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Early and Late Trisporoids Differentially Regulate β-Carotene Production and Gene Transcript Levels in the Mucoralean Fungi *Blakeslea trispora* and *Mucor mucedo*

Yamuna Sahadevan,[a] Mareike Richter-Fecken,[a] Kerstin Kaerger,[a,b,c] Kerstin Voigt,[b,c] Wilhelm Boland[a]*

Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena, Germany[a]; Jena Microbial Resource Collection, Hans-Knoell Institute, Jena, Germany[b]; Department of Microbiology and Molecular Biology, Institute of Microbiology, Faculty of Biology and Pharmacy, University of Jena, Jena, Germany[c]

The multistep cleavage of carotenoids in *Mucorales* during the sexual phase results in a cocktail of trisporic acid (C_{18}) sex pheromones. We hypothesized that the C_{18} trisporoid intermediates have a specific regulatory function for sex pheromone production and carotenogenesis that varies with genus/species and vegetative and sexual phases of their life cycles. Real-time quantitative PCR kinetics determined for *Blakeslea trispora* displayed a very high transcript turnover in the gene for carotenoid cleavage dioxygenase, *tsp3*, during the sexual phase. An *in vivo* enzyme assay and chromatographic analysis led to the identification of β-apo-12′-carotenal as the first apocarotenoid involved in trisporic acid biosynthesis in *B. trispora*. Supplementation of C_{18} trisporoids, namely D'orenone, methyl trisporate C, and trisporin C, increased *tsp3* transcripts in the plus compared to minus partners. Interestingly, the *tsp1* gene, which is involved in trisporic acid biosynthesis, was downregulated compared to *tsp3* irrespective of asexual or sexual phase. Only the minus partners of both *B. trispora* and *Mucor mucedo* had enhanced β-carotene production after treatment with C_{20} apocarotenoids, 15 different trisporoids, and their analogues. We conclude that the apocarotenoids and trisporoids influence gene transcription and metabolite production, depending upon the fungal strain, corresponding genus, and developmental phase, representing a "chemical dialect" during sexual communication.

Mucorales fungi belonging to the subphylum *incertae sedis Mucoromycotina* (formerly classified in the class *Zygomycetes*) comprise 9 families, 51 genera, and around 205 species (1). These basal fungi are fast-growing soil saprotrophs that feed on dead and decaying organic matter. The asexual phase predominates their life cycle, with multinucleated haploid sporangiospores that germinate to mycelia of either the plus or minus mating type in heterothallic strains. Adversities, like environmental stresses or nutrient depletion, especially of nitrogen and phosphorus (2), lead to the sexual phase, in which complementary mating partners in close proximity exchange metabolites and form special aerial hyphae known as zygophores. The fusion of zygophores results in thousands of nuclei from both parents in the morphologically modified structure known as the progametangia. During further structural modifications and dormancy, which may extend from months to a year, most of the nuclei undergo degradation. Finally, gametangia bearing two nuclei from opposite partners fuse to form a thick-walled dikaryotic sexual spore known as the zygospore. Mitosis followed by meiosis results in four haploid products in a sporangium that develops from the zygospore (3). One *Mucorales* species in particular, *Blakeslea trispora*, has been commercially exploited for its potential to produce an excess of carotenoids, like β-carotene and lycopene, during its sexual phase of the life cycle (4, 5). According to a new report by Global Industrial Analysts, Inc., the worldwide market for carotenoids is projected to reach $1.3 billion by 2017. Hence, microbial carotenoids have been in the limelight as an ecofriendly sustainable alternative source for synthetics. Mucoralean carotenoids are not only under investigation for their biotechnological applications but also to help in understanding how they influence the sexual phase in those fungi.

Apocarotenoids are the unsaturated nonpolar isoprenoids formed by the oxidative cleavage of carotenoids (6). The sex hormone trisporic acid and its trisporoid precursors are C_{18} apocarotenoids that regulate the sexual phase in plus and minus mating partners among heterothallic species, such as *Mucor mucedo*, *B. trispora*, *Mucor circinelloides*, and *Phycomyces blakesleeanus* (7–9). They were first discovered as stimulants of carotenogenesis-inducing positive feedback regulation, rather than as sex hormones. Both carotenoids and trisporoids were detected in quantifiable amounts during the sexual phase under dark conditions in *M. mucedo* and *B. trispora*, while *P. blakesleeanus* induced carotenogenesis upon exposure to blue light (10). However, trisporic acids extracted from *B. trispora* mated cultures grown in the dark exhibited reduced carotenogenic activity on exposure to light (11). Hence, we chose *B. trispora* and *M. mucedo*, which prefers darkness for enhanced carotene production, as the best-suited organisms for studies on the carotenoid catabolic pathway among *Mucorales* fungi.

In fungi, carotenogenesis takes place via the mevalonate pathway. Phytoene synthase and lycopene cyclase (CarRA), along with phytoene dehydrogenase (CarB), convert the first colorless linear C_{40} carotenoid, phytoene (Fig. 1), to yellow-pigmented β-carotene (12, 13). Little is known about the physiological functions of

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Address correspondence to Wilhelm Boland, boland@ice.mpg.de.
* Present address: Wilhelm Boland, Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena, Germany.
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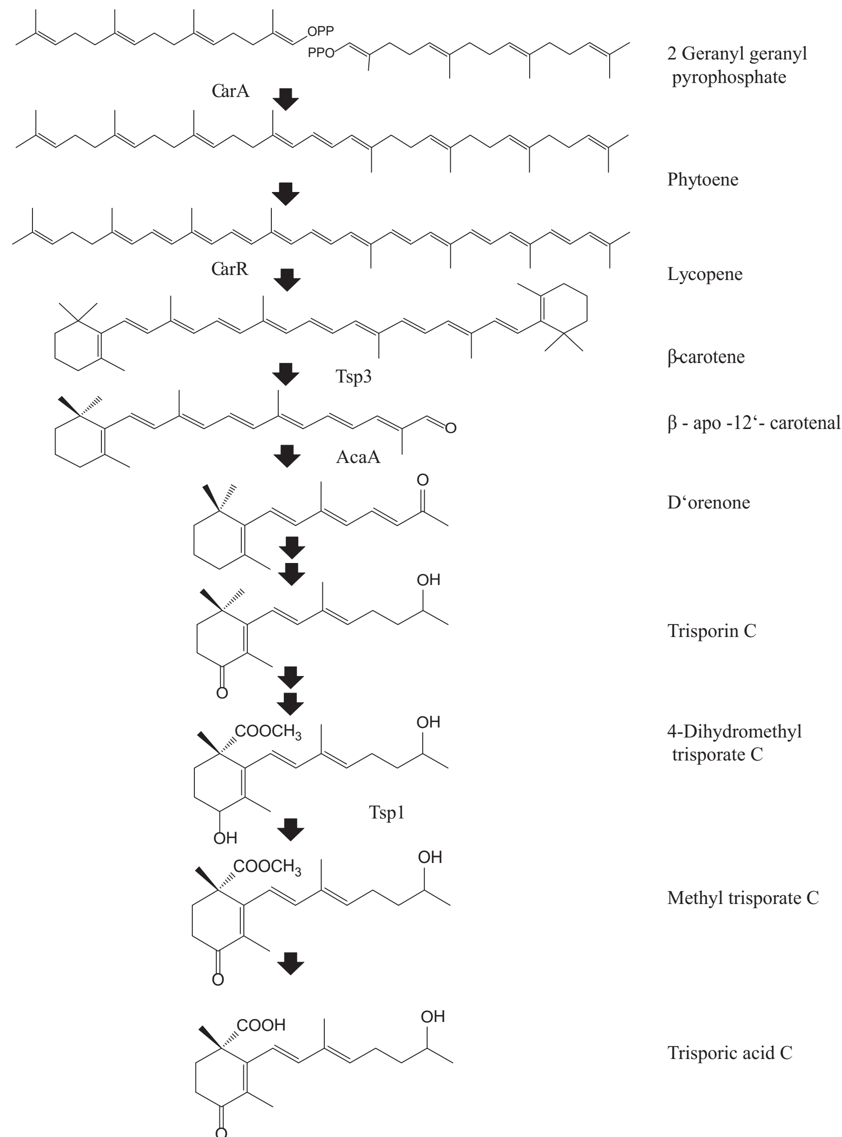

