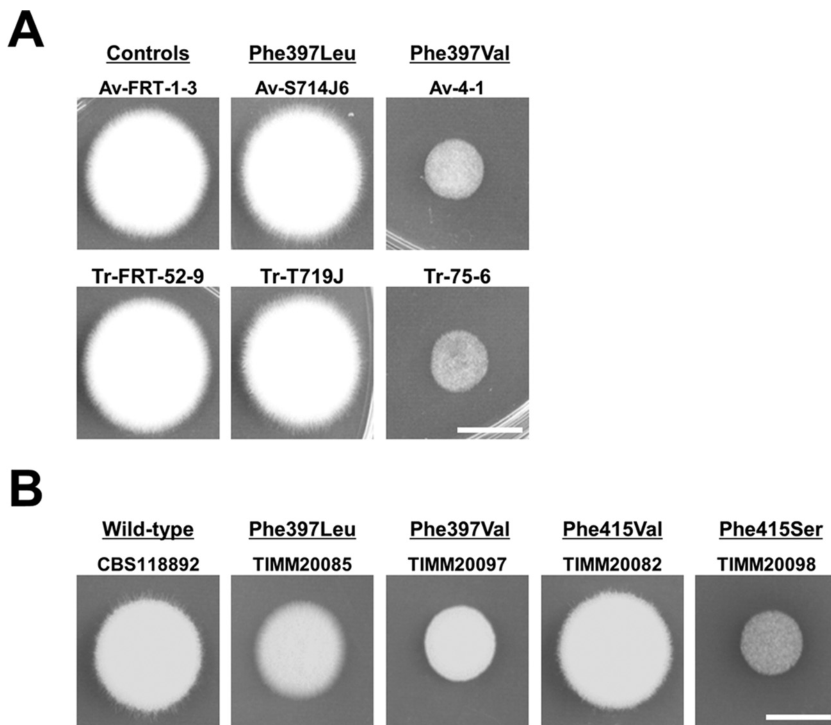

FIG 4 The growth properties of *A. vanbreuseghemii* transformants with the substitution Phe^{397}Val in the SQLE protein (A) and *T. rubrum* clinical isolates harboring the Phe397Val or Phe415Ser substitution in the SQLE protein (TIMM20097 or TIMM20098, respectively) in comparison to a Leu or Val residue, respectively (TIMM20085 and TIMM20082, respectively) (B) on solid medium. Aliquots of 10 μl of conidial suspensions containing 1×10^5 cells were spotted onto SDA and incubated at 28°C for 4 days (A) or 5 days (B). The wild-type *T. rubrum* CBS118892 was used as control. Bar, 1.0 cm.

Comparative analysis of the *SQLE* gene expression levels among terbinafine-resistant dermatophyte strains. To examine whether the resistance shown by *A. vanbreuseghemii* transformants was due to a point mutation in their *SQLE* genes or to different expression levels of polymorphic alleles in the heterologous host, quantitative real-time reverse transcription-PCR (qRT-PCR) was conducted. The *SQLE* gene expression levels in four *T. rubrum* clinical isolates and six *A. vanbreuseghemii* transformants, harboring the corresponding mutated *SQLE* genes or the wild-type alleles, were evaluated. Relative *SQLE* gene expression levels in the two control strains (Av-FRT-1-3 and Tr-FRT-52-9) did not show a significant difference from the level of their parent strain 1062Av1401 (Table 3). Likewise, no statistically significant differences in the *SQLE* gene expression levels were found between four *A. vanbreuseghemii* transformants and their respective parent strains, Av-FRT-1-3 and Tr-FRT-52-9 (Table 3). These results clearly indicate that resistance to terbinafine in *A. vanbreuseghemii* transformants was due to the respective point mutations. In addition, no statistically significant differences in *SQLE* gene expression levels were found between the four *T. rubrum* clinical isolates and the wild-type *T. rubrum* strain CBS118892 (Table 1).

