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# Expression of *T4HR1*, a 1,3,6,8-Tetrahydroxynaphthalene Reductase Gene Involved in Melanin Biosynthesis, Is Enhanced by Near-Ultraviolet Irradiation in *Bipolaris oryzae*

**Nozomi Tanaka[1], Yohei Haruki[1,2], Makoto Ueno[1], Sakae Arase[1], Junichi Kihara[1*]**

[1]Faculty of Life and Environmental Science, Shimane University, Shimane, Japan
[2]Present Address: Faculty of Agriculture, Tottori University, Tottori, Japan
Email: [*]j-kihara@life.shimane-u.ac.jp

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## Abstract

***Bipolaris oryzae* is the causal agent of brown spot disease in rice and produces the dark pigment melanin. We isolated and characterized *T4HR1* gene encoding 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) reductase, which converted 1,3,6,8-THN to scytalone in the melanin biosynthesis from *B. oryzae*. A sequence analysis showed that the *T4HR1* gene encoded a putative protein of 268 amino acids showing 50% - 99% sequence identity to other fungal 1,3,6,8-THN reductases. Targeted disruption of the *T4HR1* gene showed a different phenotype of mycelial color due to an accumulation of shunt products compared to those of wild-type on PDA plates using tricyclazole as a melanin biosynthesis inhibitor. A quantitative real-time PCR analysis showed that the expression of *T4HR1* transcripts was enhanced by near-ultraviolet (NUV) irradiation and regulated by transcriptional factor *BMR1*, similar to three other melanin biosynthesis genes (polyketide synthase gene [*PKS1*], scytalone dehydratase gene [*SCD1*], and 1,3,8-THN reductase gene [*THR1*]) in the melanin biosynthesis of *B. oryzae*. These results suggested that common transcriptional mechanisms could regulate the enhanced gene expression of these melanin biosynthesis genes by NUV irradiation in *B. oryzae*.**

## Keywords

***Bipolaris oryzae*, Gene Expression, Melanin Biosynthesis, Near-Ultraviolet,**

[*]Corresponding author.

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**1,3,6,8-Tetrahydroxynaphtalene Reductase**

## 1. Introduction

Melanin is a dark-pigmented polymer that protects organisms against environmental stress, and its production is also widespread in the fungal kingdom [1]-[3]. In general, melanin accumulates in fungal cell walls and has been believed to confer tolerance to environmental stresses such as UV radiation [4]-[7]. In fungi, light modulates processes, such as the reproduction structures and pigment biosynthesis, and the molecular bases of fungal photoreceptors as well as those of photomorphogenesis by blue and red light, have been widely studied as detailed in a number of published reviews [8]-[12].

*Bipolaris oryzae* (Breda de Haan) Shoemaker [anamorph of the ascomycetous fungus *Cochliobolus miyabeanus* (Ito et Kuribayashi) Drechslerex Dastur], the causal agent of brown leaf spot disease in rice, produces 1,8-dihydroxynaphthalene (DHN)-melanin [13] [14], and it produces its asexual spores (conidia) by the induction of near-ultraviolet irradiation [15] [16]. DHN-melanin biosynthesis in *B. oryzae* starts with a polyketide synthaseusing acetate as a precursor (**Figure 1**). A hydroxynaphthalene reductase converts 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) to scytalone. The dehydration of scytalone by scytalone dehydratase forms 1,3,8-trihydroxynaphthalene (1,3,8-THN). 1,3,8-THN reductase converts the 1,3,8-THN to vermelone, which is further dehydrated to 1,8-DHN. Finally, the oxidative polymerization of 1,8-DHN gives DHN-melanin. We have identified polyketide synthase gene (*PKS1*) [17], scytalone dehydratase gene (*SCD1*) [18], and 1,3,8-THN reductase gene (*THR1*) [19] in *B. oryzae* and observed that the expression of these melanin biosynthesis genes was specifically enhanced by near-ultraviolet (NUV: 300 - 400 nm) irradiation. The 1,3,6,8-THN reductase gene has not