FIG 1 Schematic diagram of the putative biosynthetic pathway and genes encoding respective proteins involved in β-carotene metabolism. The enzymes depicted left of the arrows lead to β-carotene biosynthesis, while those to the right of the arrows are involved in trisporic acid biogenesis. CarRA is a bifunctional enzyme having 2 domains, CarR and CarA, that lead to biogenesis of β-carotene. The first apocarotenoid formed by carotenoid cleavage oxygenase (TSP3) is further cleaved down to C_{18} trisporoid compounds. A double black arrow indicates more than one step is involved in formation of the subsequent metabolite.

enzymes or the products of β-carotene cleavage and trisporic acid biogenesis within the *Mucorales*. The hypothetical biosynthetic pathway in *P. blakesleeanus* commences with oxidative cleavage of β-carotene at the $C_{11'}$—$C_{12'}$ as the caroteneoid cleavage dioxygenase (CarS) releases β-apo-12′-carotenal (C_{25}), which further cleaves to the first C_{18} trisporoid (β-apo-13-carotenone) D'orenone at the C_{13}—C_{14} bond by apocarotenoid cleavage oxygenase (AcaA) (14). CarS mutants in *Phycomyces* fail to produce those apocarotenoids present in the wild type (15). No functional characterization has been reported for the CarS homologue in *Blakeslea trispora*, designated TSP3 (16). All of the C_{18} apocarotenoids (Fig. 1) from D'orenone (β-apo-13-carotenone) to methyl trisporate in the trisporic acid biosynthetic pathway are known as trisporoids (17–19). Few enzymes are known so far, such as 4-dihydromethyltrisporate dehydrogenase (TSP1), 4-dihydrotrisporin dehydrogenase (TSP2) (20–22), and a putative esterase enzyme from the minus partner that converts methyl trisporate to trisporic acids in both homothallic *Mucorales* (*Zygorhynchus moelleri*) and heterothallic *Mucorales* (23). The high-mobility group (HMG) transcription factor genes *sexM* and *sexP* are associated with the minus and plus mating-type loci in *P. blakesleeanus* and *M. mucedo* (24, 25). Technically, it is difficult to carry out functional gene analyses via classical genetic approaches in either *B. trispora* or *M. mucedo* without sequenced genome data. Moreover, these two organisms possess multinucleated spores, and no reports are available on successful generation of mitotically stable transformants.

The collaborative biogenesis of trisporic acids and induction of carotenogenesis during the sexual phase takes place by the exchange of trisporoids between complementary mating partners.

The role of individual trisporoids as stimulators of the feedback loop are not known. Hence, we explored biochemically how the synthetic intermediates and natural trisporoids differentially modulate β-carotene production in plus and minus mating types among *M. mucedo* (*Mucoraceae*) and *B. trispora* (*Choanephoraceae*), which belong to 2 different families (26). Functional characterization of the carotenoid cleavage dioxygenase (TSP3) was performed to identify the first apocarotenoid product formed in *B. trispora*. We conducted time series transcriptional analyses of *carRA*, *tsp3*, and *tsp1* in *B. trispora*, with these genes treated independently with 3 trisporoids in plus and minus partners. The data were then compared to those for untreated cultures, which naturally produce trisporoids, to better understand the chemical dialect and synergy of pheromones involved in sexual communication.

MATERIALS AND METHODS

Strains and culture conditions. FSU 331 (+) and FSU 332 (−) were the strains of *B. trispora* used, while FSU 621 (+) and FSU 620 (−) strains of *M. mucedo* were obtained from the Jena Microbial Resource Collection (JMRC) at the Friedrich Schiller University and Hans Knoell Institute. Preinoculum cultures were prepared on solid agar plates (9 mm) of induction medium (IM) (27) by plating a single disc of fungal mycelium (1-mm diameter) and growing the individual strains for 80 h. Spores were collected in distilled water and counted by using a hemocytometer. Carotene induction assays were carried out with 10^3 spores ml^{-1} on solid IM agar plates for both species. For transcript analysis, *Blakeslea* strains of each mating type were inoculated with 10^8 spores ml^{-1} for faster growth in 50 ml IM, at 23 to 24°C, in the dark, with a shaker speed of 220 rpm for 144 h. For mated culture experiments, a 1:1 ratio of spore inocula was used.

Carotene induction assay. Three independent solid agar cultures of each of the mating types of *B. trispora* and *M. mucedo* were treated with 20 different stimulants suspended in 200 ml $liter^{-1}$ ethanol by using an aerosol spray flask. Three solvent control cultures treated with ethanol were maintained for each strain. The cultures were further incubated for 44 h at 22°C in the dark before extraction. The fresh weight of the biomass was determined, and samples treated with 300 μl of chloroform were sonicated for 10 min at 38°C. The chloroform phase was then completely removed, and 20 μl per sample was analyzed by reverse-phase high-performance liquid chromatography (HPLC; pump 525, autosampler 560, diode array detector [DAD] 440; software from Kroma System 2000 and Kontron Instruments). Separation was carried out using a CC 250/4 Nucleosil 120-5 C_{18} column (Macherey-Nagel, Düren, Germany) with acetone-water (70:30) at a flow rate of 1 ml min^{-1} at 22°C. The elution profile was monitored with a DAD 440 (Kontron Systems) with an absorbance maximum for β-carotene in acetone of 453 nm. Concentrations were calculated based on calibration against a β-carotene standard. The recovery of β-carotene was determined from mycelial extracts to which known amounts of β-carotene (Sigma) and β-apo-8′-carotenal (Sigma) as the internal standard had been added before extraction. The average recovery was approximately 90% of the initial amount added. The procedure was carried out under dim light.