DISCUSSION

Specific point mutations in squalene epoxidase genes causing terbinafine resistance. Drug resistance of fungi has been increasing at an alarming rate over the past few decades. An understanding of the underlying molecular mechanisms is indispensable for successful therapies. In the current study, all 17 terbinafine-resistant *Trichophyton* clinical isolates were revealed to harbor squalene epoxidase (*SQLE*) gene alleles with point mutations, leading to amino acid substitutions at one of four amino acid positions (Leu^{393}, Phe^{397}, Phe^{415}, and His^{440}) within the SQLE protein. The single amino acid substitutions Leu393Phe and Phe397Leu in the SQLE protein have been

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previously reported, marking the first two cases of terbinafine-resistant *T. rubrum* strains isolated from patients (6, 9).

In a previous study, mutants of *Saccharomyces cerevisiae* resistant to terbinafine were generated by chemical, UV, and PCR-based mutagenesis, and molecular analysis revealed point mutations in the *SQLE* gene, leading to amino acid substitutions at one of five amino acid positions, Leu^{231}, Phe^{402}, Phe^{420}, Pro^{430}, and Phe^{433}, in the SQLE protein (16). The residues Phe^{402} and Phe^{420} in *S. cerevisiae* SQLE correspond to Phe^{397} and Phe^{415} in *T. rubrum* SQLE, respectively. No amino acid substitutions equivalent to Leu^{231}, Pro^{430}, and Phe^{433} in *S. cerevisiae* SQLE were identified in the 17 terbinafine-resistant *Trichophyton* clinical isolates of the present study. Amino acid substitutions corresponding to Phe397Leu in the *Trichophyton* SQLE protein were also found in equivalent positions in the SQLE protein of *Aspergillus fumigatus* and *Aspergillus nidulans* terbinafine-resistant strains (17). Accordingly, point mutations at one of a few sites in the *SQLE* gene confer resistance to terbinafine in different fungi.

Identification of precise amino acid substitutions responsible for resistance to drugs is helpful in the determination of the interaction between the drugs and their targets. The atomic three-dimensional (3D) modeling of the *S. cerevisiae* SQLE protein was built based on previously available experimental findings, and, furthermore, it would screen the detailed amino acids critical for binding terbinafine to the enzyme (18). Among the 50 amino acids screened in that study, Phe^{402}, Cys^{416}, Phe^{417}, Phe^{420}, Tyr^{90}, and Val^{92} seem to interact especially strongly with this agent. Similar to the *S. cerevisiae* SQLE protein, all of the amino acid substitutions found in the SQLE protein of the terbinafine-resistant *Trichophyton* clinical isolates reported in the present study and previously (6, 9) were also localized in the C-terminal region of SQLE. However, the *A. vanbreuseghemii* transformants carrying amino acid substitutions at Phe^{415}, equivalent to Phe^{420} in the *S. cerevisiae* SQLE, were 4- to 8-fold more susceptible to terbinafine than those carrying amino acid substitutions at Leu^{393} and Phe^{397}, equivalent to Leu^{398} and Phe^{402} in the *S. cerevisiae* SQLE protein (Table 3). Hence, there is a possibility that together with Phe^{397}, Leu^{393} rather than Phe^{415} would make greater contributions to the SQLE-terbinafine firm contacts in *Trichophyton* species. In addition, His^{440} in the *Trichophyton* SQLE protein was shown to confer resistance to terbinafine for the first time in the present study. However, there is a valine (Val^{447}) in the equivalent position of the *S. cerevisiae* SQLE protein, which was not screened by the 3D modeling study as an amino acid critical for binding terbinafine to the enzyme. These results suggest a subtle conformational difference of the terbinafine binding pocket within the enzyme between *S. cerevisiae* and *Trichophyton* SQLE proteins, possibly leading to the difference in their susceptibilities to this agent. Alternatively, an amino acid substitution may cause conformational changes in the enzyme that result in reduced drug affinity.

