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Comparative Genomics and Transcriptomics Depict Marine Algicolous *Arthrinium* Species as Endosymbionts That Help Regulate Oxidative Stress in Brown Algae

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The whole genome and transcriptome analyses were performed for prediction of the ecological characteristics of *Arthrinium* and the genes involved in gentisyl alcohol biosynthesis. Whole genome sequences of *A. koreanum* KUC21332 and *A. saccharicola* KUC21221 were analyzed, and the genes involved in interspecies interaction, carbohydrate-active enzymes, and secondary metabolites were investigated. Three of the seven genes associated with interspecies interactions shared by four *Arthrinium* spp. were involved in pathogenesis. *A. koreanum* and *A. saccharicola* exhibit the enzyme profiles similar to those observed in plant pathogens and endophytes rather than saprobes. Furthermore, six of the seven metabolites of known clusters identified in the genomes of the four *Arthrinium* spp. are associated with plant virulence. These results indicate that *Arthrinium* spp. are potentially pathogenic to plants. Subsequently, different conditions for gentisyl alcohol production in *A. koreanum* were established, and mRNA extracted from cultures of each condition was subjected to RNA-Seq to analyze the differentially-expressed genes. The gentisyl alcohol biosynthetic pathway and related biosynthetic gene clusters were identified, and gentisyl alcohol biosynthesis was significantly downregulated in the mannitol-supplemented group where remarkably low antioxidant activity was observed. These results indicate that gentisyl alcohol production in algicolous *Arthrinium* spp. is influenced by mannitol. It was suggested that the algicolous *Arthrinium* spp. form a symbiotic relationship that provides antioxidants when the photosynthetic activity of brown algae decreases in exchange for receiving mannitol. This is the first study to analyze the lifestyle of marine algicolous *Arthrinium* spp. at the molecular level and suggests a symbiotic mechanism with brown algae. It also improves the understanding of fungal secondary metabolite production *via* identification of the gentisyl alcohol biosynthetic gene clusters in *Arthrinium* spp.

Keywords: algicolous fungi, biosynthetic gene cluster, comparative genomics, gentisyl alcohol, whole genome sequence

INTRODUCTION

Arthrinium spp. are filamentous ascomycetes that have been isolated from various environments such as bamboo, sedges, soil, seaweeds, egg masses of sailfin sandfish, corals, and beach sand (Elissawy et al., 2017; Heo et al., 2018; Wang et al., 2018; Pintos et al., 2019). They have been reported to thrive as saprophytes, endophytes, and pathogens. Among saprophytes, few species (ex. *A. phaeospermum*) have been identified as wood-decaying fungi (Astiti and Suprapta, 2012). In contrast, many *Arthrinium* spp. (*A. arundinis*, *A. garethjonesii*, *A. hydei*, *A. hyphopodii*, *A. neosubglobosa*, *A. phaeospermum*, *A. sacchari*, *A. saccharicola*, etc.) have been isolated from the inner tissue of a variety of hosts including bamboos, candle bush (*Senna alata*), seeds of white leadtree (*Leucaena leucocephala*), yacon (*Smallanthus sonchifolius*), cock's-foot (*Dactylis glomerata*), Malabar nut (*Adhatoda vasica*), Japanese sedge (*Carex konomugi* Ohwi), and brown algae (*Sargassum fulvellum*) (Sánchez Márquez et al., 2007; Khan et al., 2009; Maehara et al., 2010; Ramos et al., 2010; Shamsi et al., 2013; Shen et al., 2014; Lezcano et al., 2015; Dai et al., 2016; Pansanit and Pripdeevech, 2018; Suradkar and Hande, 2018). *A. arundinis* was isolated from a lichen (*Cladonia* sp.) (Wang et al., 2017). Moreover, the pathogenicity of at least four *Arthrinium* spp. (*A. arundinis*, *A. phaeospermum*, *A. sacchari*, and *A. xenocordella*) has been reported in various hosts, including wheat (damping-off), barley (kernel blight), bamboos (blight, brown culm streak, culm rot, and foot rot), rosemary (leaf spot), olive trees (leaf necrosis), aloe (flower malformation), and legumes (fruit blight) (Khan and Sullia, 1980; Suxuan et al., 1999; Mavragani et al., 2007; Li et al., 2013; Piccolo et al., 2013; Bagherabadi et al., 2014; Chen K. et al., 2014; Aiello et al., 2018; Jiang et al., 2018). Therefore, *Arthrinium* spp. may be endophytic or pathogenic rather than saprotrophic.

In general, symbiosis of endophytes is based on conferring competitive advantages to their hosts and there are various possibilities for the types of benefits provided by *Arthrinium* to the specific host (Rudgers et al., 2004). First, it has been observed that *A. phaeospermum* produces gibberellin, a plant growth-promoting compound, and promote mycorrhizal formation in pink rock-rose (Khan et al., 2009; Sabella et al., 2015). Additionally, *A. arundinis* accelerates strawberry seed germination by promoting their coat destruction (Guttridge et al., 1984). These *Arthrinium* spp. benefit host plant growth. On the contrary, at least five *Arthrinium* spp. (*A. arundinis*, *A. aureum*, *A. phaeospermum*, *A. saccharicola*, and *A. serenense*) exhibit antibiotic activity against over 39 microorganisms (Oka et al., 1993; Alfatafta et al., 1994; Vijayakumar et al., 1996; Aissaoui et al., 1999, 2001; Sato et al., 2000; Calvo et al., 2005; Miao et al., 2006; Bloor, 2008; Ramos et al., 2010; Heo et al., 2018; Pansanit and Pripdeevech, 2018; Hinterdobler and Schinnerl, 2019). Among them, the antibiotics apiosporamide, arthrichitin and arthrinic acid, and griseofulvin and terpestacin were isolated and identified from *A. arundinis*, *A. phaeospermum*, and an unidentified *Arthrinium* species, respectively (Oka et al., 1993; Alfatafta et al., 1994; Vijayakumar et al., 1996; Bloor, 2008; Elissawy et al., 2017). Thus, it is speculated that few *Arthrinium* spp. help the host defend against harmful microorganisms by producing antimicrobial compounds. Additionally, various roles of *Arthrinium* spp. in symbiosis have been suggested and verified. However, their relationship with brown algae and sponges, which are their major hosts in the marine environment, has not been extensively studied, perhaps due to the difficulty of conducting empirical experiments using marine organisms. Marine algicolous species have been proposed to help maintain the redox equilibrium in the host by producing antioxidants to remove excess reactive oxygen species (ROS), which are generated by the absorption of ultraviolet (UV) radiation by dissolved organic matter in seawater (Mopper and Kieber, 2000). A previous study has confirmed that most marine *Arthrinium* spp., including *A. saccharicola* and *A. koreanum* isolated from gulfweeds (*Sargassum fulvellum*) and egg masses of sailfin sandfish (*Arctoscopus japonicus*) that spawn on them, demonstrate high antioxidant activity and produce gentisyl alcohol (Heo et al., 2018). Therefore, the physiological and ecological characteristics of *Arthrinium* were investigated at the molecular level and genes related to gentisyl alcohol biosynthesis were investigated by analyzing the genome and transcriptome. This is the first comparative genome analysis of the genus *Arthrinium*.