cDNA cloning. The *in vivo* carotenoid cleavage assays were carried out in *Escherichia coli* strain JM109, as it possesses a stable genotype pβ-carotene plasmid engineered with a gene cluster of 4 enzymes of *Erwinia carotovora*, i.e., CrtB (geranyl geranyl diphosphate), CrtE (phytoene synthase), CrtI (phytoene desaturase), and CrtY (lycopene cyclase) (28–30). The pβ-carotene plasmid is a pACYC177 low-copy-number plasmid with constitutive expression and includes a kanamycin resistance marker (14, 31). The cDNA of *B. trispora* was amplified with the full-length *tsp3* primers (see Table S1 in the supplemental material) by using Accuprime *Taq* polymerase (Invitrogen) and subjected to 3′A-overhang addition on postamplified product by following the manufacturer's instructions. The product was further ligated into the pBAD/Topo Thio vector (Invitrogen), which has an ampicillin resistance marker. The integrity of the product was verified by sequencing.

***In vivo* enzyme assay.** The pβ-carotene plasmid was cotransformed with pBAD-TSP3 in *E. coli* JM109 chemically competent cells. Overnight cultures were grown at 28°C with a shaker speed of 250 rpm in Luria-Bertani (LB) broth and induced with 0.08%, 0.2%, or 2% arabinose (Sigma) at an optical density at 600 nm of 0.5. Samples were collected at hour 0, 4, 16, and 24 after arabinose induction. A positive control without the pβ-carotene plasmid and with 2% arabinose induction and a negative control without TSP3 were maintained along with a no-arabinose control to check for leaky expression (see Fig. S2 in the supplemental material). Cell pellets were obtained by centrifugation at 2,599 × *g* (rotor radius, 92.79 mm) at 4°C for 20 min and resuspended in HPLC-grade acetone. After centrifugation, the supernatant was dried and dissolved in the HPLC solvent mixture, followed by HPLC and LC-mass spectrometry (MS) analysis.

Chromatography and mass spectrometry. HPLC was conducted on an HP1100 system equipped with a photodiode array detector and an automatic sample injector. The separations were carried out by using a Bischoff C_{30} reverse-phase column (250 mm by 4.6 mm by 3 μm) with methanol (A) and methyl tertiary butyl ether (B) as the solvents (32). The column was developed at a flow rate of 1 ml/min with 20% B initially for up to 5 min. A gradient was maintained within 5 min to 90% B, with a 1-min hold time, and then switched to the initial 20% B until the end of the run time. The standard compound β-carotene was purchased from Sigma-Aldrich (Seelze, Germany), and β-apo-12′-carotenal was obtained from Carotenature (Lupsingen, Switzerland). Both standards and samples were treated with the same solvent mixture with an injection volume of 15 μl and monitored at three wavelengths, 420, 450, and 461.4 nm. The chromatographic spectra were acquired by using the Chemstation software package. Mass spectrometry was carried out with an LCQ mass spectrometer with an APCI interface (Finnigan MAT, Bremen, Germany). The capillary temperature was set at 160°C, and the vaporizer temperature was 450°C.

Transcript analysis. Three independent liquid culture experiments with plus, minus, and mated (+/−) cultures of *Blakeslea trispora* were carried out for the transcript analysis of genes involved in carotenoid metabolism. RNA was extracted from about 100 mg of the stored frozen sample by using TRIzol reagent (Invitrogen). Genomic DNA contamination was avoided by including a Turbo DNase treatment (Ambion). The quality and integrity of RNA samples were ensured by including a formaldehyde–Tris-acetate-EDTA (TAE) agarose gel run prior to cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen) (33, 34). The target genes were *carRA* (phytoene synthase and lycopene cyclase), the carotenoid cleavage gene *tsp3* (carotenoid cleavage oxygenase), and *tsp1* (4-dihydromethyltrisporate dehydrogenase). Among the four housekeeping genes, *gpd* (glyceraldehyde phosphate dehydrogenase), *EF-1*α (α subunit of translation elongation factor 1), *pyrG* (orotidine-5′-monophosphate decarboxylase), and *act-1* (actin) were selected for normalization and relative quantification of data. *act-1* had the most stable gene expression and hence was chosen as the internal standard for data analysis, based on the *Minimum Information for Publication of Quantitative Real-Time PCR Experiments* guidelines (35). Moreover, we performed transcript analysis of *carRA* in mated cultures of *B. trispora* to choose the optimal growth phase as the basal time point for determining the relative fold change in gene expression (data not shown) (36). Primers with amplicon sizes ranging between 91 and 165 bp were designed using Primer-BLAST software from NCBI. Vector NTI software was used to select the best available primer pair with the least number of potential secondary structures in the amplicon and with a GC content of 46 to 70%. In order to estimate PCR efficiency, all selected primers (synthesized by Eurofins [Ebersberg, Germany]) were run with pooled samples in 5-fold dilution series over five points (data not shown) (37). Built-in software from Stratagene (Mx 3000P) was used to construct a standard curve for each primer