Transfer of terbinafine resistance from one isolate to another. To investigate the relationship between these amino acid substitutions and terbinafine tolerance, point mutations leading to several amino acid substitutions found in the SQLE protein of the *Trichophyton* clinical isolates were introduced into the endogenous *SQLE* gene of the dermatophyte *Arthroderma vanbreuseghemii* using genetic manipulation tools. As shown in Table 3, all of the *A. vanbreuseghemii* transformants expressing mutated SQLE proteins showed significant reductions in susceptibilities to terbinafine, demonstrating that the amino acid substitutions in the SQLE protein are a major cause of the terbinafine resistance in *Trichophyton* clinical isolates. Interestingly, transformants with the Phe397Val substitution displayed retarded growth (Fig. 4A). The clinical isolates TIMM20097 and TIMM20098 harboring the Phe397Val and Phe415Ser substitutions, respectively, also grew slowly compared to growth of the wild-type *T. rubrum* and the isolates TIMM20085 and TIMM20082 harboring the Phe397Leu and Phe415Val substitutions, respectively (Fig. 4B). These results suggest the possibility that the Phe397Val and Phe415Ser substitutions in the SQLE protein affect both the susceptibility to terbinafine and the squalene epoxidase activity. The report of fluconazole resistance in *Candida albicans* associated with reduced affinity of sterol 14-α-demethylase (target of

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fluconazole) supports this hypothesis (19). Similarly, the growth of TIMM20098 was more retarded than that of TIMM20097 (Fig. 4B). This may explain why *A. vanbreuseghemii* transformants harboring the Phe415Ser substitution could not be successfully generated.

The discrepancy in terbinafine tolerance of our clinical isolates with the same Phe397Leu mutation, particularly TIMM20092 displaying higher tolerance, could be explained by additional mechanisms of resistance (Table 1). This hypothesis is supported by the abundant work on resistance to azole antifungal agents in *Candida albicans*. The fungal target of these agents is a cytochrome P450 encoded by the gene *ERG11*. Azole resistance of clinical isolates of *C. albicans* was mediated by various mechanisms, including missense mutations in *ERG11* and overexpression of genes encoding multidrug transporters, and combined effects of such mechanisms were observed in the same azole-resistant clinical isolate (20). It is possible that overexpression of genes coding for efflux pumps are also involved in terbinafine tolerance in some of our clinical isolates. Moreover, disruption of a multidrug transporter in *T. rubrum* resulted in an increased sensitivity to terbinafine among various tested antifungal compounds, which was 2-fold higher than that of the nonmutated strains cloned (21).

Selectable markers used in site-directed mutagenesis may also have effects on the susceptibility of generated mutants. A recent study on succinate dehydrogenase (Sdh), the primary molecular target of the novel antifungal agent ME1111, showed that the selectable marker cassette harboring the *E. coli* hygromycin B phosphotransferase gene could increase the susceptibility to the drug (22). The finding was evidenced by the fact that the generated control strains harboring the wild-type gene and the selectable marker cassette were more susceptible to ME1111 than the recipient host strain or reference strain. Hence, there is a possibility that the selectable marker, which was retained in the downstream region of the ORFs of each subunit gene, could confer reduced expression of such genes. To obviate the possibility, the point mutations in the present study were introduced into the endogenous *SQLE* gene of *A. vanbreuseghemii* using an FLP recombinase-mediated site-specific recombination system derived from *S. cerevisiae* (Fig. 2A and B). Following transformation of a recipient strain using each *SQLE*-targeting vector, the selectable marker (*E. coli* neomycin phosphotransferase gene) cassette was excised from the *SQLE* locus in the transformants by FLP recombinase-mediated site-specific recombination between the flanking FRT sequences. Nevertheless, an FRT site remained between the coding sequence of the gene and its downstream region. The FRT sequence is an inverted repeat and thus may affect transcription, mRNA secondary structure, and transcript stability. However, differences in transcription levels of the target gene with and without the added FRT sequence were found to be not statistically significant (Table 3). Moreover, as shown in Table 3, the generated marker-free control strains (Av-FRT-1-3 and Tr-FRT-52-9) showed susceptibilities to terbinafine and itraconazole that are nearly identical to those in the parent strain (1062Av1401) and the reference strain (*T. interdigitale* ATCC MYA-4439). The inserted wild-type gene could serve as a proper control for comparison with the mutated alleles.