MATERIALS AND METHODS

Fungal Culture and DNA/RNA Extraction

Two algicolous *Arthrinium* species, *A. saccharicola* KUC21221 and *A. koreanum* KUC21332, were obtained from the Korea University Culture (KUC) collection. *Arthrinium* species found in the egg mass of *A. japonicus* are known to have originated from an adjacent brown alga, as *A. japonicus* spawns on *S. fulvellum*, one of the major endophytic hosts of *Arthrinium* spp. (Heo et al., 2018). For genome sequencing, they were cultured in 1-L Erlenmeyer flasks containing 500 mL potato dextrose broth for 7 days at 25°C in dark. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions with slight modifications (Kohler et al., 2011). To improve the accuracy of genome annotation, total RNA was extracted from the same cultures using the RNeasy Plant Mini Kit (Valencia, CA, United States) according to the manufacturer's instructions. The DNA and RNA qualities were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States) with a DNA 1000 chip.

Arthrinium koreanum KUC21332 was inoculated on malt extract agar (MEA, Bacto, Sparks, MD, United States). The fungus was cultured for 7 days, and three agar plugs with mycelium were used as the inocula. To assess the difference in gentisyl alcohol production according to the nitrogen source [peptone, sodium glutamate, KNO_3, and $(NH_4)_2SO_4$], the fungus was cultured in 250-mL Erlenmeyer flasks containing 50 mL medium (40 g glucose, 10 g nitrogen source, 0.5 g $MgSO_4$, 0.5 g KH_2PO_4, and 0.5 g K_2HPO_4 in a liter of distilled water) for 10 days in dark. To determine the carbon source (glucose, sucrose, maltose, soluble starch, and mannitol) and to perform RNA-Seq, it was cultured in the medium (40 g carbon source, 10 g sodium glutamate, 0.5 g

$MgSO_4$, 0.5 g KH_2PO_4, and 0.5 g K_2HPO_4 in a liter of distilled water) for 3 days in dark. Total RNA was extracted in the same way as described above.

Library Construction and Whole Genome Sequencing

A DNA library with approximately 20-kb fragment sizes was constructed and the WGS was acquired using the PacBio Sequel platform at Macrogen Co., Ltd., (Seoul, South Korea). Meanwhile, a single molecule real-time (SMRT) library was constructed and sequenced with a single SMRT cell. The reads were trimmed, corrected, and filtered. Data on high-quality and error-corrected Illumina reads were used as input for the program Proovread v2.14.0 to correct the potential sequencing errors in the PacBio long reads (Hackl et al., 2014). Assembler Falcon was used for *de novo* assembly of the corrected PacBio reads (Chin et al., 2016). The assemblies were finalized and manually corrected after polishing using the paired-end Illumina reads and Pilon v1.21 (Walker et al., 2014).

To improve the accuracy of genome annotation, mRNA in the samples was sequenced. The RNA-Seq library was constructed and Illumina HiSeq 4000 sequencing was performed at Macrogen Co., Ltd., (Seoul, South Korea). Trimmed and corrected RNA-Seq reads were aligned to the reference genome using HISAT2 v2.1.0 (Kim et al., 2019). The genes were predicted using Augustus v3.3.3, BRAKER v2.1.5, GenMark-ES v4.61, and GlimmerM v2.5.1 (Majoros et al., 2003; Stanke et al., 2006, 2008; Ter-Hovhannisyan et al., 2008; Hoff et al., 2016, 2019). The final consensus gene model was constructed using all predictions using EVidenceModeler v1.1.1 (Haas et al., 2008). Repeats were masked using RepeatMasker v4.1.0 and RepeatModeler v2.0[1]. Annotation completeness was evaluated using BUSCO v4.1.2 on fungi_odb10, ascomycota_odb10, and sordariomyceta_odb10 gene sets (Seppey et al., 2019). Orthologous protein families with the reference genome datasets were identified using Orthofinder v2.4.0 with default setting except for an inflation parameter ($I = 2.5$) (Emms and Kelly, 2019).

Genome Annotation

The proteins were annotated by predicting functional domains from Pfam using InterProScan (Hunter et al., 2009; El-Gebali et al., 2019). To further facilitate functional interpretation, proteins were aligned to the non-redundant database of NCBI[2]. Gene ontology (GO) terms were mapped using InterProScan v5.29-68.0 (Gene Ontology Consortium, 2012; Jones et al., 2014). Carbohydrate-active enzymes (CAZymes) were analyzed using dbCAN2 (hmmer) (Potter et al., 2018; Zhang et al., 2018). Gene clusters related to secondary metabolism were analyzed using antiSMASH Fungi v5.1.2, and secondary metabolite regions were identified using strictness "relaxed" (Blin et al., 2019).

Antioxidant Assay

To obtain fungal extracts, the fungal cultures were filtered with 0.45 μm syringe filters (Chromafil PE- 20/15 MS, Macherey-Nagel, Düren, Germany) and extracted with 50 mL of ethyl acetate for 24 h. The ethyl acetate layer was collected and dried at 35°C using a rotary evaporator, and the obtained extracts were stored at −18°C until use.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich Inc., St. Louis, MO, United States) was dissolved in 80% methanol at 150 μM. The 198 μL of DPPH solution and 22 μL of the fungal extracts (10 mg/mL DMSO) was mixed in each well in a 96-well plate. The plate was allowed to reach a steady state for 30 min at room temperature in dark. The absorbance was measured at 540 nm using a microplate reader (Sunrise^{TM}, Tecan Group Ltd., Port Melbourne, VIC, Australia).

RNA-Seq, Transcriptome Assembly, and Differentially Expressed Gene Analysis

To analyze differentially-expressed genes (DEGs), RNA-Seq libraries were constructed using the Illumina TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, United States) and were sequenced on the Illumina NovaSeq 6000 platform at Macrogen Co., Ltd., (Seoul, South Korea). Quality check of sequenced reads was performed using the FastQC v0.11.7[3].