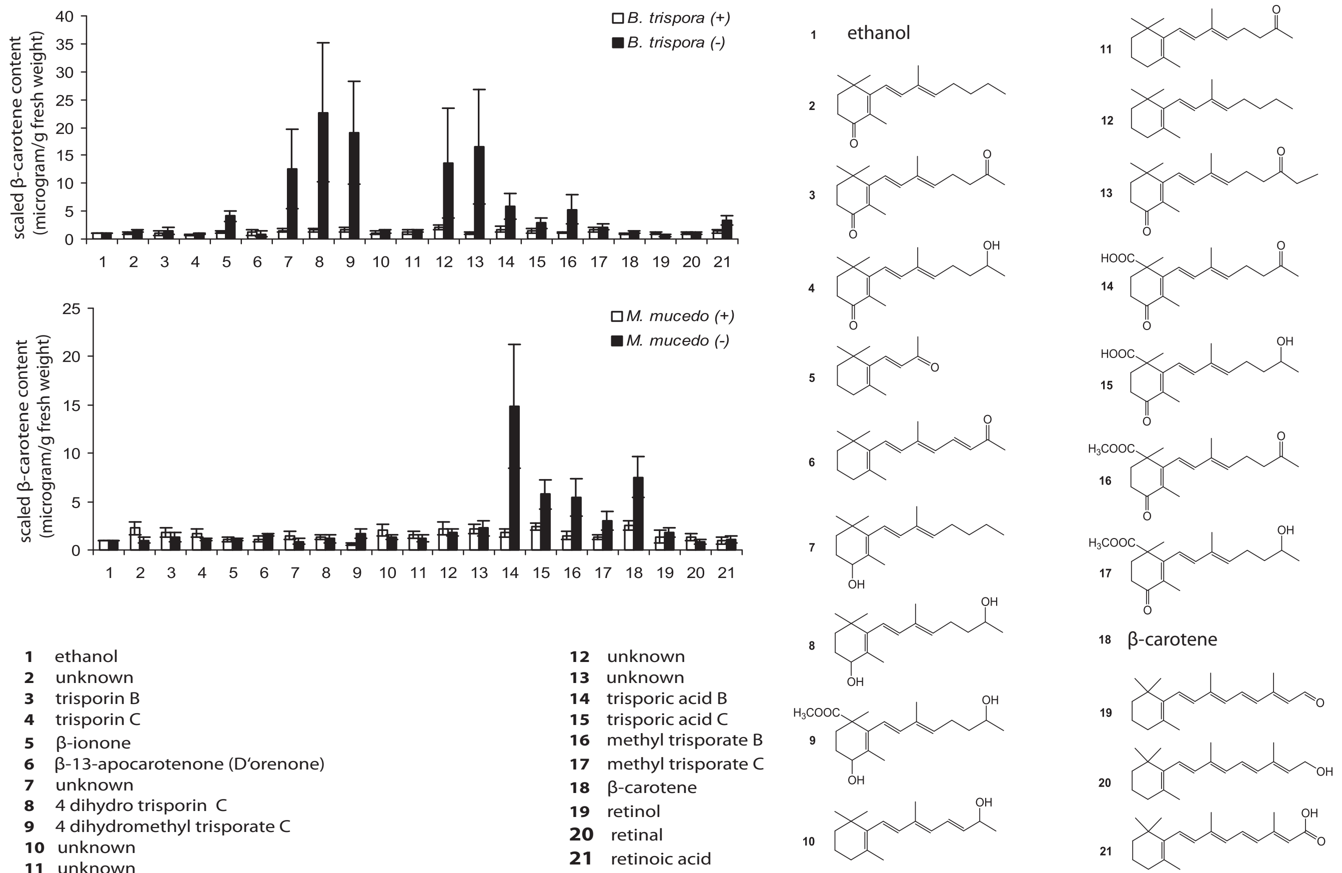

FIG 2 β-Carotene production in *B. trispora* and *M. mucedo* treated with (14 μg/plate) apocarotenoids and trisporoids. Graphs were plotted based on mean values from 3 replicates, and error bars indicate standard errors of the means. Among the 21 stimulants, the compounds that are synthetics and have never been reported as natural products were not given names.

pair, and the efficiency was determined. Quantitative PCR (qPCR) assays were always carried out using three technical replicates for each of the three independent biological replicates, and a no-reverse transcriptase control (NRT) was included for every sample along with a no-template control (NTC) for every primer pair used in each run, using the Brilliant II SYBR green qPCR kit (Agilent). A high annealing temperature of 60°C with 35 cycles was optimized as the run condition in order to minimize nonspecific binding and competition for the substrate. The details for the primers used for the real-time analysis are provided in Table S1 of the supplemental material.

RESULTS

Trisporoids and probable intermediates (synthetics) differentially influence β-carotene production in *B. trispora* and *M. mucedo*. The individual mating partners of *B. trispora* secrete trisporoids along with a mixture of trisporic acids that stimulate carotenogenesis with the minus partner and hardly influence the plus partner (38). This finding motivated us to perform carotene induction assays using individual apocarotenoids on heterothallic *B. trispora* and *M. mucedo* to assess whether the "chemical dialect" enforced by those metabolites are unique among species that seem to have similar mechanisms of trisporoid synthesis in the dark.

Among the 21 test compounds, including a control solvent of ethanol (compound 1), trisporic acid B (TSAB) (compound 14) (Fig 2) was the most active inducer of carotenogenesis in *M. mucedo* (−), even at the low concentration of 14 μg ml^{-1} in solid agar plates of fungal cultures (data not shown). Therefore, preincubated (for 90 h) individual mating partners were treated with the stimulants at 14 μg ml^{-1} per plate, incubated for an additional 44 h, and extracted to quantitatively analyze β-carotene production. All of those compounds enlisted, compounds 2, 7, 10, 11, 12, and 13, were synthesized in our laboratory (39). We presumed they are the probable trisporoid intermediates, based on our synthetic approach, as none of them had yet been isolated or reported as a natural product from any mucoralean members, suggesting their low abundance, instability, or quick biotransformations to sex hormones. Hence, supplementing those synthetic trisporoids at an arbitrary concentration was the only choice to gain an understanding of their impact on physiological traits in fungi. Likewise, trisporin B (compound 3) and C (compound 4), β-apo-13-carotenone (D'orenone) (compound 6), and compound 11 had no significant impact on carotene production in the investigated strains. Meanwhile, chain elongation by one carbon generated the synthetic analogue (compound 13) of trisporin B (compound 3) as an efficient enhancer of carotenogenesis in *B. trispora* (−). Unknown compounds 7, 12, and 13, 4-dihydrotrisporin C (compound 8), and its methyl derivative, 4-dihydromethyl trisporic acid C (compound 9), enhanced the metabolite level only in *B. trispora* (−).

Trisporic acid B (compound 14) made a stronger impact than trisporic acid C (TSAC) (compound 15) on β-carotene produc-