High frequency of terbinafine-resistant isolates. The frequency of our terbinafine-resistant clinical isolates of about 1% is strikingly high, considering that the average size of genomes of dermatophytes is about 22 Mb (23). The various sizes of the amplicons obtained by specifically amplifying subrepeat elements in the ribosomal DNA nontranscribed spacer attested that different isolates with identical mutations did not belong to the same strain. Therefore, we could exclude the possibility of the propagation of a particular resistant strain in several patients. We also excluded the possibility that resistance was developed during our screening on SDA containing terbinafine for two reasons. (i) *Trichophyton* isolates were from patients with tinea pedis and/or onychomycosis who did not respond to terbinafine treatment. In three cases where a remaining portion of the sample was stored, the *SQLE* gene could be amplified by targeting total DNA extracted from nails, which was found to harbor a mutation identical to that identified in cultured *Trichophyton* (data not shown). (ii) The development of resistance

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to terbinafine in *T. rubrum* was found to occur at a rare frequency *in vitro* (10). Moreover, no resistant *T. rubrum* strain was generated from a terbinafine-sensitive strain used as a negative control, which was always added with a terbinafine-resistant strain as a positive control in each test plate during the screening procedure.

To explain this high frequency of terbinafine-resistant strains, it should be considered that treatments with terbinafine involve a prolonged exposure to the antifungal drug, which could favor the selection of resistant strains. This hypothesis is supported by the finding that fluconazole resistance in *C. albicans* is correlated with the total dose of fluconazole administered to patients (24). The emergence of *C. albicans* strains that are less susceptible to this antifungal agent is favored by the uptake of a total dose of fluconazole of more than 5 g (24). Retrospective analysis of the follow-up of patients revealed that 8 of the 17 patients harboring a terbinafine-resistant *Trichophyton* isolate had already been treated with terbinafine when the skin and/or nail sample was sent to the laboratory for mycological analysis. One patient was apparently not pretreated. Complete data were not available for eight patients. Prospective studies would be of interest to confirm the possible emergence of resistant strains during long-term treatment.

MATERIALS AND METHODS

Strains and medium. *Trichophyton interdigitale* ATCC MYA-4439 and *T. rubrum* CBS118892 were obtained from the American Type Culture Collection (Manassas, VA) or CBS-KNAW Culture Collections (Utrecht, Netherlands). *Arthroderma vanbreuseghemii* (formerly *T. mentagrophytes*) 1062Av1401 (25), which lacks a homolog of the human *Ku80* (26), was used as a recipient strain for genetic manipulation. Microconidium formation was induced at 28°C using modified 1/10 Sabouraud dextrose agar (SDA) (27) supplemented with 500 μg/ml cycloheximide (Wako Chemical) and 50 μg/ml chloramphenicol (Sigma-Aldrich). *Agrobacterium tumefaciens* EHA105 (28) was maintained as previously described (29). *Escherichia coli* DH5α (Nippon Gene) was used for molecular cloning.

Screening of *Trichophyton* isolates resistant to terbinafine. Over a 3-year period (2013 to 2016), 1,644 *T. rubrum* and 412 *T. interdigitale* isolates were obtained from clinical samples sent to the CHUV for mycological analysis. The samples were mainly collected from patients with tinea pedis or tinea unguium. All clinical isolates were tested for fungal growth on SDA containing 0.2 μg/ml terbinafine, a quantity equivalent to twice that of the MIC for *T. rubrum* and *T. interdigitale* under these conditions (30). Examination of fungal growth was performed after 7, 10, and 14 days. Growing strains were kept and stored in Lausanne and Tokyo in SD broth supplemented with glycerol and dimethyl sulfoxide (DMSO) at −80°C pending further analysis. Isolated terbinafine-resistant strains were deposited in the culture collection of the Teikyo University Institute of Medical Mycology (TIMM) under the identification numbers given in Table 1.