The RNA-Seq data were analyzed using the "new Tuxedo" pipeline according to a published protocol (Pertea et al., 2016). Briefly, the raw reads were quality filtered using the program Trimmomatic v0.39 and aligned to each genomic DNA reference obtained in this study using HISAT2 v2.1.0 (Bolger et al., 2014; Kim et al., 2019). Data on the transcripts and their expression levels were assembled and estimated using the StringTie v1.3.4 (Kovaka et al., 2019). The DEG analysis was conducted using the DESeq2 (Love et al., 2014). To reduce systematic bias that could affect biological meaning in comparison between samples, the size factor was estimated using count data and normalized using median of ratios method. Between the experimental groups, there was no significant difference ($p = 0.564$) in the expression level of pyruvate kinase (GO:0004743), which catalyzes the final step of glycolysis, indicating that the difference in secondary metabolism was independent of the amount of available energy (Abdel Fattah et al., 2010; Zhang et al., 2016).

Statistical Analysis

Statistical analyses were performed with R version 3.5.3 (R Development Core Team, 2013). Non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (adonis2) were performed using the package "vegan" (Oksanen et al., 2013).

RESULTS

Genome Sequencing, Assembly, and Annotation

In the present study, the genomes of two marine *Arthrinium* spp., *A. koreanum* KUC21332 and *A. saccharicola* KUC21221, isolated from the inner tissue of a brown alga *Sargassum fulvellum* and egg mass of *A. japonicus*, respectively, were

[1]http://repeatmasker.org

[2]http://www.ncbi.nlm.nih.gov

[3]http://www.bioinformatics.babraham.ac.uk/projects/fastqc

analyzed. *A. koreanum* demonstrates a genome size of 48.75 Mbp (GC content: 52.09%), including 14,381 gene models, and *A. saccharicola* exhibits a 55.08-Mbp genome (GC content: 50.07%) with 14,773 models (**Table 1**). Both genomes are larger than those of *A. phaeospermum* AP-Z13 (48.45 Mbp) and *A. arundinis* NRRL 25634 (47.67 Mbp), with *A. saccharicola* genome being the largest among the four species. The number of gene models of both species is similar to *A. phaeospermum* (14,055 models), while that of *A. arundinis* NRRL 25634 is higher (16,992 models). The genome assembly completeness of both species ranges from 96.3–98.8%.

Genome Comparison of Four *Arthrinium* spp.

The four *Arthrinium* spp. shared 10,001 genes (**Figure 1A**). *A. koreanum* shared more genes (615) with *A. arundinis* compared to the genes shared with *A. saccharicola* (212) and *A. phaeospermum* (146). *A. saccharicola* shared more orthogroups (672) with *A. phaeospermum* compared to those shared with *A. arundinis* (208) and *A. koreanum* (212). Compositions of shared and specific genes were analyzed by gene ontology (GO) categories and have been illustrated in **Figure 1B**. The genes that only existed in *A. koreanum* (circle XII) were unidentified. Approximately half (mean 51.2%) of the genes were involved in biological process (BP), and 37.0 and 11.5% were involved in molecular function (MP) and cellular component (CC), respectively. Metabolic process (GO:0008152), cellular process (GO:0009987), and localization (GO:0051179) accounted for 48.6, 29.0, and 13.1% of the total genes in BP, respectively. Additionally, there were seven genes involved in interspecies interactions between organisms (GO:0044419), all of which were common in at least two *Arthrinium* spp. Four of them were shared by all four *Arthrinium* spp., of which two were involved in the defense response to gram-negative bacteria (GO:0050829), one was responsible for anti-bacterial defense response (GO:0042742), and the other were involved in pathogenesis (GO:0009405). One gene shared by *A. arundinis*, *A. koreanum*, and *A. phaeospermum* was involved in the DNA restriction-modification system (GO:0009307). One gene shared by *A. arundinis*, *A. saccharicola*, and *A. phaeospermum*, and the other shared by *A. koreanum* and *A. saccharicola*, were involved in pathogenesis (GO:0009405). In the case of CC, cellular anatomical entity (GO:0110165), protein-containing complex (GO:0032991), and intercellular (GO:0005622) accounted for 84.6, 7.8, and 7.2% of the total, respectively. In the case of MF, catalytic activity (GO:0003824) and binding (GO:0005488) accounted for 46.9 and 40.1% of the total genes, respectively, followed by transporter activity (GO:0005215) and molecular function regulator (GO:0098772), accounting for 7.5 and 3.6%, respectively.

Additionally, the genes encoding CAZymes and plant cell wall degrading enzymes (PCWDEs) and compare to those of other ascomycetes exhibiting various lifestyles. Five, eight, and twelve species were selected for dark septate endophytes (DSEs), plant pathogens, and saprobes, respectively, by referring to the Mycocosm group of the Joint Genome Institute and previous studies (**Supplementary Table 1**; Galagan et al., 2003; Dean et al., 2005; Güldener et al., 2006; Hane et al., 2007; Van Den Berg et al., 2008; Ellwood et al., 2010; Andersen et al., 2011; Rouxel et al., 2011; Gianoulis et al., 2012; Ohm et al., 2012; O'Connell et al., 2012; Grigoriev et al., 2013; Koike et al., 2013; Traeger et al., 2013; Nordberg et al., 2014; Xu et al., 2015; David et al., 2016; Jourdier et al., 2017; Knapp et al., 2018). DSE is a paraphyletic group (Sordariomycetes, Pezizomycetes,

TABLE 1 | Assembly and genome features of *Arthrinium koreanum* KUC21332 and *A. saccharicola* KUC21332.