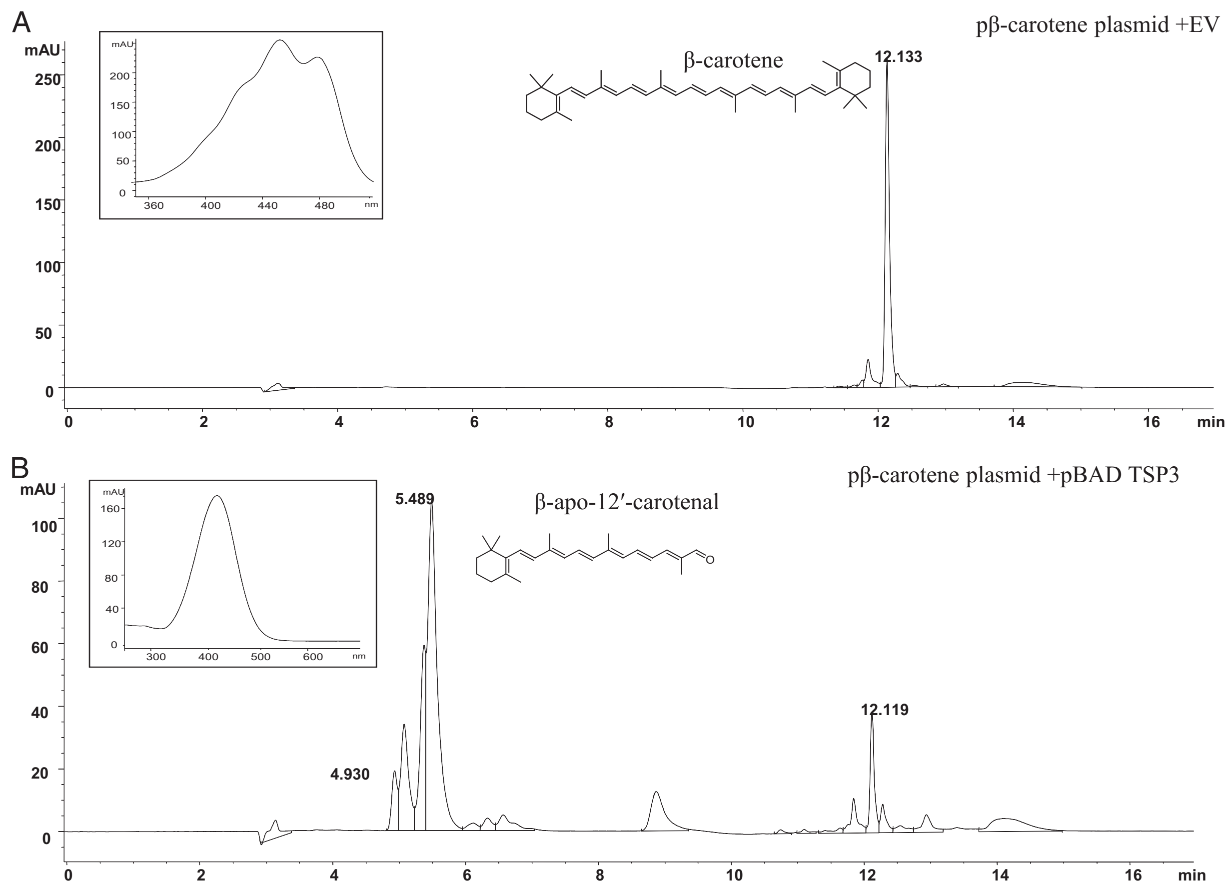

FIG 3 HPLC chromatogram from the *in vivo* enzyme assay with TSP3 coexpressed along with the β-carotene-producing plasmid after 24 h and the EV-empty pBAD vector. The inset show the UV-visible spectra for β-carotene substrate (A) and β-apo-12′-carotenal (B).

tion in minus partners of *M. mucedo* and *B. trispora*. The plus mating partners did not respond to the methyl esters (compounds 16 and 17) in either *Mucor* or *Blakeslea*, respectively, while the carotenoid levels were enhanced in the minus mating partners of both organisms. Interestingly, in *Mucor* the effects of the free acid TSAC (compound 15) on carotene production were comparable to those of methyl TSAB (compound 16). Externally added β-carotene (compound 18) had no effect on *B. trispora* but stimulated carotenogenesis in *M. mucedo* (−). The C_{20} apocarotenoids, namely, retinal (compound 19), retinol (compound 20), and retinoic acid (compound 21), had no impact on carotenogenesis in either *Mucorales* species. This finding correlated with the results of supplementation experiments in *Mucor rouxii* in which retinal and retinol were used (40). β-Ionone (compound 5) triggered a weak stimulation of carotenogenesis only for *B. trispora* (−) and had no influence on *M. mucedo*.

β-apo-12′-Carotenal (C_{25}) as the first apocarotenoid cleavage product in *B. trispora*. *In vivo* enzyme assays were carried out in *E. coli* cells with the β-carotene-overproducing pβ-carotene plasmid coexpressed with pBAD-TSP3. Among the coexpression *E. coli* broth cultures, 0.08% and 0.2% arabinose induction indicated notable bleaching effects in the pelleted cells (see Fig. S1 in the supplemental material). LC-MS analysis of cells harvested and resuspended in acetone at 0 and 4 h did not indicate the presence of β-carotene or any other apocarotenoid, while after 24 h, the acetone extracts of pelleted cells showed the presence of the C_{25} compound β-12′-apocarotenal, along with the initial substrate, β-carotene, by HPLC (Fig. 3). The maximum absorption wavelength (λ_{max}) was 422 nm for β-apo-12′-carotenal and 450 nm for β-carotene. The compounds were identified using authentic standards. β-apo-12′-Carotenal showed the characteristic UV absorption and the expected molecular ion (M^+H^+) at *m/z* 351.10 (see Fig. S2 in the supplemental material).

Time series analyses of carotenoid metabolic gene expression in *Blakeslea trispora*. Unlike *M. mucedo*, *B. trispora* produces 1,000 times more trisporic acids, and a putative nonheme carotenoid cleavage oxygenase (TSP3) was reported as the first enzyme involved in sex hormone synthesis (16). Hence, we performed transcript analysis in *B. trispora* for *carRA*, *tsp3*, and *tsp1*, genes involved in three different stages of carotenoid metabolism, and to identify how the developmental phases induce temporal trends in gene expression. The fold change in target gene expression normalized to that of the internal standard, β-actin, relative to expression at time zero (12 h after incubation) was carried out for the time series analysis for up to 144 h with a 24-h interval, as per the $2^{-\Delta CT}$ method (41). *carRA* (Fig. 4A) was significantly upregulated in *B. trispora* (−), with a 160-fold increase ($P < 0.001$) after 144 h of incubation, while low and constitutive transcript levels up to 25-fold higher ($P = 0.002$) were observed with the plus strain. There was no statistically significant difference in *carRA*