Trichophyton total DNA extraction and *SQLE* gene analysis. *Trichophyton* total DNA was extracted from fresh fungal cultures on SD agar medium and nail samples as previously described using a DNeasy Plant minikit (Qiagen) (31). A diameter of approximately 0.5 cm of growing mycelium was used. Before DNA extraction, nail fragments (20 to 100 mg) were incubated overnight in 500 μl of sodium sulfide dissolving solution (10% [wt/vol] Na_2S, 25% [vol/vol] ethanol). After centrifugation at 8,000 $\times$ *g* for 2 min, the sample precipitate containing fungal elements was washed twice with distilled water. The *SQLE* gene of the terbinafine-resistant clinical isolates was amplified by PCR with High Fidelity DNA Polymerase (Roche Diagnostics), the primer pair TrSQLE-F1 and TrSQLE-R1, and chromosomal DNA as the template. Nucleotide sequences of these primers are shown in Table 4.

***Trichophyton rubrum* strain typing.** Strain typing based on the length of the parts containing the tandem repetitive subelements (TRSs), TRS-1 and TRS-2, in nontranscribed spacers of ribosomal DNA (ribosomal DNA intergenic spacer regions) was performed as described by Jackson et al. (32). The primer pair TrNTSF-2 and TrNTSR-4 and the pair TrNTSC-1 and TrNTSR-1 were used to amplify the TRS-1 and the TRS-2 regions, respectively (32). Strains were characterized by the size of both TRS-1 and TRS-2 amplicons, which are polymorphic for length.

Construction of transformation vectors harboring wild-type and mutated *SQLE*. Genomic data of the *SQLE* locus that shares homology with the *T. rubrum SQLE* gene (*TrSQLE*) (GenBank accession number TERG_05717) were identified in *A. vanbreuseghemii* based on GenBank accession number AB690298 and our private draft genome of the strain TIMM2789. *A. vanbreuseghemii* SQLE (designated AvSQLE) differs in six amino acids at distinct positions (Val^{24}, Thr^{52}, Glu^{174}, Asp^{352}, Ala^{392}, and Ile^{483}) from TrSQLE (GenBank accession number EGD89476). To generate the *SQLE* ORF fragment encoding the wild-type TrSQLE protein, six point mutations were introduced into the *AvSQLE* gene by overlap extension PCR with the corresponding six pairs of primers (listed in Table 4); the resultant fragment was designated *sSQLE* (where *s* indicates synthetic gene). The *SQLE* gene alleles harboring point mutations leading to Leu393Phe, Leu393Ser, Phe397Leu, Phe397Ile, Phe397Val, Phe415Ser, Phe415Val, or His440Tyr in AvSQLE and TrSQLE were also generated from the wild-type *AvSQLE* gene by overlap extension PCR with the corresponding pair of primers (listed in Table 4). All the amplified fragments were sequenced to verify the introduction of correct nucleotide substitutions at the targeted sites.

To construct a series of *AvSQLE* locus-targeting binary vectors, two types of DNA fragments were amplified by PCR; the SQLEa fragment contained the 5′ untranslated region (UTR) of the *AvSQLE* gene

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TABLE 4 PCR primers used in this study