Genome features	*A. koreanum* KUC21332	*A. saccharicola* KUC21221	*A. phaeospermum* AP-Z13	*A. arundinis* NRRL 25634
Genome assembly				
Genome size (Mbp)	48.75	55.08	48.45	47.67
Depth	117	118	-	-
Number of contigs	50	37	19	686
Max contig length (Kbp)	8,999.76	8,166.37	5,249.37	935,02
N_{50} contig (Kbp)	3,570.80	3,269.72	3,733.26	234.56
L_{50} contig	5	6	6	61
GC content (%)	52.09	50.07	53.05	52.00
Number of gene models	14,381	14,773	14,055	16,992
Mean gene length (bp)	1,569.8	1,498.9	1,521.5	1,580.3
Number of exons	40,319	40,066	37,886	42,806
Mean exons per gene	2.8	2.7	2.7	2.5
Mean exon length (bp)	499.0	491.4	498.6	566.0
Assembly completeness (BUSCO database)				
Fungi_odb10 (%)	98.4%	98.8%	94.5%	99.6%
Ascomycota_odb10 (%)	98.2%	98.2%	91.6%	99.0%
Sordariomyceta_odb10 (%)	97.3%	96.3%	89.3%	98.0%
Genome reference	This study	This study	Li et al., 2020	Nordberg et al., 2014

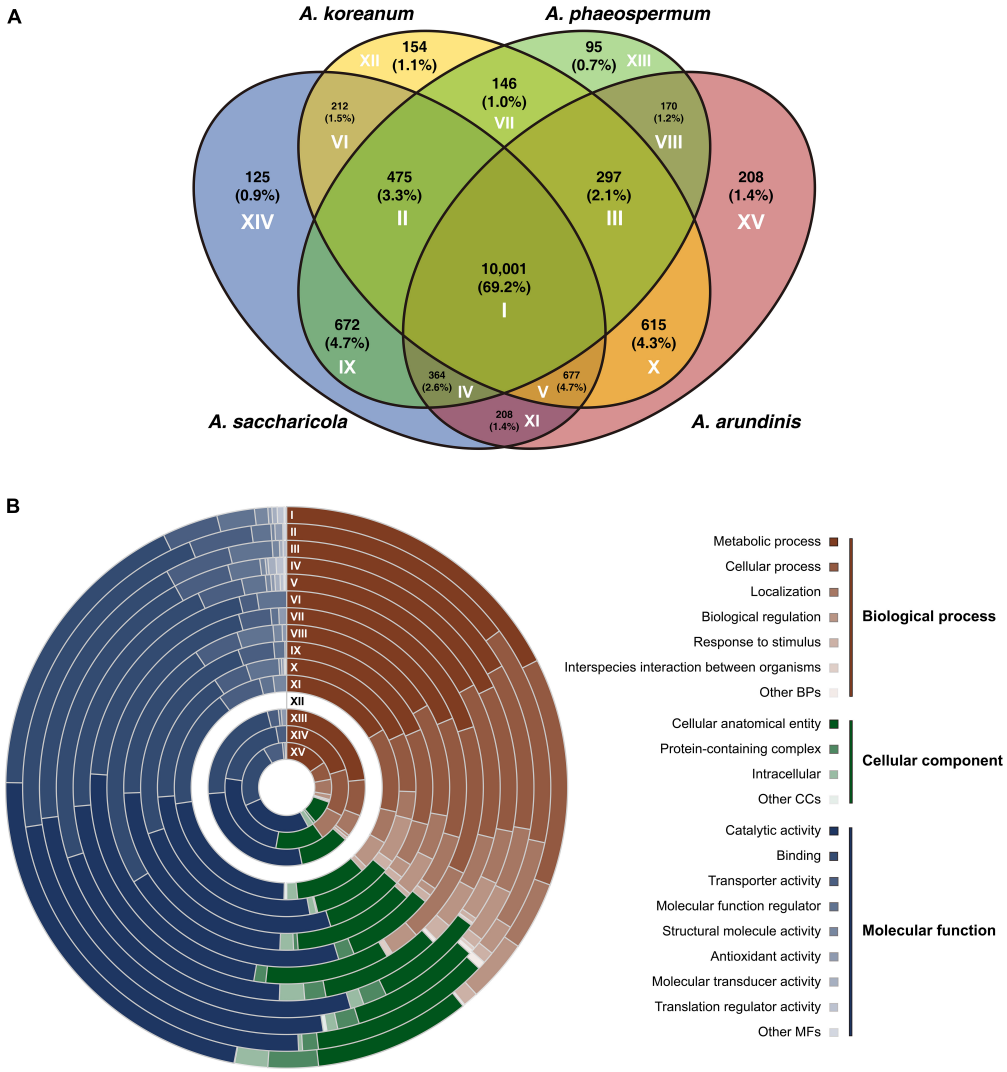

FIGURE 1 | Genome comparison between *Arthrinium koreanum*, *A. saccharicola*, *A. phaeospermum*, and *A. arundinis*. **(A)** Venn diagram showing the number of shared and specific gene orthologs. **(B)** Compositions of gene families according to gene ontology categories. Circles I-XV correspond to areas with same Roman numerals in panel **(A)**.

Dothideomycetes, Leotiomycetes, Eurotiomycetes, etc.) of endophytic fungi belonging to Ascomycota, and was selected because it represented endophytic ascomycetes. The sequence data of *A. arundinis* NRRL 25634, *Entoleuca mammata* CFL468, *Pestalotiopsis* sp. NC0098, and *Truncatella angustata* MPI-SDFR-AT-0073 were produced by the United States Department of Energy Joint Genome Institute[4] in collaboration with the user community.

The *Arthrinium* spp. had a total of 601-626 CAZymes, of which 307–323, 96–104, 9–11, 62–68, 11–14, and 113–117 were associated with glycoside hydrolase (GH), glycosyltransferase (GT), polysaccharide lyase (PL), carbohydrate esterase (CE), carbohydrate-binding module (CBM), and auxiliary activity (AA), respectively. The average number of CAZyme by lifestyle was 713, 553, and 397 for DSE, plant pathogen, and saprobe, respectively. *Arthrinium* spp. had 127–134 of PCWDEs, while DSE, plant pathogen, and saprobe had an average of 168, 130, and 65 PCWDEs, respectively. In the NMDS plot generated using the CAZyme profile of these species, *Arthrinium*-DSE-plant pathogen was clustered together, and the saprobe was widely distributed at a distance from this cluster. Based on the permutational multivariate analysis of variance, significant dissimilarities were observed between clusters according to lifestyles, and *A. koreanum* and *A. saccharicola*, whose lifestyles have not been identified, showed significant dissimilarity only with the saprobe (**Figure 2**). The NMDS plot generated using the PCWDE profile showed similar results, except that the dissimilarity between the DSE and the plant pathogen cluster was not significant. Further, among PCWDEs, GH53, GH127, PL4, and CE12 were significant predictors in the

[4]https://www.jgi.doe.gov

dimension 2 direction, and the representative enzymes of these CAZyme families are arabinogalactan endo-β-1,4-galactanase (EC 3.2.1.89), non-reducing end β-L-arabinofuranosidase (EC 3.2.1.185), rhamnogalacturonan endolyase (EC 4.2.2.23), and acetylxylan esterase (EC 3.1.1.72), respectively.