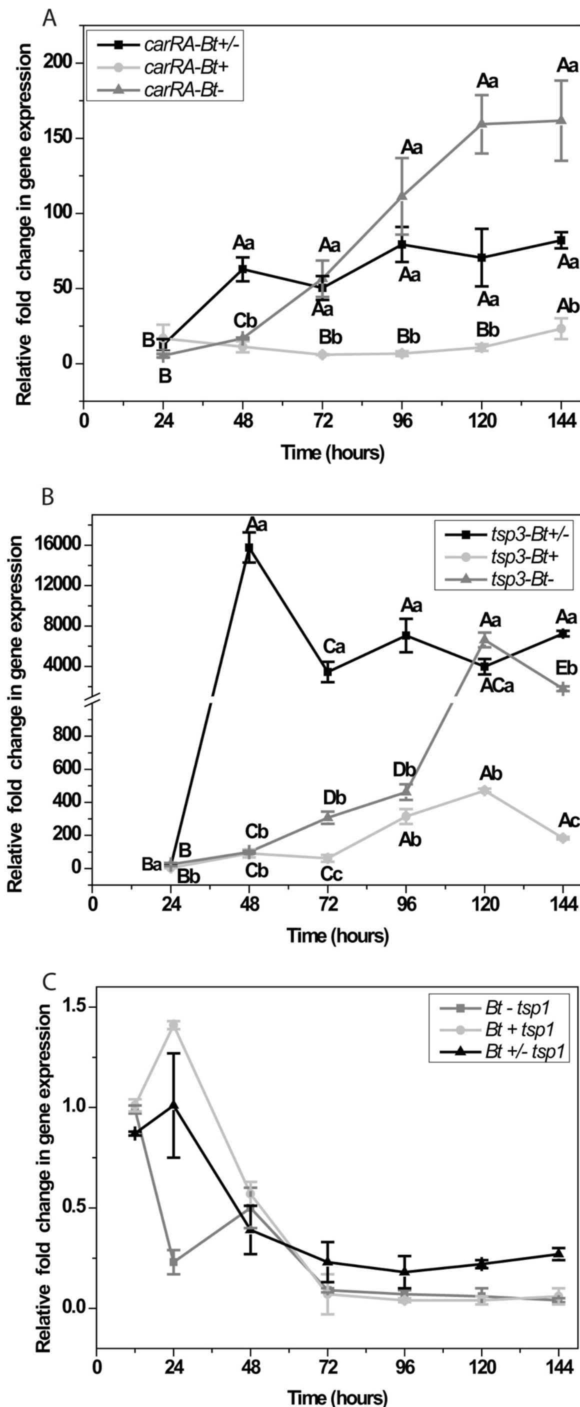

FIG 4 Real-time qRT-PCR analyses of the dynamics of *carRA* (A), *tsp3* (B), and *tsp1* (C) in *Blakeslea trispora* plus (Bt+), minus (Bt−), and mated (Bt+/−) cultures. The $2^{-\Delta CT}$ method was used for the relative quantification of gene expression, using actin as the internal standard. Graphs are plotted with the mean values ± standard errors of the means from 3 biological replicates. Statistical analysis was conducted by using a 2-way ANOVA with 2 independent factors, time and mating type, along with the dependent variable, the log-transformed (log 10) values of the relative fold change. The small letters indicate significant differences ($P < 0.05$) based on Tukey's test (*post hoc*) at each time point among the 3 mating types. Capital letters indicate significant differences ($P < 0.05$) between different time points for a single mating type. No significant difference was observed in *tsp1*, irrespective of time and mating type.

transcript levels beyond 48 h of growth in mated (+/−) or minus cultures. The expression of *tsp3* was 16,000-fold higher at 48 h in (+/−) cultures and declined to 7,000-fold after 144 h of incubation (Fig. 4B). In plus cultures, the gene *tsp3* was constitutively upregulated for up to 72 h and showed a progressive increase, with a maximum 400-fold increase at 120 h, declining to 200-fold at 144 h. A statistically significant difference was observed for the 6 time points under investigation and among the 3 culture types, i.e., plus, minus, and mated cultures, based on their 3 independent biological replicates in *tsp3* transcripts ($n = 18$; $P = 0.028$). Curiously, the mRNA level of *tsp1*, the penultimate enzyme proposed in trisporic acid biogenesis, exhibited downregulation irrespective of asexual or sexual phase in *B. trispora* (Fig. 4C). Our results indicated that *carRA* and *tsp3* transcripts vary during the asexual and sexual developmental phases of fungal growth in submerged liquid induction medium, based on their diverse physiological functions.

Individual trisporoids differentially regulate gene expression in *B. trispora* mating partners. Until now, all research on biological functions of trisporic acids and trisporins was limited either to carotenogenesis or the potential for the development of sexual structures known as zygophores in *M. mucedo* or *P. blakesleeanus*. We speculated that the prominent fluctuations of *tsp3* transcripts in *B. trispora* during the sexual phase imply the synergistic effects of *de novo* trisporoids regulating a positive feedback metabolic loop (Fig. 4B). In order to understand the genetic potential of individual trisporoids as sexual stimulants, real-time PCR transcript analysis of *carRA* and *tsp3* were conducted in plus and minus strains after treating them with methyl trisporate C (compound 17), D'orenone (compound 6), and trisporin C (compound 4), which are formed at early and late stages of trisporic acid biogenesis (Fig. 5). The trisporoids were treated at 12 h after spore incubation, and temporal trends for the relative fold change in gene expression were determined for up to 60 h of incubation as mentioned above, but here the time interval of sample collection was shortened to 12 h.

Ethanol, methanol, and isopropanol are active solvent stimulants of microbial carotenogenesis (4). Therefore, the sparingly water-soluble trisporoids were used in acetone solution (50 μM, final concentration) on both mating partners grown independently in 50 ml liquid induction medium at 12 h after incubation. The three tested trisporoids induced statistically significant differences in transcripts (Fig. 5A and C) at each of the 4 given time points in the plus partners ($P < 0.001$; $n = 4 \times 3$, or 12), whereas in minus partners, transcript levels differed significantly among the three treatments at 24 h ($P = 0.002$), 48 h ($P = 0.001$), and 60 h ($P = 0.007$). *carRA* gene expression varied with each of the trisporoid treatments among the 4 time points ($n = 4 \times 3$, or 12) with D'orenone ($P = 0.016$), methyl trisporate C (MTSPC; $P < 0.001$), trisporin C (TSPC; $P = 0.043$) in minus strains and D'orenone ($P < 0.001$), MTSPC ($P = 0.003$), and TSPC ($P = 0.033$) in plus strains (Fig. 5A and B). However, MTSPC and D'orenone imposed unique temporal trends (Fig. 5B) of differential expression in minus strains. The first C_{18} trisporoid, D'orenone, and methyl trisporate C induced carotene cleavage in the plus mating type (Fig. 5C) but had no impact on carotenogenesis (Fig. 2). The impact of trisporins (compounds 3 and 4) were negligible in both mating partners, in comparison to that of D'orenone or β-apo-13-carotenone (compound 6).