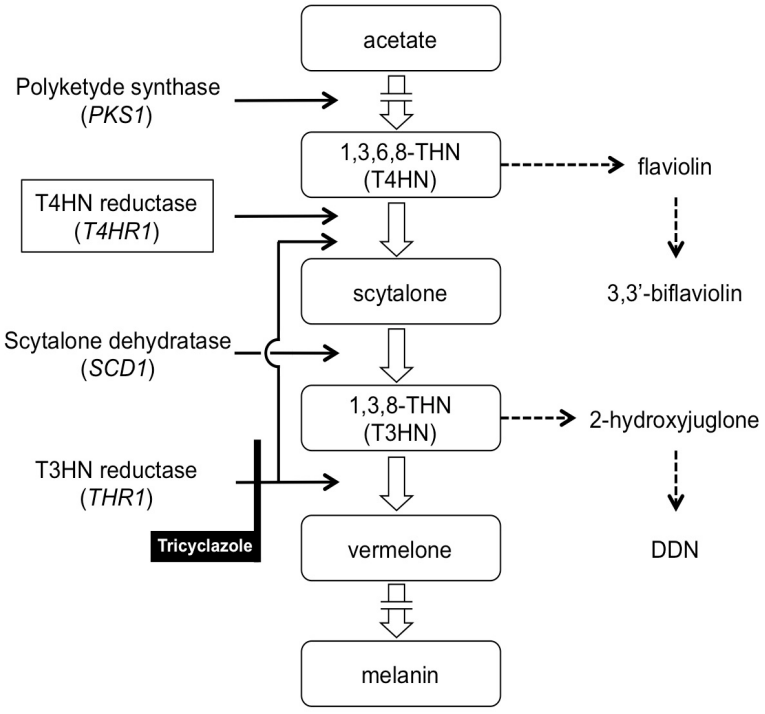


**Figure 1.** The melanin biosynthesis pathway of *Bipolaris oryzae*. *PKS1*, polyketide synthase gene (AB176546); *T4HR1*, 1,3,6,8-tetrahydroxynaphthalene reductase gene (AK424235, in this study), *SCD1*, scytalone dehydratase gene (AB100172); *THR1*, 1,3,8-trihydroxynaphthalene reductase gene (AB083402). Open arrows and dotted arrows indicate the main and shunt pathways, respectively. Tricyclazole inhibits T3HN reductase activity. DDN, 3,4-dihydro-4,8-dihydroxy-1(2H)naphthalenone.

yet been identified and characterized in *B. oryzae*, although 1,3,6,8-THN reductase genes have been identified and characterized in *Magnaporthe grisea* [20] [21], *Colletotrichum orbiculare* [22], *Sordaria macrospora* [23], and *Cochliobolus heterostrophus* [24].

In previous studies, to reveal the mechanisms underlying the photomorphogenetic response to NUV irradiation in *B. oryzae*, we focused on the regulatory genes induced by NUV irradiation, and we reported that NUV irradiation specifically enhanced the expression of a novel NUV-inducible gene (*UVI-1*) [25] and the photolyase gene [26]. Recently, we also reported 46 newly identified genes that were enhanced by NUV irradiation in *B. oryzae*, following our use of suppression subtractive hybridization methods [27]. Interestingly, one of the 46 genes, NUV12, showed significant similarities to 1,3,6,8-THN reductase in some fungi, as mentioned above.

Here we reported the cloning of a 1,3,6,8-THN reductase gene (*T4HR1*) from *B. oryzae*. We also demonstrated that the expression of the *T4HR1* was enhanced by NUV irradiation and was dependent on the transcription factor *BMR1*, which was similar to the expressions of three other melanin biosynthesis genes in *B. oryzae*.

## 2. Materials and Methods

### 2.1. Fungal Strains and Growth Conditions

*Bipolaris oryzae* strain D9/F6-69 and Δ*BMR1* (stock culture at the Laboratory of Plant Pathology, Shimane University) were used as the wild-type and *BMR1* disruptant strain [28], respectively. The experimental fungus was grown on potato dextrose agar (PDA) in 9-cm petri plates (Iwaki, Tokyo, Japan). The plates were inoculated centrally with a mycelial plug. The cultures were incubated at 25˚C ± 1˚C for 4 days in continuous darkness and then subjected to light treatments. Black-light (BLB) lamps (FL-BLB; Toshiba Electric, Tokyo, Japan), which emitted wavelengths of 300 to 400 nm (mainly 352 nm), were used as the source of NUV radiation. The irradiance of NUV radiation was measured by an USB4000-UV-VIS miniature fiber optic spectrophotometer (Ocean Optics, Dunedin, FL, USA). The average irradiance of the NUV radiation was 280 $mW \cdot m^{-2}$.

### 2.2. Cloning and Sequence Analysis of *T4HR1* Gene

Genomic DNA was extracted from mycelia grown in potatodextrose broth as described [25]. The target DNA of NUV17 was amplified by polymerase chain reaction (PCR) and purified using the NucleuSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). A cDNA library constructed in the λgt10 vector was screened using a digoxigenin (DIG)-labeled NUV17 fragment from *B. oryzae*. DNA labeling, hybridization and detection were carried out using a nonradioactive system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The DNA sequence analysis was performed on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). Both strands were completely analyzed by overlapping at every junction. A computer analysis of the DNA sequence data was performed using GENETYX®-Mac (GENETYX Co., Tokyo, Japan). Comparisons between DNA and the predicted aminoacid sequence and a phylogenetic analysis were carried out using the BLAST and CLUSTALW network programsat the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp).