Identification of Gene Clusters Related to Secondary Metabolism and Gentisyl Alcohol Biosynthesis

The analysis of biosynthetic gene clusters (BGCs) of the four *Arthrinium* spp. showed that the highest number of BGCs was found in *A. arundinis* (84), followed by *A. koreanum* (76), and *A. phaeospermum* (69) and *A. saccharicola* (69) (**Supplementary Figure 1**). The secondary metabolite backbone genes consisted of 23–28 type I polyketide synthase (T1PKS) genes, 16–21 non-ribosomal peptide synthetase (NRPS) genes, 13–18 terpene genes, 0–3 indole genes, and 1–2 type III polyketide synthase (T3PKS) genes. Six BGCs were annotated with 100% similarity in the four *Arthrinium* spp. NRPS and two T1PKS BGCs, known to produce dimethylcoprogen, alternapyrone and ACR toxin I, respectively, were observed in all four species, and another two T1PKS BGCs, known to produce (R)-mellein and alternariol, were commonly found in *A. koreanum* and *A. arundinis* (Fujii et al., 2005; Izumi et al., 2012; Chen et al., 2013; Chooi et al., 2015a,b). Additionally, a T1PKS BGC, known to produce pyranonigrin E, was observed in *A. phaeospermum* (Andersen et al., 2011).

Arthrinium koreanum KUC21332 and *A. saccharicola* KUC21221, the gentisyl alcohol-producing species, commonly harbor 6-MSA BGCs (42.3 and 42.5 kb, respectively), which show 40% similarity with patulin BGC, while *A. phaeospermum* AP-Z13 does not harbor one (**Figure 3**). The nine genes that they commonly harbor in their BGCs are *GsaA*-*GsaI*. *GsaH* is orthologous to *PatK*, the backbone gene encoding 6-methylsalisylic acid (6MSA) synthase that catalyzes the first step of patulin biosynthesis (conversion of acetyl-CoA and malonyl-CoA to 6MSA); *GsaE* is orthologous to *PatG* encoding 6MSA decarboxylase that catalyzes the second step (conversion of 6MSA to m-cresol); *GsaD* is orthologous to *PatH* encoding m-cresol methyl hydroxylase that catalyzes the third step (conversion of m-cresol to m-hydroxybenzyl alcohol); *GsaF* is orthologous to *PatI* encoding m-hydroxybenzyl alcohol hydroxylase that catalyzes the fourth step (conversion of m-hydroxybenzyl alcohol to gentisyl alcohol); *GsaA* is orthologous to *PatL* encoding C6 transcription factor that activates gene expression; *GsaB* and *GsaG* are orthologous to *PatO* and *PatJ* encoding isoamyl alcohol oxidase and a putative dioxygenase, respectively, that catalyze the sixth step (conversion of gentisaldehyde to isoepoxydon); and *GsaC* and *GsaI* have been predicted to encode major facilitator superfamily (MFS) transporter (77.98% identical to EKG18982.1) and aldehyde reductase (73.39% identical to KAF6798856.1), respectively (Li et al., 2019). *A. arundinis* NRRL 25634 also harbors a 6-MSA BGC (44.9 kb) similar to patulin BGC (46% similarity), with two additional genes named *GsaJ* and *GsaK*, orthologous to *PatM* and *PatC* encoding ATP-binding cassette (ABC) transporter and MFS transporter, respectively; PatM and PatC were proposed to be involved in the extracellular patulin secretion (Li et al., 2019). In the other two *Arthrinium* spp., GsaC may replace the function of PatC, and the function of GsaI in the BGC should be further studied.

By measuring the DPPH radical-scavenging activity of *A. koreanum* KUC21332 extract using different organic (peptone and sodium glutamate) and inorganic nitrogen compounds (KNO_3 and $(NH_4)_2SO_4$) as a nitrogen source, the highest activity (90.6–98.3%) was observed with sodium glutamate (**Figure 4A**). The DPPH radical-scavenging activity was also determined using a monosaccharide (glucose), disaccharides (sucrose and maltose), a polysaccharide (soluble starch), and a sugar alcohol (mannitol) as the carbon source and sodium glutamate as the nitrogen source. As the activities rapidly increased within 2 days in the nitrogen source test, the carbon source test was conducted using 3-day cultures. The highest activity (79.2%) was observed with glucose, which was approximately 25 times higher than the lowest activity (3.2%) observed with mannitol (**Figure 4B**). To verify whether the *Gsa* BGC was responsible for gentisyl alcohol production, DEG analysis was performed with the transcriptomes from the mannitol- and glucose-supplemented cultures. The expression of *GsaA*, *GsaC*, *GsaG*, and *GsaH* was found to be significantly lower in the mannitol-supplemented group compared to that in the glucose-supplemented group. The fold-change values of the four genes in the glucose-/mannitol-supplemented group were 2.03, 27.28, 8.53, and 5.10 ($q < 0.001$ for all; the Wald test with Benjamini–Hochberg correction), respectively.

DISCUSSION

To understand the lifestyle of *Arthrinium* spp. at the molecular level, the full-length genomes of *A. koreanum* KUC21332 and *A. saccharicola* KUC21221 were analyzed and those of *A. phaeospermum* AP-Z13 and *A. arundinis* NRRL 25634 were comparatively analyzed. Since few species, including *A. arundinis* and *A. phaeospermum*, are plant pathogens and many species have been isolated from the internal tissues of plants or marine algae, genes related to interspecies interaction, CAZymes, and secondary metabolites were analyzed, focusing on pathogenicity and endogenous symbiosis. The seven genes associated with interspecies interaction and shared by the four *Arthrinium* spp. were involved in pathogenesis and defense processes against bacteria and invading foreign DNA *via* other biological agents. This suggests that *A. arundinis* and *A. phaeospermum*, which are known to be pathogenic to various plants, and *A. koreanum* and *A. saccharicola*, can establish abnormal conditions in other organisms. In the NMDS plot generated with CAZyme profiles of other ascomycetes with different lifestyles, *A. arundinis* and *A. phaeospermum* were clustered together with plant pathogens, and *A. koreanum* and *A. saccharicola* were distributed close to them (**Figure 2**). The overlap of DSEs and plant pathogens indicates that pathogenesis is an unbalanced symbiosis of endophytes (Kogel et al., 2006). According to the results of permutational multivariate analysis of variance, *A. koreanum* and *A. saccharicola* showed CAZyme profiles similar to plant