At 48 h after supplementation (60 h after incubation) with

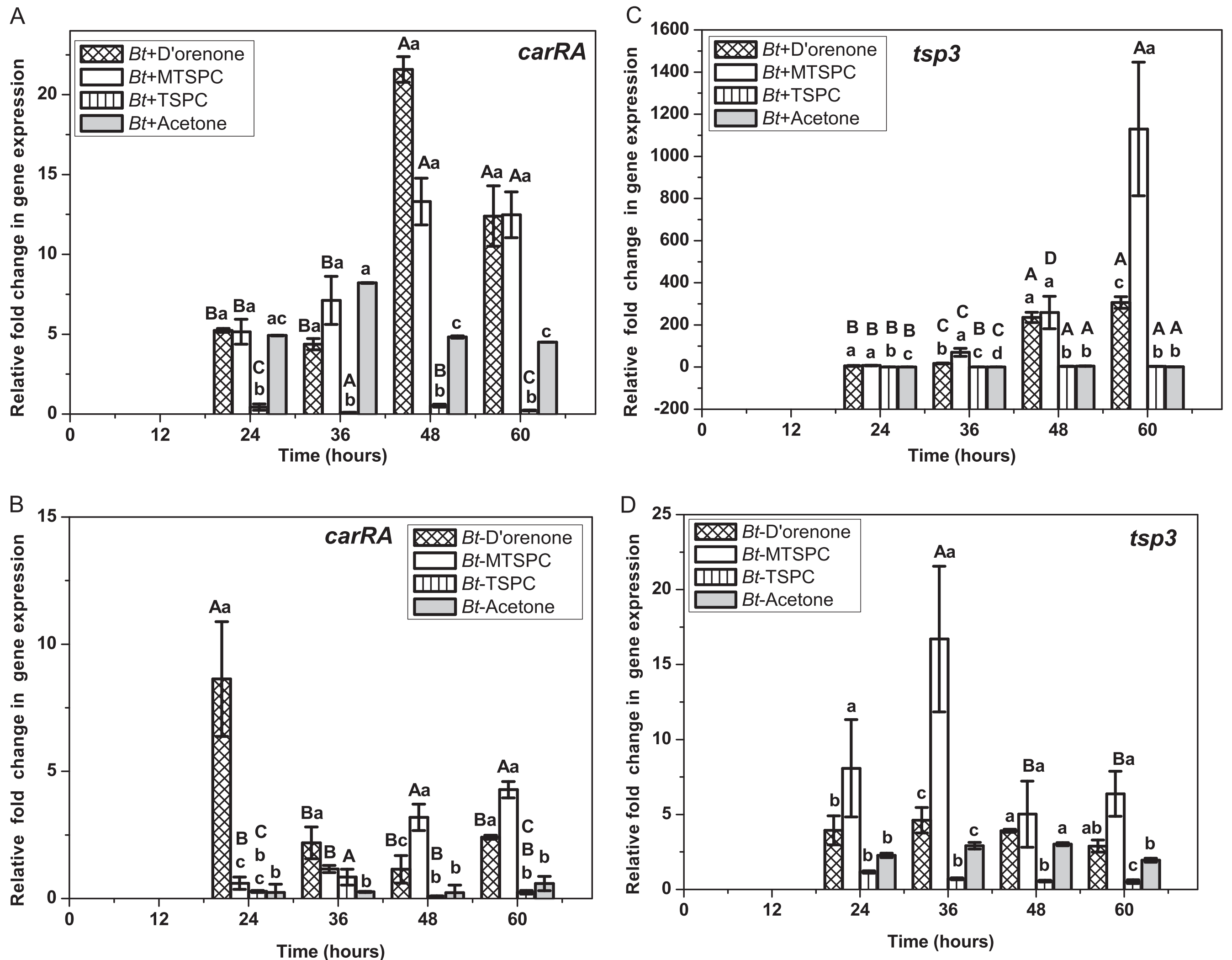

FIG 5 Transcript data analysis for *carRA* (A and B) and *tsp3* (C and D) after treatment with trisporoids in plus and minus *B. trispora* cultures. Graphs were plotted as means ± standard errors of the means for 3 independent biological replicates. A statistically significant difference was observed among the 4 trisporoids treatments at the 4 different time points only with *B. trispora* (−) *tsp3* (D) gene expression (ANOVA; $P = 0.018$; $n = 4 \times 4 \times 3$). Student -Newman-Keul's *post hoc* comparison showed a significant difference for D'orenone with MTSPC ($P = 0.044$), MTSPC with TSPC ($P = 0.015$), and MTSPC with acetone ($P = 0.037$). All treatments showed statistically significant differences ($P < 0.001$; $n = 4 \times 3$) among each other at the 4 designated time points (in small letters); capital letters indicate statistically significant differences for each treatment ($P = 0.002$; $n = 4 \times 3$) at 24 h, 48 h, and 60 h).

D'orenone, the *tsp3* transcript levels in *B. trispora* (+) rose 325-fold (Fig. 5C). All treatments induced statistically significant differences ($P = 0.009$; $n = 4 \times 3$) after 24 h, 36 h ($P = 0.003$), 36 h ($P = 0.027$), and 48 h ($P = 0.029$). The influence of each trisporoid on the *B. trispora* (+) *tsp3* transcript levels differed significantly among the different time points ($n = 4 \times 3$), i.e., D'orenone ($P = 0.003$), MTSPC ($P = 0.001$), and TSPC ($P < 0.001$). Contrary to its impact on *carRA*, MTSPC was the major activator of *tsp3* expression in both mating types, with a maximum 1,200-fold increase in plus partners at 60 h after incubation (Fig. 5C). The maximum expression in *B. trispora* (−) was at 36 h by both MTSPC and D'orenone, with 17.5-fold and 4.8-fold increases, respectively (Fig. 5D). *tsp3* gene expression significantly varied among the treatments, including the control-only treatment for *B. trispora* (−), based on consideration of the different time points as a single variable (analysis of variance [ANOVA], $P = 0.018$; $n = 4 \times 4 \times 3$).

DISCUSSION

The cocktail of trisporic acids, designated A, B, C, D, E based on the substitution pattern at C_{15} and functional groups attached to the ring (C_4), was discovered in the 1960s, and since then this cocktail has been extensively studied as the exclusive sex hormones in mucoralean fungi. These C_{18} apocarotenoid hormones regulate the sexual phase and positive feedback metabolism of β-carotene. Except for the zygophore-inducing effects of trisporins in *P. blakesleeanus*, no information was available about the function of individual trisporoid metabolites among *Mucorales*. We adopted the chemical approach by treating 4 different genotypes with diverse trisporoids and probable intermediates involved in the pathway to assess their potentials in feedback regulation. Our data support the hypothesis that the mode of action of each trisporoid varies among genotypes and growth phases, and this in turn led to the concept of a "chemical dialect."

Apocarotenoid precursors as signaling molecules in *Mucorales*. Peptide hormones act as signaling molecules in *Ascomycota* and *Basidiomycota*, while nonpeptide apocarotenoids known as trisporoids are the exclusive sex pheromones within the order *Mucorales* of the subphylum *Muoromycotina* (42). No information is yet available about their receptors of pheromone perception and hence signals transduction pathways. Among the enlisted stimulants used for carotene induction assay, trisporins, methyl trisporates, and dihydromethyl trisporates were the natural pheromones isolated from *Phycomyces*, *M. mucedo*, and *Blakeslea*. The addition of excess amounts of trisporin B (10^{-4} M) triggered the early phases of mating in *P. blakesleeanus* but failed to produce mature zygospores (43). Methyl trisporates extracted from *B. trispora* were highly active in bioassays for zygophore induction on the minus but not on the plus partners in *M. mucedo* and *P. blakesleeanus* (44). Synthetic trisporoids possessing a polar hydroxyl group at C_4, i.e., compounds 7, 8, and 9, were the most active stimulators of carotenogenesis in *Blakeslea*. Why those compounds did not activate *M. mucedo* is so far unclear (Fig. 2), and experiments should be done on additional *Mucorales* members to figure out the mechanism of action. The induction effect of synthetic analogues (12, 13) in *B. trispora* is curious, and further studies on the biological functions of these metabolites are essential to make valid conclusions. The inefficiency of stimulants on β-carotene production in plus strains of both *Blakeslea* and *M. mucedo* (Fig. 2) supports the hypothesis that a suppressor gene (44) is associated with the plus mating partners. All compounds represented a mixture of E/Z isomers and were of 95% purity. In natural trisporoid extracts, both isomers were detected. Therefore, the isomer mixture of each compound mimics the natural situation but this may not be true for the concentrations.