### 2.3. Disruption of *T4HR1* Gene

The *T4HR1* gene deletion vector was constructed based on the double-joint PCR strategy [29]-[31] as shown in **Figure 2(a)**. Fragment t4hr1 A (left side, 397-bp) and Fragment t4hr1 B (right side, 415-bp) of the *T4HR1* locus were amplified from *B. oryzae* genomic DNA using primers T4HRAF/T4HRAR and T4HRBF/T4HRBR, respectively. A 3.0-kb *hph* cassette (P*trpC*-*hph*-T*trpC*) containing hygromycin-resistant gene was also amplified from pSH75 [32] with primers T4HRfushpF/T4HRfushpR. The PCR was performed using KOD Plus DNA polymerase (Toyobo Life Science, Tokyo, Japan) and a Thermal Cycler Gene Atlas (Astec, Fukuoka Japan). These amplified fragments were joined together in the second-round PCR using TaKaRa EX Taq HS (TaKaRa Bio, Otsu, Japan), the product of which acted as the template for the final amplification with primers T4HRAF/T4HRBR. The final 3.8-kb double-joint PCR product (*T4HR1* disruption fragment) was purified using the NucleuSpin Gel and PCR Clean-up kit before transformation. The wild-type strain D9/F6-69 was transformed with the 3.8-kb double-joint PCR productas described [19]. Transformants were selected on PDA containing 100 $\mu g mL^{-1}$ hygromycin B (Wako Pure Chemical Industries, Osaka, Japan).

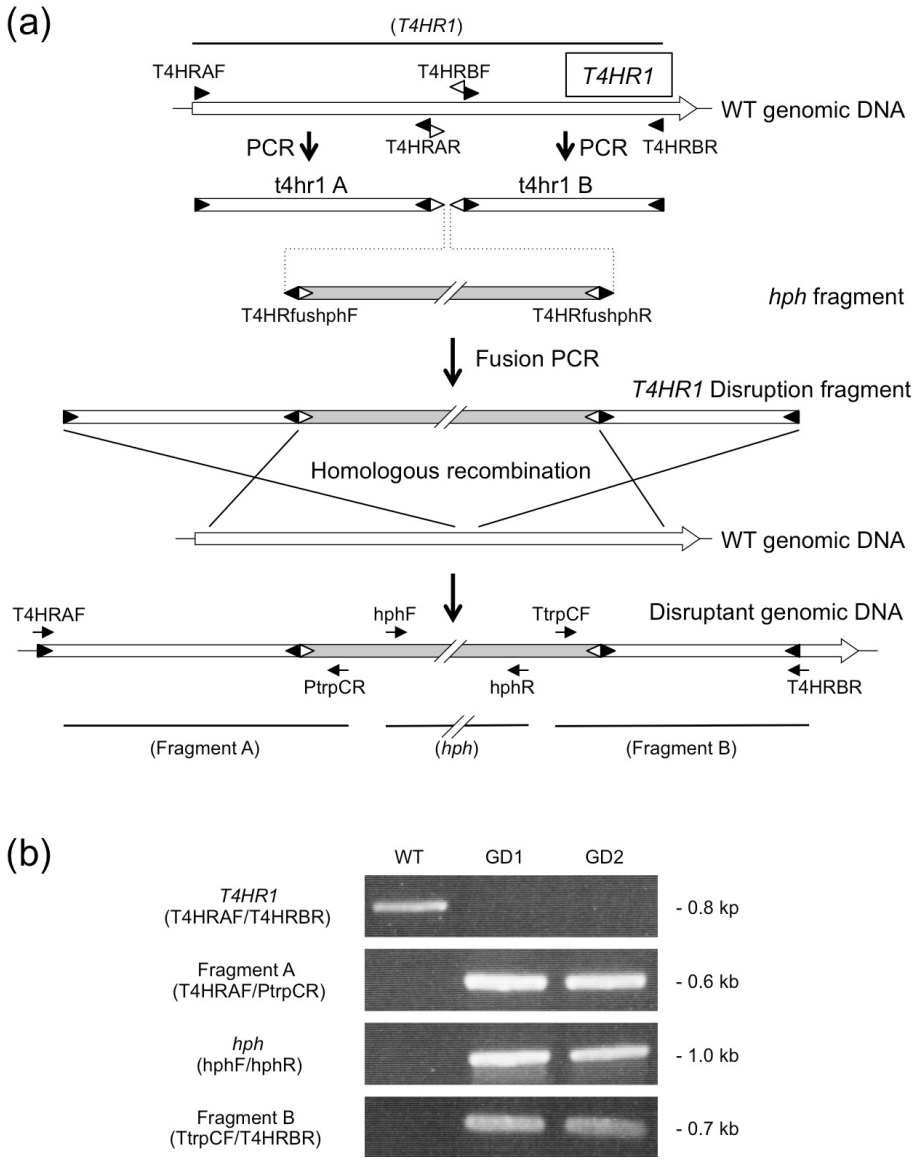


**Figure 2.** Targeted disruption of *T4HR1*. (a) Strategy of targeted disruption. Fusion PCR was used to construct the *T4HR1* gene replacement fragment. The sequences of these primers are listed in **Table 1**. Predicted structures of the *T4HR1* locus before (WT genomic DNA) and after homologous recombination (disruptant genomic DNA) are also indicated. (b) PCR analysis of gene replacement in *T4HR1* in wild-type (WT) and *T4HR1* disruptants (GD1 and GD2). Each PCR was carried out using the primer pair indicated in the parentheses. The sequences of these primers are also listed in **Table 1**.