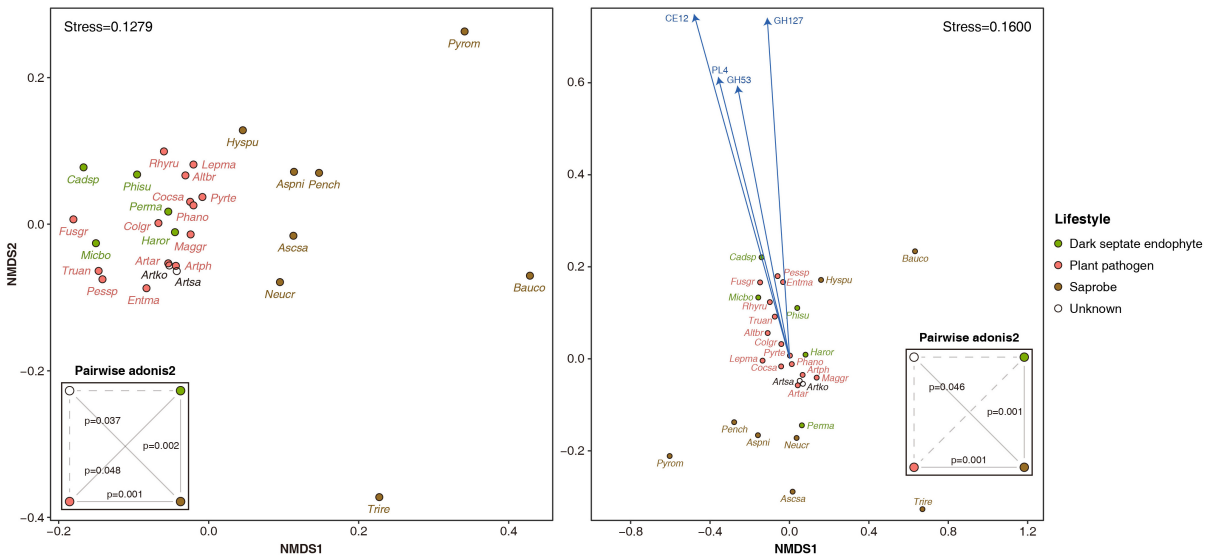

FIGURE 2 | Non-metric multidimensional scaling plot generated using profiles of carbohydrate active enzymes (CAZymes, left) and plant cell wall degrading enzymes (PCWDEs, right) of four *Arthrinium* spp. and other 25 ascomycetes with different lifestyles. Fitted variables are scaled by Pearson correlation coefficient ($p < 0.05$). Dissimilarities between CAZyme and PCWDE profiles by lifestyle based on permutational multivariate analysis of variance were summarized in the small boxes titled pairwise adonis2. The solid and dotted lines in the pairwise adonis2 boxes indicate significant ($p < 0.05$) and insignificant ($p > 0.05$), respectively. Altbr, *Alternaria brassicicola*; Artar, *Arthrinium arundinis*; Artko, *A. koreanum*; Artph, *A. phaeospermum*; Artsa, *A. saccharicola*; Ascsa, *Ascocoryne sarcoides*; Aspni, *Aspergillus niger*; Bauco, *Baudoinia compniacensis*; Cadsp, *Cadophora* sp.; Cocsa, *Cochliobolus sativus*; Colgr, *Colletotrichum graminicola*; Entma, *Entoleuca mammata*; Fusgr, *Fusarium graminearum*; Haror, *Harpophora oryzae*; Hyspu, *Hysterium pulicare*; Lepma, *Leptosphaeria maculans*; Maggr, *Magnaporthe grisea*; Micbo, *Microdochium bolleyi*; Neucr, *Neurospora crassa*; Pench, *Penicillium chrysogenum*; Perma, *Periconia macrospinosa*; Pessp, *Pestalotiopsis* sp.; Phano, *Phaeosphaeria nodorum*; Phisu, *Phialocephala subalpina*; Pyrte, *Pyrenophora teres*; Pyrom, *Pyronema omphalodes*; Rhyru, *Rhytidhysteron rufulum*; Trire, *Trichoderma reesei*; Truan, *Truncatella angustata*.

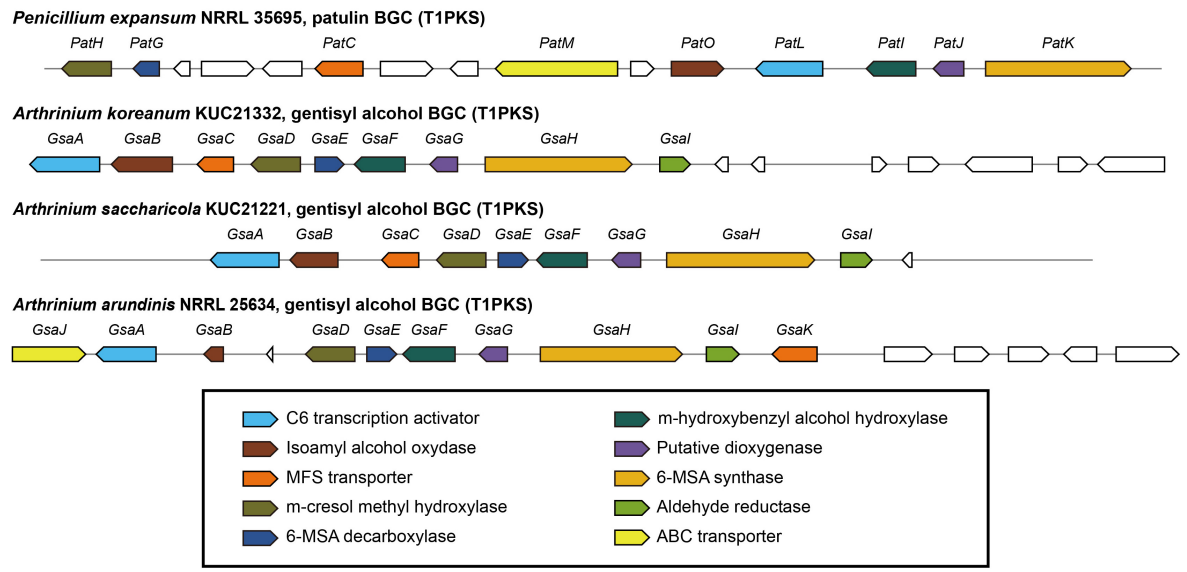

FIGURE 3 | Predicted gentisyl alcohol biosynthetic gene clusters (BGCs) of *Arthrinium* spp. matched to the patulin BGC of *Penicillium expansum*. Transcription direction is indicated by arrowheads.