Purpose	Primer	Sequence (5′–3′)[b]	Note
Amplification of *SQLE* genes for qRT-PCR	qRT_erg1_2-F	CCAGACTGATGGCAAGCAAGA	
	qRT_erg1_2-R	ATAAGCTCCAGGCCCCAGAA	
	qRT_erg(Tr)1_2-F	CCAGACTGATGGCAAACAAGA	
Amplification of *SQLE* genes from clinical isolates	TrSQLE-F1	ATGGTTGTAGAGGCTCCTCCC	
	TrSQLE-R1	CTAGCTTTGAAGTTCGGCAAA	
Amplification of the DNA fragments containing the 5′ UTR of *AvSQLE* and the *SQLE* ORF	AvSQLE-F15/SpeI	TCACGAAGCTA**ACTA**GTACCTGAAAGATGAC	Inactivation of the KpnI site
	AvSQLE-R23/ApaI	AAAAAGGGCCCCTAGCTTTGAAGTTCGGCAAATA	
Amplification of the 3′ UTR fragment of *AvSQLE*	AvSQLE-F13/BamHI	CAAGGATCCACAGATAGGCTTATCTCTAGCTCT	
	AvSQLE-R16/KpnI	CAGGGTACCTCCGTTCATAGTCAACGAACGTCTCG	
Generation of the s*SQLE* ORF encoding wild-type TrSQLE[a]	AvSQLE-F21	TCCGCGAAGCCCAAGG**T**ATACCGCGACGAA	Ala24Val
	AvSQLE-R18	TTCGTCGCGGTAT**A**CCTTGGGCTTCGCGGA	
	AvSQLE-F22	GGCATTGCTGGATGT**A**CGCTGGCCGTTGCGTT	Ala52Thr
	AvSQLE-R19	AACGCAACGGCCAGCG**T**ACATCCAGCAATGCC	
	AvSQLE-F23	TCCACGCACACAGGGGA**G**GTCCTTGGAGTTCAATG	Asp174Glu
	AvSQLE-R20	CATTGAACTCCAAGGAC**C**TCCCCTGTGTGCGTGGA	
	AvSQLE-F24	ATGTCGTTCTCCTCCGG**G**ATCTACTTAGTCCAGA	Asn352Asp, hybridization probe
	AvSQLE-R21	TCTGGACTAAGTAGAT**C**CCGGAGGAGAACGACAT	
	AvSQLE-F25	TCAATATTCTTGCCCAG**G**CCTTATACTCTATATTC	Ser392Ala
	AvSQLE-R22	GAATATAGAGTATAAGG**C**CTGGGCAAGAATATTGA	
	AvSQLE-F26	GGTCATCCTTCCTTTC**A**TATTTGCCGAACTT	Val483Ile
	AvSQLE-R24	AAGTTCGGCAAATA**T**GAAAGGAAGGATGACC	
Generation of point mutations in AvSQLE and TrSQLE[a]	AvSQLE-F27	TTGCCCAGTCCTT**C**TACTCTATATT	Leu393Phe (AvSQLE)
	AvSQLE-R27	AATATAGAGTA**G**AAGGACTGGGCAA	
	AvSQLE-F29	TTCTTGCCCAG**G**CCTT**C**TACTCTATATT	Leu393Phe (TrSQLE)
	AvSQLE-R29	AATATAGAGTA**G**AAGG**C**CTGGGCAAGAA	
	AvSQLE-F35	TTGCCCAGTCCT**C**ATACTCTATATT	Leu393Ser (AvSQLE)
	AvSQLE-R35	AATATAGAGTAT**G**AGGACTGGGCAA	
	AvSQLE-F36	TTCTTGCCCAG**G**CCT**C**ATACTCTATATT	Leu393Ser (TrSQLE)
	AvSQLE-R36	AATATAGAGTAT**G**AGG**C**CTGGGCAAGAA	
	AvSQLE-F28	TACTCTATATT**A**GCCGCTGGTG	Phe397Leu (both SQLE proteins)
	AvSQLE-R28	CACCAGCGGC**T**AATATAGAGTA	
	AvSQLE-F31	TACTCTATA**A**TCGCCGCTGGTG	Phe397Ile (both SQLE proteins)
	AvSQLE-R31	CACCAGCGGCGA**T**TATAGAGTA	
	AvSQLE-F30	TACTCTATA**G**TCGCCGCTGGTG	Phe397Val (both SQLE proteins)
	AvSQLE-R30	CACCAGCGGCGA**C**TATAGAGTA	
	AvSE-F37	GGCTGCTTCAGGTATT**C**CCAACTTGGACTTATA	Phe415Ser (both SQLE proteins)
	AvSE-R37	TATAAGTCCAAGTTGG**G**AATACCTGAAGCAGCC	
	AvSQLE-F14	GGCTGCTTCAGGTAT**G**TCCAACTTGGACTTATA	Phe415Val (both SQLE proteins)
	AvSQLE-R8	TATAAGTCCAAGTTGGA**C**ATACCTGAAGCAGCC	
	AvSQLE-F29	CCTTGGTTCTATTACGA**T**ATTTTTACTCCGTAGCC	His440Tyr (both SQLE proteins)
	AvSQLE-R29	GGCTACGGAGTAAAAAT**A**TCGTAATAGAACCAAGG	