## 2.4. Quantitative Real-Time PCR Analysis

Colonies grown on a PDA medium for 4 days in darkness were exposed to BLB light as a source of NUV radiation for 1 h. To peel off the mycelia from the agar medium, the agar surface of a petri plate (15 mL PDA) was overlaid with a single layer of dialysis tubing (8 cm in flat width; EIDIA Co., Tokyo, Japan) that had been autoclaved at 121˚C for 15 min. Mycelia were scratched from dialysis tubes by a sterilized stainless steel spatula, frozen in liquid nitrogen, and ground with a mortar and pestle to a fine frozen powder. Total RNA was isolated from the frozen mycelial powder by phenol/chloroform extraction, followed by LiCl precipitation according to the published protocols [33]. DNA contamination was eliminated by treatment with RNase-free DNase I (TaKaRa Bio). cDNA was prepared from the total RNA as a template using Ready-To-Go You-prime First-Strand

Beads (GE Healthcare UK, Buckinghamshire, UK) with anoligo $(dT)_{15}$ primer (Promega, Madison, WI). Real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa Bio) and a Thermal Cycler Dice Real-Time System TP800 (TaKaRa Bio). The primers used in the reaction (**Table 1**) were designed by Primer3 at the website http://frodo.wi.mit.edu. The PCR conditions were as follows: initial denaturation (95˚C, 20 sec), followed by 40 cycles of denaturation (95˚C, 5 sec) and annealing/extension (60˚C, 30 sec) according to the manufacturer's instructions. Post-PCR melting curves confirmed the specificity of single-target amplification, and the fold expression of each gene relative to the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD*) was determined.

## 3. Results and Discussion

### 3.1. Cloning of the 1,3,6,8-THN Reductase Gene from *B. oryzae*

Using suppression subtractive hybridization, we previously identified 46 genes (NUV01-NUV46) whose expressions were enhanced by NUV irradiation in *B. oryzae* [27]. Among these clones, a 363-bp fragment of NUV12 showed similarity to other fungal 1,3,6,8-THN reductases. Thus, we screened the cDNA library of *B. oryzae* using the DIG-labeled 363-bp fragment as a probe by plaque hybridization. From about 50,000 plaques, three positive clones were identified. One of the phage clones, designated cT4HR1, was sequenced and the cloned cDNA was designated *T4HR1* (DDBJ Accession No. AK424235). The *T4HR1* gene contained an open reading frame (ORF) encoding 268 amino acids.

To confirm the existence of an intron of the *T4HR1* gene, we also screened the genomic library of *B. oryzae*, using the DIG-labeled *T4HR1* cDNA probe by colony blot hybridization. One positive clone was isolated and named pUCT4HR1. A comparison of both sequences of the *T4HR1* cDNA and the genomic DNA revealed no intron (data not shown). A Southern blot analysis showed that the *T4HR1* gene was present as a single copy in the *B. oryzae* genome (data not shown).

**Table 1.** PCR primers used in this study.

| Gene | Primer name | Sequence (5' to 3') |
|---|---|---|
| *T4HR1* | T4HRAF | GGTCGTCACAGTACCCACTG |
| | T4HRAR | GGAACAACTGGCATGAATTCTGACCAACGAAGAATTGC |
| | T4HRBF | TCCTTCTTTCTAGAGGATCCATTGAGGATAACGGCCGTA |
| | T4HRBR | CCCATCTTCTGACACCACAC |
| | T4HRfushphF | GCAATTCTTCGTTGGTCAGAATTCATGCCAGTTGTTCC |
| | T4HRfushphR | TACGGCCGTTATCCTCAATGGATCCTCTAGAAAGAAGGA |
| | T4HRF* | ATTGAGGATAACGGCCGTAT |
| | T4HRR* | AGCGTGGTCCTTGACACCAA |
| *PKS1* | PKS17002F* | GACCCAGCTCCCATGAAGTG |
| | PKS17196R* | ACCGTGATCCCCCTTCATC |
| *SCD1* | SCD69F* | GGAGGTGATGGGCTGTCAA |
| | SCD188R* | TCCAGTCCTTGCTGTCGTA |
| *THR1* | THR481F* | GATTGGTGGCCGTATCATCCT |
| | THR685R* | ACCTTCTTCTCACCAGCATCGA |
| P*trpC* | PtrpCR | CCGAGTTTGTTTCGGAGAAG |
| T*trpC* | TtrpCF | CCTGGGTTCGCAAAGATAAT |
| *hph* | hphF | ATGAAAAAGCCTGAACTCACCG |
| | hphR | CTATTCCTTTGCCCTCGGACGA |
| *GPD* | GPD161F* | AACGGCAAGACCATCCGTT |
| | GPD241R* | GACGACGTAGTAAGCGCCAGT |

*Primers used in the quantitative real-time PCR analysis.