pathogens and DSEs rather than saprobes. Additionally, the total number of CAZymes and PCWDEs in *Arthrinium* spp. was similar to that of plant pathogens, followed by that of DSEs. These results indicate that their lifestyles are on the mutualism-parasitism-continuum. Furthermore, metabolites of known clusters identified in the genomes of four *Arthrinium* spp. are associated with virulence, except for pyranonigrin E, which is found only in *A. phaeospermum*. A previous

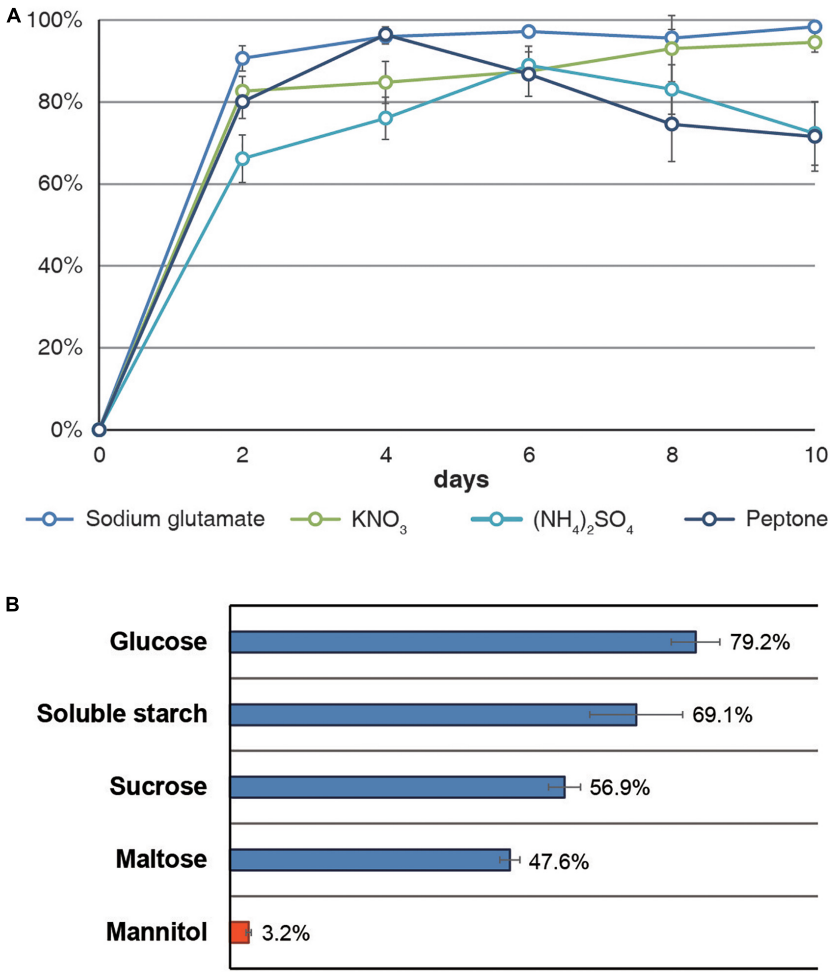

FIGURE 4 | DPPH radical-scavenging activities of *Arthrinium koreanum* KUC21332 culture extracts. **(A)** Activities by nitrogen source with glucose as a carbon source. **(B)** Activities by carbon source after being cultured for 3 days with sodium glutamate as a nitrogen source.

study has suggested that alternapyrone and dimethylcoprogen play roles in plant pathogenesis (Gluck-Thaler et al., 2020). Dimethylcoprogen, a fungal extracellular siderophore, was reported to be responsible for the pathogenicity of *Alternaria alternata* to citrus (Chen L. H. et al., 2014). ACR toxin I is a toxin of *Alternaria alternata*, which is essential for its pathogenicity, and alternariol is a colonization and virulence factor of this fungus during plant infection (Izumi et al., 2012; Wenderoth et al., 2019). (R)-mellein has been identified as a non-host-specific phytotoxin of various plant pathogens, such as *Parastagonospora nodorum*, *Botryosphaeria obtusa*, *Phoma tracheiphila*, *Neofusicoccum parvum*, and *Sphaeropsis sapinea* (Chooi et al., 2015a). These results indicate that *Arthrinium* spp., including *A. koreanum* and *A. saccharicola*, are potential plant pathogens. Since most sources of *Arthrinium* spp. did not show disease symptoms, they were presumed to be opportunistic pathogens that were pathogenic depending on the host condition.

Meanwhile, *Arthrinium* spp. are reportedly not pathogenic in brown algae, an endogenous host in the marine environment, and no abnormal or detrimental state has been reported in brown algae when *Arthrinium* spp. were isolated from their internal tissues. Instead, most algicolous *Arthrinium* strains showed high antioxidant activity, and it was hypothesized that antioxidant production was a strategy of marine *Arthrinium* spp. in endophytic symbiosis with brown algae (Heo et al., 2018). The antioxidant assay and DEG analysis based on different nutrient sources, including glutamic acid and mannitol, support this hypothesis. Glutamic acid is an accessible nitrogen source for algicolous *Arthrinium* spp., which is the most abundant amino acid accounting for about 13% of the total protein content of *Sargassum* spp., the most common endophytic host of *Arthrinium* spp., and mannitol is the main carbon storage material in brown algae, accounting for 20–30% of the dry weight of brown algae (Reed et al., 1985; Peng et al., 2013). According to the antioxidant assay results, the activity was the highest in the sodium glutamate-supplemented group, but all other groups showed high activity as well (**Figure 4A**). On the contrary, the activity was markedly different for the carbon source. The activity was barely observed with mannitol as a carbon source and sodium glutamate as a nitrogen source, whereas glucose-,

starch-, sucrose-, and maltose-supplemented groups showed high activity (47.6–79.2%) (**Figure 4B**). Through the DEG analysis, it was confirmed that the low antioxidant activity of the mannitol-supplemented group was due to the downregulation of gentisyl alcohol biosynthesis. These results suggest that gentisyl alcohol production in algicolous *Arthrinium* spp., such as *A. koreanum*, is primarily influenced by mannitol rather than glutamic acid. In marine environments, mannitol functions as an osmoprotectant and acts as an antioxidant that prevents peroxidation of cellular components by ROS, which is generated by the absorption of UV rays by dissolved organic matter in seawater (Groisillier et al., 2014). Since the residual mannitol amount in brown algae reflects the photosynthetic activity in brown algae, it is expected that algicolous *Arthrinium* spp. produce gentisyl alcohol to compensate for the reduced antioxidant activity when the mannitol content decreases at night. Additionally, deterioration of the cellular function or vitality of brown algae due to aging can also cause a situation where algicolous fungi are not sufficiently supplemented with mannitol. Likewise, gentisyl alcohol produced in response to mannitol deficiency in these fungi can help regulate the redox equilibrium of brown algae, when the ability of the algal host to control the redox equilibrium is poor. It is suggested that algicolous *Arthrinium* spp. establish a symbiotic relationship that enhances the viability of brown algae by providing antioxidants when the photosynthetic activity decreases in exchange for receiving mannitol.