[a]Introduction of nucleotide substitutions into *SQLE* genes by overlap extension PCR.
[b]Restriction sites are underlined, and substitutions are in boldface.

and each *SQLE* ORF, and the SQLEb fragment contained the 3′ UTR of the *AvSQLE* gene (Fig. 2A). Each fragment was doubly digested with the SpeI/ApaI or the BamHI/KpnI enzymes, respectively, and subsequently subcloned into the binary vector pAg1-*AbKu70*/T2 (Table 2; see Fig. S1 in the supplemental material), resulting in the replacement of the 5′ UTR and 3′ UTR fragments of the *Arthroderma benhamiae Ku70* gene (ARB_06096) within the vector. Depending on the *SQLE* ORF contained in the SQLEa fragment, four types of vectors were generated: pAg1-*AvSQLE*/T and pAg1-*sSQLE*/T harboring the *AvSQLE* ORF and the *sSQLE* ORF encoding the wild-type TrSQLE protein, respectively, and the pAg1-*mAvSQLE*/T and pAg1-*msSQLE*/T (where *m* indicates mutated gene constructs) series harboring the ORF of each mutated *SQLE* allele, which contained a point mutation leading to the Leu393Phe, Leu393Ser, Phe397Leu, Phe397Ile, Phe397Val, Phe415Ser, Phe415Val, or His440Tyr substitution in AvSQLE and TrSQLE proteins, respectively (Table 2).

The PCRs were performed using PrimeSTAR HS DNA polymerase or Extaq DNA polymerase (TaKaRa Bio). If necessary, the amplified fragments were gel purified with a QIAEX II gel extraction kit (Qiagen). All the internal ApaI, BamHI, KpnI, and SpeI sites contained in the amplified fragments were inactivated by overlap extension PCR with a corresponding pair of primers (Table S1).

Fungal genetic transformation. *A. vanbreuseghemii* 1062Av1401 was transformed by the *A. tumefaciens*-mediated transformation (ATMT) method as described previously (29). After cocultiva-

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tion, nylon membranes were transferred onto SDA containing 250 μg/ml G418 (Sigma-Aldrich) and 10 μM $CuSO_4 \cdot 7H_2O$, and overlaid with 10 ml of SDA supplemented with the same concentration of G418 and $CuSO_4 \cdot 7H_2O$. The plates were further overlaid after 48 h with 10 ml of SDA containing 350 μg/ml G418 and 10 μM $CuSO_4 \cdot 7H_2O$ and then incubated for 4 to 5 days. The colonies regenerating on the selective medium were considered putative G418-resistant clones and transferred onto solid morpholinepropanesulfonic acid (MOPS)-buffered RPMI 1640 medium (RPMI 1640A) supplemented with 500 μg/ml cycloheximide, 50 μg/ml chloramphenicol, 200 μg/ml cefotaxime sodium (Sanofi-Aventis) (if necessary), and 20 μM bathocuproine disulfonate (BCS) (Dojindo Laboratories) and passaged several times. The *T. rubrum ctr4* promoter (P_{ctr4}) is a conditional promoter that is repressed in the presence of copper. Chelation of copper by bathocuproine sulfate activates the P_{ctr4}, leading to the induction of *Penicillium chrysogenum flp* (*Pcflp*) gene expression. Expression of PcFLP recombinase leads to excision of the selectable marker via PcFLP-mediated site-specific recombination between the flanking FRT sequences.