The trisporic acid biosynthetic pathway is quite complex because of the diverse metabolites (Fig. 1) along with the morphological and physiological changes that take place among the mating partners. Molecular genetics approaches were limited to early steps in the mevalonate pathway due to the inadequacy of protein and DNA database information on downstream processes. Transcript-level analysis and relative quantification were previously carried out for the carotenogenic genes *carRA* and *carB* in individual mating partners and mated cultures of *B. trispora* incubated for up to 3 days, with a basal time point of 24 h after inoculation for data analysis (45, 46). Hence, we focused upon an extended time series transcript analyses of the 3 functional genes in *B. trispora*, one involved in β-carotene production (*carRA*), and 2 others in trisporic acid biogenesis (*tsp3* and *tsp1*) for testing the null hypothesis, that trisporoids generated by mating partners do not differentially regulate gene transcription during the asexual and sexual developmental phases (Fig. 4A, B, and C). A statistically significant difference in *carRA* gene upregulation was observed only at 48 h among *B. trispora* (−) and mated cultures, and this may be because of activation of molecular signaling cascades involved in early steps of the TSA pathway in the sexual phase. Constitutive gene expression beyond 72 h in the mated phase supports the ongoing trisporoid-regulating feedback loop, while it is reasonable to have higher transcript levels for minus strains that produce more β-carotene and negligible trisporic acids compared to the plus strain or mated culture in *B. trispora*. The plus strain known to produce 0.1% of trisporic acids compared to mated cultures (100%) in *B. trispora* had progressive transcript turnover in *carRA* on a temporal trend, and this supports the hypothesis that trisporoids synergistically enhances carotenogenesis at the genetic level (Fig. 4A).

The carotenoid cleavage oxygenase (*tsp3*) of *B. trispora* had transcript turnover when plus and minus strains were grown in the dark upon treatment with 14 μg ml^{-1} trisporic acid B (16). In our time series experiments without any stimulation, *tsp3* transcripts showed significant upregulation over the growth phases, extending up to 144 h or 6 days in plus and minus strains. The striking increase of 16,000-fold at 48 h (Fig. 4B) exclusively in mated cultures and the further decline to 4,000-fold by 72 h exemplifies the transcriptional bursts associated with dynamic biological networks controlled by molecular signals in eukaryotes (47). A stable state of equilibrium might have been acquired through the activity of functional proteins and metabolites involved in the TSA pathway beyond 72 h, thereby maintaining a constitutive transcript level. Through *in vivo* enzyme assays we identified β-apo-12′-carotenal, formed by TSP3 activity, which suggested that the specificity of β-carotene cleavage at $C_{11'}$—$C_{12'}$ (see Fig. S2 in the supplemental material) is conserved among the 2 members in the order *Mucorales*, namely, *B. trispora* and *P. blakesleeanus*. Since both species are not closely related, even belonging to different families (*Choanephoraceae* and *Phycomycetaceae*), it can be presumed that this specific carotene cleavage is conserved throughout the whole order.

Both mating partners of *M. mucedo* expressed equal transcript levels and translation of TSP1, but a slightly induced activity was only associated with the *M. mucedo* (−) partner after 60 to 80 min of trisporic acid treatment, suggesting a role for posttranslational gene regulation (21, 48). TSP1 transcript turnover was constitutive in both *B. trispora* (Fig. 4C) and *P. blakesleeanus* (Kerstin et al., unpublished data) irrespective of sexual or asexual phases. However, we did not observe any significant changes in transcript levels of *B. trispora tsp1*, even with developmental phases during sexual stages (Fig. 4C), suggesting posttranscriptional modifications. Hence, we did not perform further transcript analysis of *tsp1* after trisporoid treatment.

Trisporins (compounds 3 and 4) activate zygophore development at millimolar concentrations (43), and methyl trisporates (compounds 16 and 17) enhance carotenogenesis even at micromolar levels (49); these are the exclusive morphogenetic factors in the mucoralean sexual phase, while β-apo-13-carotenone (D'orenone) (compound 6) is an unexplored compound. The latter compound did not stimulate carotenogenesis in solid cultures of either *Blakeslea* or *Mucor* treated with a lower concentration of 14 μg ml^{-1} (Fig. 2), but at 50 μM it induced transcript levels of *carRA* and *tsp3* in *Blakeslea* (Fig. 5). Such discrepancies with transcript levels and secondary metabolites are known in other members of the *Mucorales* (50). The biological function of D'orenone is known to be as an apocarotenoid that inhibits root elongation in *Arabidopsis* and is a member of the strigolactone biosynthetic pathway (51). The inefficiency of trisporin C, either in carotenogenesis at 14 μg ml^{-1} (Fig. 2) or in transcription at an even higher concentration of 50 μM (Fig. 5) throws light on its differential effects and dose dependency, which varies with genus, species, or strain, as proposed in the pheromone-action-unitary theory (44). It is interesting that the early trisporoid triggered *carRA* transcripts while late trisporoid MTSPC influenced *tsp3*. In general, the higher transcript levels in mated cultures without trisporoid stimulation (Fig. 4A and B) for the corresponding genes explain

the synergistic effects of these metabolite cocktails that are naturally present in fungal partners. Trisporoids and apocarotenoids as signaling molecules determine trends in the transcription of genes associated with the carotenoid metabolic network throughout the developmental phase of *B. trispora*.

Fungal communication plays a vital role in growth, development, morphogenesis, mating, activation of virulence factors, and pathogenesis. The heterothallic *Mucorales*-like *Rhizopus stolonifer* and *Mucor piriformis* are causal agents for postharvest diseases in fruits and vegetables (52). *Mucor* sp., *Rhizopus oryzae*, and *Lichtheimia* sp. are potential pathogens that cause rapidly emerging mucoromycosis in immunocompromised humans in tropical and temperate areas (53–55). Hence, it is important to unravel the molecular mechanisms of trisporoid signaling in *Mucorales* in order to determine the potential role of sexual phase in pathogenesis and survival within the host. The current study has demonstrated a broad diversity of dynamics and biological effects of the early and late trisporoids on gene expression and secondary metabolism in plus, minus, and mated cultures of the two mucoralean representatives. The elucidation of similarities and differences in the expression of genes involved in carotenoid degradation and the participation of their gene products in sexual and asexual interactions are additional essential elements of the complex language between different zygomycetes (see Table S2 in the supplemental material). For the future, it is necessary to establish the genetic basis of differences between the plus and minus strains in response to individual trisporoids. *M. mucedo* treated with trisporoid stimulants had higher transcript levels of *sexM* than of *sexP* (25). Parallel studies in *B. trispora* and other members (including homothallic ones) of the order *Mucorales* will promote the elucidation of genetic variations and contrasts among partners that are otherwise morphologically indistinguishable.

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