Gentisyl alcohol biosynthetic pathway in *Arthrinium* spp. was also identified and the related BGCs were reported. The known gentisyl alcohol biosynthetic pathways were reportedly a part of the aculin A and patulin biosynthetic pathways of *Aspergillus aculeatus* and *Penicillium expansum*, respectively (Petersen et al., 2015; Li et al., 2019). Both pathways share the reactions of conversion of acetyl-CoA and malonyl-CoA to gentisyl alcohol, an intermediate product. It was expected that the gentisyl alcohol biosynthetic pathway of *Arthrinium* spp. might consist of genes orthologous to the genes in aculin A and patulin biosynthetic pathways. Indeed, 6-MSA BGCs similar to *P. expansum* patulin BGCs were observed. Considering that *A. koreanum* KUC21332 and *A. saccharicola* KUC21221 produced gentisyl alcohol as a major secondary metabolite instead of patulin, it was expected that the biosynthetic genes responsible for the later steps of patulin biosynthesis were absent. As expected, orthologs of the other six genes (*PatA*, *PatB*, *PatD*, *PatE*, *PatF*, and *PatN*) of patulin BGC were not present in the 6-MSA BGCs of the *Arthrinium* spp.; PatN, PatF, PatD, and PatE encode isoepoxydon dehydrogenase, neopatulin synthase, alcohol dehydrogenase, and glucose-methanol-choline oxidoreductase (patulin synthase) that catalyze the seventh to final reactions (from isoepoxydon to patulin) (Li et al., 2019). This confirms that enzyme-catalyzed steps after production of gentisyl alcohol in the patulinw biosynthetic pathway of *Penicillium* are absent in the suggested gentisyl alcohol biosynthetic pathway of *Arthrinium* (**Figure 5**). According to the DEG analysis results, *GsaA, GsaC,* and *GsaH* expression levels were downregulated. *GsaG* was neglected as its substrate, gentisaldehyde, cannot be produced because of the absence of responsible genes. *GsaA* expression downregulation in the mannitol-supplemented group was suspected to be responsible for the suppression of gentisyl alcohol biosynthesis,

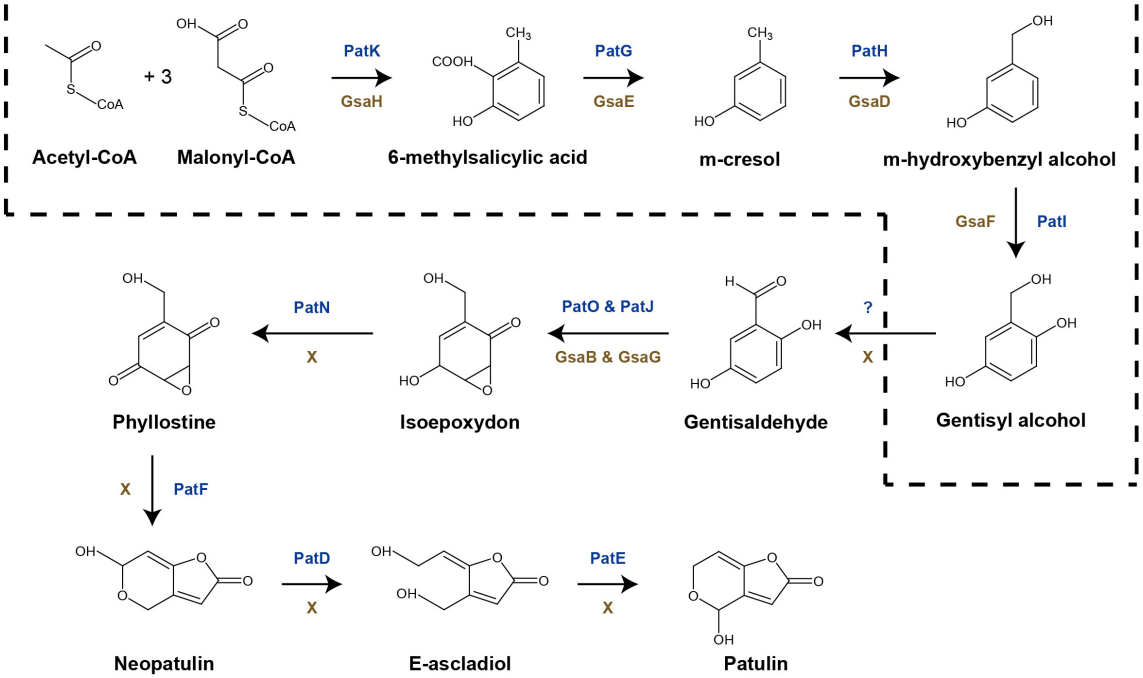

FIGURE 5 | Proposed scheme of gentisyl alcohol biosynthetic pathway in *Arthrinium* spp. (dotted box) expressed in the patulin biosynthetic pathway.

since GsaA acts as a specific transcription factor in this pathway. *GsaH* and *GsaC* expression downregulation decreases 6-MSA and MFS transporter production, respectively, thereby reducing the production and extracellular secretion of gentisyl alcohol. This result is consistent with the results of the antioxidant assay, thus demonstrating that the 6-MSA BGC is responsible for gentisyl alcohol biosynthesis. The expression levels of other genes in the BGC were not significantly different, indicating that *GsaC* and *GsaH* were targeted in gentisyl alcohol biosynthesis regulation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA692538.

AUTHOR CONTRIBUTIONS

YH and HL: conceptualization. YH, S-YO, and S-IH: methodology. S-YO and S-IH: validation. YH: formal analysis and writing—original draft preparation. KK, SKw, YY, and DK: investigation. J-JK: project administration and resources. S-YO and JK: writing—review and editing. YH and S-YO: visualization. HL, SKa, and J-JK: supervision. JK and J-JK: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.753222/full#supplementary-material

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Conflict of Interest: YH and SKa are employed by COSMAX BTI.

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