Screening of the desired transformants. The desired transformants were finally screened by PCR, Southern blotting analyses, and nucleotide sequencing. Total DNA was extracted according to a method described previously (29). Aliquots of 50 to 100 ng of the total DNA were used as templates in the PCRs. For Southern blotting, aliquots of approximately 10 μg of the total DNA were digested with an appropriate restriction enzyme, separated by electrophoresis on 0.8% (wt/vol) agarose gels, and transferred onto Hybond-N^+ membranes (GE Healthcare Ltd.). Southern hybridization was performed using an ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare Ltd.) according to the manufacturer's instructions.

Drug susceptibility testing. MICs were determined according to the broth microdilution method of the Clinical and Laboratory Standards Institute (15).

Total RNA extraction and qRT-PCR. Plugs from fresh cultures of five *T. rubrum* strains (CBS118892, TIMM20083, TIMM20084, TIMM20086, and TIMM20087) and seven *A. vanbreuseghemii* strains (1062Av1401, Av-FRT-1-3, AvS38A, AvS714J6, Tr-FRT-52-9, Tr-T31C, and Tr-T719J) grown on SDA were inoculated in about 15 ml of RPMI 1640 broth and cultivated at 28°C on a rotary shaker at 200 rpm. After 3 days, the growing mycelia from each strain were collected, frozen, and ground under liquid nitrogen with a Multi-Beads shocker (Yasui Kikai) at 1,800 rpm for 10 s, which was repeated 3 times. Total RNA was extracted using an RNeasy Plant minikit (Qiagen) and was treated with DNase I (Invitrogen). First-strand cDNA was synthesized using a high-capacity RNA-to-cDNA kit (Applied Biosystems). The quantitative real-time reverse transcription-PCR (qRT-PCR) analysis was performed using Fast SYBR green PCR master mix on an ABI PRISM 7500 Fast real-time PCR system (Applied Biosystems) under standard conditions, according to the manufacturer's recommendations. Two sets of primers were used, one to amplify *SQLE* alleles derived from *A. vanbreuseghemii* (qRT_erg1_2-F and qRT_erg1_2-R) and the other to amplify *SQLE* alleles derived from *T. rubrum* [qRT_erg(Tr)1_2-F and qRT_erg1_2-R] (Table 4). Dissociation curves of the q PCR-amplified products were plotted to confirm the absence of nonspecific products or primer dimers. Normalization was done to the 18S rRNA gene using two primers, 18S-1-F and 18S-1-R (33), and relative quantification of gene expression was calculated according to the $2^{-\Delta\Delta CT}$ (where C_T is threshold cycle) method. Expression levels of *SQLE* genes examined in six *A. vanbreuseghemii* transformants and four *T. rubrum* clinical isolates are indicated as relative fold changes compared to levels in the *A. vanbreuseghemii* parent strain 1062Av1401 or the wild-type *T. rubrum* strain CBS118892, respectively. Statistical significance of *SQLE* gene expression levels among strains was evaluated using Student's *t* test.

Nucleotide sequence accession number. The nucleotide sequence of the *A. vanbreuseghemii SQLE* (*AvSQLE*) locus containing the ORF was deposited in the GenBank under accession number KU242352.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00115-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

We thank Junko Ito (Chiba University Medical Mycology Research Center, Chiba, Japan) for technical support in the drug susceptibility tests of dermatophyte strains by the microdilution method of the Clinical and Laboratory Standards Institute (CLSI M38-A2).

This study was partly supported by the National BioResource Project—Pathogenic Microbes in Japan and the Joint Usage/Research Program of the Medical Mycology Research Center, Chiba University (grant number 16-26).

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