### 3.2. Comparison of the Predicted *T4HR1* Protein Sequence with Those of Other Fungal 1,3,6,8-THN Reductases

The deduced amino acid sequence of *T4HR1* revealed high similarity to the fungal 1,3,6,8-THN reductase involved in the melanin biosynthesis of *Cochliobolus heterostrophus* [24], *Setosphaeriaturcica* (EU494943), *Neurospora crassa* (Q7S3E8), *Sordaria macrospora* [23], *Colletotrichum orbiculare* [22], and *Magnaporthe grisea* [20] [21], all of which produce DHN melanin (**Figure 3**). These sequence similarities among the predicted *T4HR1* protein of *B. oryzae* and other fungal 1,3,6,8-THN reductases suggest that the predicted *T4HR1* protein of *B. oryzae* may have the same function as other fungal 1,3,6,8-THN reductases in melanin biosynthesis.

```
Bo    1 -------------MVVTVPTASLEGQVALITGAGRGIGRGCAIELGKRGASVVVNYVSSE  47
Ch    1 -------------MVVTVPSASLEGQVALITGAGRGIGRGCAIELGKRGASVVVNYVSSE  47
St    1 -------------MVVNVPTASLEGQVALITGAGRGIGRGCAIELGKRGASVIVNYVSSE  47
Nc    1 -------------MPISIPTASLEGKVALVTGAGRGIGRGVALELARRGASVIVNYVSSA  47
Sm    1 -------------MPISIPTASLEGKVALVTGAGRGIGRGVALELARRGASVIVNYVSSA  47
Co    1 MAPSATDGAPLEQTAGYDAARPFVGKVALITGAGRGIGRGIALELGKRGANVVVNYGSSS  60
Mg    1 ----MAPSADITSSGPSDASKPLAGKVALVTGAGRGIGRGIAIELGRRGASVVVNYGSSS  56
                     .    ......*.***.********** *.**..***.*.***.**

Bo   48 GPANEVCEIIRGFNNGAKAVAIKADVSKVSEIHRLFEEAKQAFGKINIVMSNSGTESWDK 107
Ch   48 GPANEVCEIIRGFNNGAKAVAIQADVSKVSEIHRLFEEAKQAFGKINIVMSNSGTESWDK 107
St   48 GPANEVCEIIRGFNNGAKAVAIKADVSKVSEIQRLFEEAKKAFGKINIVMSNSGTESWDK 107
Nc   48 GPANEVVKEIESLHNGARAIAIQADVRRVSEIDRLFAEAKRAFGRIDIVMSNSGTESWDK 107
Sm   48 GPANEVVKEIESYNNGARAKAIQADVRKVSEIDRLFAEAKQAFGRIDIVMSNSGTESWDK 107
Co   61 KAAEEVVAEIQKS--GAKAVALKADISKPLEVVDLFDRAVAHFGRMDFVISNSGKEVWCP 118
Mg   57 KAAEEVVAELKKL--GAQGVAIQADISKPSEVVALFDKAVSHFGGLDFVMSNSGMEVWCD 114
        ..*.**. ..   ..**...*..**.....*. .** .*. .** ...*.****.*.*..

Bo 108 TEEISEEKYDHVFNLNTRAQFFVGQTAYKYIEDNGRIILMSSIAAGLLGVKDHALYNASK 167
Ch 108 TEEISEEKYDHVFNLNTRAQFFVGQTAYKYIEDNGRIILMSSIAAGLLGVKDHALYNASK 167
St 108 TEEITEEKYDHVFNLNARAQFFVGQTAYKYIEDNGRIILMSSIAAGLLGVKDHALYNASK 167
Nc 108 TEDVTEEKYDYVFDLNAKAQFFVGQAAYKHLERNGRVILMTSIAAGLMGVRNHALYNASK 167
Sm 108 TEEITEEKYDYVFDLNAKAQFFVGQAAYKHLEQNGRVILMTSIAAGLMGVKNHALYNASK 167
Co 119 EEDVDEALFQNIFDLNCRGQFFVAQQGLKHCSRGGRILLTSSVAASMGGIPNHALYAGSK 178
Mg 115 ELEVTQELFDKVFNLNTRGQFFVAQQGLKHCRRGGRIILTSSIAAVMTGIPNHALYAGSK 174
        .......... .*.** ..****.* ..*. . .**..*..*.**.. *...****..**

Bo 168 MAVIGFIKAFATDFGKRGVTVNGVAPGGIKSDMFTQNAWHYIPGGTPEWPAEKIESLMAS 227
Ch 168 MAVIGFIKAFATDFGKRGVTVNGVAPGGIKSDMFTQNAWHYIPGGTPEWPAEKIESLMAS 227
St 168 MAVIGFIKAFATDFGKRGITVNGVAPGGIKSDMFTQNAWHYIPGGTPEWPAEKIESLMAS 227
Nc 168 MAVQGFVKAFATDFGDKGITVNGVAPGGIKSDMFAENAWHYIPGGTPDLGKDKIEKMMAE 227
Sm 168 MAVQGFVKAFATDFGDKGITVNGVAPGGIKSDMFAENAWHYIPGGTPDLGKDKIERMMAE 227
Co 179 AAVEGFTRAFAVDCGAKGITVNAIAPGGVKTDMYDENSWHYVPDGYKGMPIEIIDQGLAN 238
Mg 175 AAVEGFCRAFAVDCGAKGVTVNCIAPGGVKTDMFDENSWHYAPGGYKGMPQEKIDEGLAN 234
        .** ** .***.*.* .*.***..****.*.**. .*.***.*.*..  . ..*.  .*

Bo 228 HCPLGRCAVPEDVARVVAFLASEDGGWVNGQVLTISGGSSQ 268
Ch 228 HCPLGRCAVPEDVARVVAFLASEDGGWVNGQVLTISGGSSQ 268 (99.2%)
St 228 HCPLGRCAVPEDVARVVAFLASEDGGWVNGQVLTISGGSSQ 268 (97.3%)
Nc 228 HCPLGRCAVPEDVARVVAFLASEDGGWVNGQIITISGGSSQ 268 (76.8%)
Sm 228 HCPLGRCAVPEDVARVVAFLASEDGGWVNGQIITISGGSSQ 268 (79.1%)
Co 239 MNPLKRVGVPADIGKAVASLCHSDCEWINGQVIKVTGGGT- 278 (49.8%)
Mg 235 MNPLKRIGYPADIGRAVSALCQEESEWINGQVIKLTGGGI- 274 (53.2%)
        ..**.*...*.*....*..*......*.***.....**...
```

**Figure 3.** Comparison of the deduced amino acid sequence of the *B. oryzae T4HR1* (Bo) with those of other fungal 1,3,6,8-THN reductases. Ch: *Cochliobolus heterostrophus* (EF060260); St: *Setosphaeriaturcica* (EU494943); Nc: *Neurospora crassa* (Q7S3E8); Sm: *Sordaria macrospora* (AM493725); Co: *Colletotrichum. orbiculare* (AB661336); Mg: *Magnaporthe grisea* (AF290182). Perfectly conserved and well-conserved positions in the alignment are indicated by an asterisk (*) and a dot (.), respectively. Similarity (% identity) of the deduced amino acids sequence of the *B. oryzae T4HR1* (Bo) and those of other fungal 1,3,6,8-THN reductases are indicated in parentheses at the end of each amino acid sequence.

### 3.3. Disruption of the *T4HR1* Gene

We investigated the effects of disrupting *T4HR1* in *B. oryzae* using transformation-mediated gene disruption (**Figure 2(a)**) as described [29]-[31]. As a result, three transformants, named GD1, GD2 and GD3, were isolated and two transformants were selected to examine homologous integration by a PCR procedure (**Figure 2(b)**). The *T4HR1* fragment (0.8 kb) was amplified using the primer pair T4HRAF/T4HRBR in the wild-type but not in GD1 and GD2 transformants. In contrast, Fragment A (0.6 kb), *hph* (1.0 kb), and Fragment B (6.7 kb), all of which are involved in the *T4HR1* disruptant fragment, were amplified in the GD1 and GD2 transformants but not in the wild-type with the primer pairs T4HRAF/PtrpCR, hphF/hphR and TtrpCF/T4HRBR, respectively. These results suggested that *T4HR1* gene was deleted by the homologous integration of the *T4HR1* disruption fragment in the GD1 and GD2 transformants.

In our previous work, the disruption of each of the melanin biosynthesis genes *PKS1*, *SCD1*, and *THR1* resulted in an albino phenotype in *B. oryzae* [17]-[19]. However, the mycelial color on PDA medium was not different between the wild-type and the *T4HR1* disruptants (**Figure 4**). The DHN melanin biosynthesis pathway has two reduction steps: the conversion of T4HN to scytalone and the conversion of T3HN to vermelone (**Figure 1**). In *M. grisea* and *C. orbicurare*, it is known that T3HN reductase converts both reduction steps [21] [22]. Thus, the conversion of T4HN to scytalone could progress by T3HN reductase with or without T4HN reductase in *B. oryzae*, and the *T4HR1* disruptants could produce melanin as well as the wild-type. To confirm the deficiency of T4HN reductase activity in the *T4HR1* disruptants, we observed the mycelial color on PDA agar using tricyclazole as an inhibitor of T3HN reductase. A muddy orange color was observed in the wild-type, suggesting that shunt products, 2-hydroxyjuglone and DDN, could accumulate in the wild-type (**Figure 4**) due to the inhibition of the T3HN reductase activity (**Figure 1**). In contrast, a vivid orange color was observed in the *T4HR1* disruptants (**Figure 4**). These results suggested that the shunt products, flaviolin and 3,3-biflabiolin, could accumulate in the *T4HR1* disruptants, because the conversion of T4HN to scytalone was completely blocked due to the lack of T4HN reductase and the inhibition of T3HN reductase in the *T4HR1* disruptants. However, the elucidation of these shunt products must be addressed in future studies to confirm this speculation.

### 3.4. *T4HR1* Expression under NUV Irradiation

We examined the time-course of the expression of the *T4HR1* transcripts compared with those of *PKS1*, *SCD1*, and *THR1* transcripts under NUV irradiation by performing a quantitative real-time PCR. We found that the *T4HR1* transcription level increased with increasing NUV irradiation time, similar to the time-course of three other melanin biosynthesis genes, *PKS1*, *SCD1* and *THR1* (**Figure 5**). These results suggest that the expression of *T4HR*1 gene is enhanced by NUV irradiation, as is the expression of other melanin biosynthesis genes, *PKS1*, *SCD1* and *THR1*, and common mechanisms regulate the transcription of these melanin biosynthesis genes. In our previous work, we found that the *BMR1* gene encoding a transcriptional factor for melanin biosynthesis was essential for the transcription of three melanin biosynthesis genes, *PKS1*, *SCD1* and *THR1* [28]. Thus, in the present study we further examined whether *T4HR1* gene expression was affected in the *BMR1* disruptant (**Figure 6**). In the wild-type strain, *T4HR1* gene was expressed in darkness and was significantly enhanced in mycelia

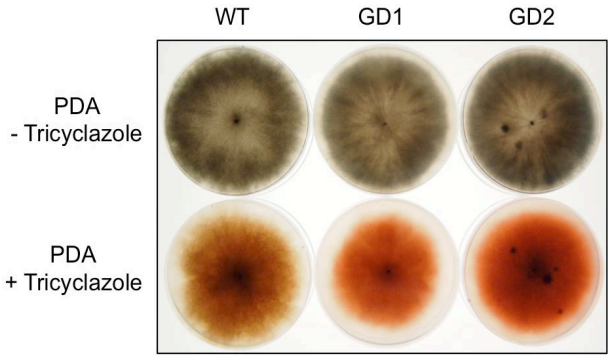


**Figure 4.** Growth of the wild-type (WT) and *T4HR1* disruptants (GD1 and GD2) on a PDA plate with (+) or without (−) tricyclazole (100 μM).

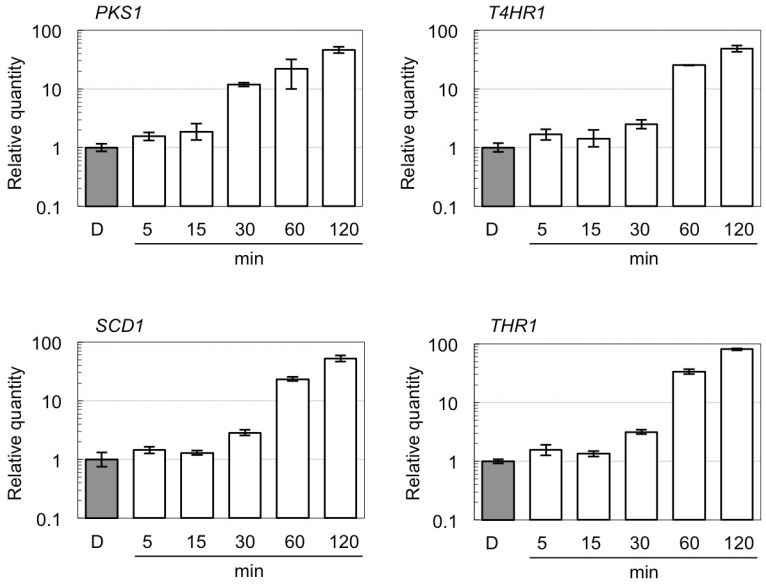


**Figure 5.** Time-course expression of the melanin biosynthesis genes under NUV irradiation. Mycelia grown for 4 days in darkness were transferred under NUV irradiation and harvested at the time indicated. The primers used in this experiment were as follows: *PKS1*: PKS7001F/PKS7196R; *T4HR1*: T4HRF/T4HRR; *SCD1*: SCD69F/SCD188R; *THR1*: THR481F/THR685R; *GPD* (for internal control): GPD161F/GPD241R. The sequences of these primers are listed in **Table 1**. The expression was normalized by dividing the average value of *GPD* as the internal control. The relative changes in each mRNA expression were determined as fold changes relative to a dark control (=1). Vertical bars: std. dev.

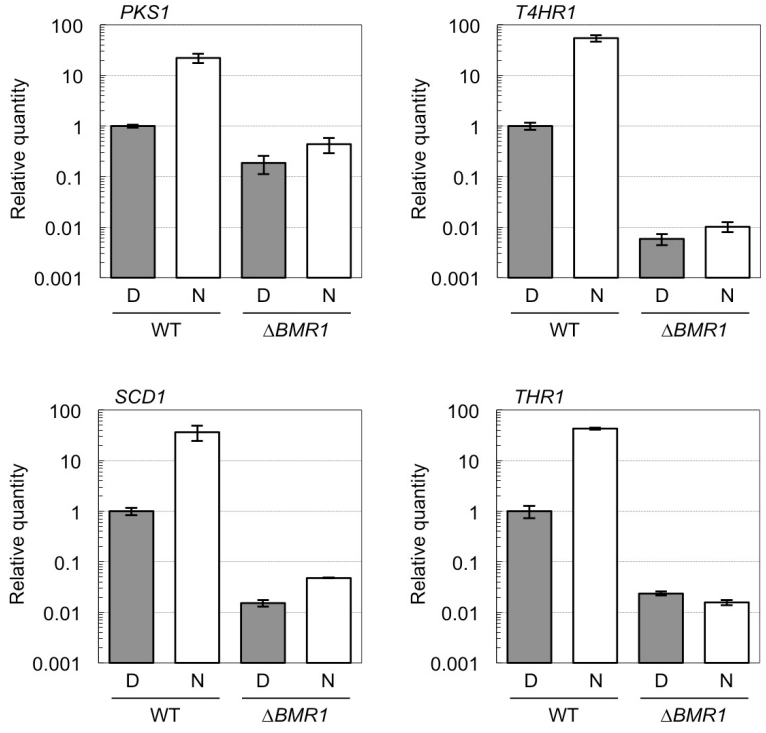


**Figure 6.** Expression of the melanin biosynthesis genes in the wild-type (WT) and *BMR1* disruptant (Δ*BMR1*). Mycelia grown for 4 days in darkness (dark) were exposed to NUV irradiation for 1 h (NUV), and total RNA was extracted. The primers used in this experiment were: *PKS1*: PKS7001F/PKS7196R; *T4HR1*: T4HRF/T4HRR; *SCD1*: SCD69F/SCD188R; *THR1*: THR481F/THR685R; *GPD* (for an internal control): GPD161F/GPD241R. The sequences of these primers are listed in **Table 1**. The expression was normalized by dividing the average value of *GPD* as the internal control. The relative changes in each mRNA expression were determined as fold changes relative to a dark control (=1). Vertical bars: std. dev.

exposed to NUV irradiation. In the *BMR1* disruptant, the expression level of *T4HR1* was reduced by approx. 100-fold compared to that of the wild-type in the dark (**Figure 5**). In addition, enhanced gene expression of *T4HR1* by NUV irradiation was not observed in the *BMR1* disruptant. These expression patterns were similar to those of the other three melanin biosynthesis genes, *PKS1*, *SCD1* and *THR1* (**Figure 6**). These results suggest that the transcriptional factor *BMR1* could be essential for constitutive expression in the dark and for the NUV irradiation-enhanced expression of *T4HR1* as well as the other melanin biosynthesis genes *PKS*1, *SCD*1 and *THR1*. We previously observed that the expression of the *BMR1* transcripts was also enhanced in mycelia exposed to NUV irradiation and over expression of the *BMR1* gene enhanced melanin biosynthesis under dark conditions through high expression levels of the melanin biosynthesis genes [28]. Thus, the NUV irradiation-enhanced *BMR1* expression would lead to an accumulation of the transcriptional factor *BMR1*, resulting in enhanced expression of the *PKS1*, *T4HR1*, *SCD1* and *THR1* transcripts by NUV irradiation.

We previously isolated and characterized the *Blue-light regulator* 1 (*BLR1*) gene, which encodes a putative blue/UVA-absorbing photoreceptor similar to white collar 1 (WC-1) of *Neurospora crassa* in *B. oryzae* [34]. In addition, it suggested that the *BLR1* protein was necessary not only for conidial formation but also for NUV radiation-enhanced photolyase gene expression in *B. oryzae* [34]. Enhanced gene expression of *T4HR1* by NUV irradiation was also observed in the *BLR1* disruptant [27], suggesting that the expression of melanin biosynthesis genes could be regulated by another putative NUV-absorbing photoreceptor which was distinct from the regulation by *BLR1* as a blue/UVA-absorbing photoreceptor.

## 4. Conclusion

We identified the 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) reductase gene in the melanin biosynthesis pathway of *B. oryzae*. The expression of *T4HR1* transcripts was enhanced by near-ultraviolet irradiation and regulated by the transcriptional factor *BMR1*, similar to three other melanin biosynthesis genes, suggesting that common transcriptional mechanisms could regulate the enhanced gene expression of these melanin biosynthesis genes by NUV irradiation in *B. oryzae*. The remaining melanin biosynthesis-related genes, such as an oxidase gene presumably involved in the oxidative polymerization of DHN, should be identified and characterized in order to completely elucidate the melanin biosynthesis pathway of *B. oryzae*. Studies of the transcriptional regulation of melanin biosynthesis genes by NUV irradiation could contribute to the elucidation of the molecular basis of the photomorphogenesis by NUV irradiation in *B. oryzae*.

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