Proteomic sensing associated with terpenoid biosynthesis of *Artemisia annua* L. in response to different artificial light spectra

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**ABSTRACT**

Artificial light has been used to control plant growth and secondary metabolite production. *Artemisia annua* L. plants were illuminated with three light-emitting diode (LED) spectra to investigate proteomic and biochemical responses. After 7 days, proteomic data revealed different protein numbers in leaves, stems, and roots under particular treatments. Results demonstrated increased accumulation of several proteins, including secondary metabolite-related proteins. The red light (R) (660 nm) highly induced terpenoid proteins. Similar biochemical profiles were observed in white light (W) (445, 544 nm) and blue light (B) (445 nm) conditions, while profiles from R treatment were different. Functional proteins of W and B treatments were involved in the MVA and MEP pathways and sesquiterpene biosynthesis. By contrast, unique proteins in R treatment were mainly expressed in sesquiterpene and tetraterpene biosynthesis. Specific relationships between biosynthetic proteins and sesquiterpenes were observed, suggesting the indispensable role of the light spectrum in regulating terpenoid biosynthesis.

**Introduction**

Qinghao (*Artemisia annua* L.), also known as sweet wormwood, is an important Chinese herb used to treat many infectious diseases (Graziose et al. 2010; Chang 2016). The plant extract exhibited wide-ranging pharmacological properties including antioxidant, antimicrobial, antiparasitic, and antiviral activities (Efferth et al. 2001; Cai et al. 2004; Sharopov et al. 2020). Phytochemical analyses revealed that leaves and crude extracts of *A. annua* contained several useful compounds including terpenoids, flavonoids, coumarins, sterols, phenols, lipids, and other hydrocarbons (Bhakuni et al. 2001; Czechowski et al. 2018). Among these, terpenoids are the largest and most diverse group, consisting of more than 40,000 plant secondary compounds (Bohlmann and Keeling 2008). Artemisinin, a useful sesquiterpene compound found in leaves of *A. annua*, plays a central role in combating the malaria-causing parasite *Plasmodium falciparum* (Klayman 1985; Dondorp et al. 2009), while the triterpene squalene showed a synergistic effect with artemisinin to promote *anti-falciparum* activity (Karaket et al. 2014).

Terpenoids contain unit(s) of 5-carbon isoprene and can be classified as hemi- (C5: isoprene), mono- (C10), sesqui- (C15), di- (C20), sester- (C25), tri- (C30), and tetr- (C40: carotenoids) terpenoids (Yazaki et al. 2017). Biosynthesis of terpenoid compounds requires the common precursor isopentenyl diphosphate (IPP) that can be synthesized by two different pathways involving mevalonic acid (MVA) and 2-C-methyl-D-erythritol 4-phosphate (MEP). The MVA pathway operates in cytosol, while the MEP pathway is localized in plastids. Crosstalk and exchange of IPP between these two pathways have been investigated in many plant species (Lange and Croteau 1999; Hemmerlin et al. 2003; Laule et al. 2003; Opitz et al. 2014). Biosynthesis of mono-, di- and tetraterpenes occurs in plastids, while cytosol is mainly responsible for sesquiterpene and tetraterpene biosynthesis (Muhlemann et al. 2014; Abbas et al. 2017). Alteration of the terpenoid biosynthetic pathway affects the target and also other related compounds.

Plants grown in their natural habitats spontaneously encounter a variety of stresses including light stresses from sunlight radiation. To maintain cellular activity and survival, plants employ molecular mechanisms underlying plant responses involving the activity of metabolic enzyme networks associated with responsive proteins (Kong and Oka-jima 2016; Chi et al. 2019). Previous studies indicated the influence of light signals on biosynthesis of secondary metabolites in several plant species. High light emission enhanced accumulation of coniferin, syringin, and flavonoids in *Arabidopsis* roots (Hemm et al. 2004), while the presence of light increased levels of several terpenoids, indole alkaloids, and loganin in *Catharanthus roseus* L. plants (Yu et al. 2018). Cited reports suggested positive impacts of light on improving plant metabolite production; however, inappropriate light supply ultimately destroyed plant photosynthetic apparatus and induced the accumulation of reactive oxygen species (ROS), leading to alterations in plant growth and development or even death (Li et al. 2009; Xie et al. 2014; Huang et al. 2019). The role of light signals in regulating metabolite production was also demonstrated in *A. annua*. Levels of bioactive compounds such as artemisinin were promoted at more than 1.5 times the control when UV-B and UV-C were irradiated before transplantation (Rai et al. 2011). Improvement in bioactive compounds occurred, with significant induction of key genes in the biosynthetic pathway after UV irradiation.

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Blue and red spectra irradiation increased the contents of artemisinin and its derivatives via upregulation of biosynthesis genes and suppression of enzymes in the competitive pathway (Zhang et al. 2018).

The implication of light quality provided a method for significant improvement of bioactive and other useful compounds in plants. Effects of different light wavelengths on transcriptomic responses of biosynthetic genes have previously been reported but data on proteomic responses of plants to light signals remain limited. Previous studies mainly emphasized the role of light in regulating light sensory proteins (photoreceptor proteins) such as UV-A/blue light receptors (Fuglevand et al. 1996; Lin 2002), red/far-red photoreceptors (Sharrock 2008; Legris et al. 2019), and UV-B photoreceptors (Hejde and Ulm 2012; Jenkins 2014), while the impacts of light signals on biosynthetic proteins have not been investigated in A. annua. Hence, here, comprehensive analyses of proteomic expression and the phytochemical fingerprint of volatile terpenoid compounds in A. annua were conducted to examine the proteomic and metabolomic responses of the plant to different light signals. Relationships between unique terpenoid proteins and putative terpenoid compounds in each specific light spectrum were also investigated.

Materials and methods

Plant material, growth conditions, and LED spectra irradiation

Artemisia annua seeds have been kindly provided by Dr. Chalermpol Kirdmanee (BIOTEC, NSTDA) since the year 2000, under the cooperative research project between Mahidol University and the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand (Supaibulwatana et al. 2004). The seeds were germinated and subsequently multiplied in the tissue culture lab at the Faculty of Science, Mahidol University. Seedlings of A. annua L. were maintained in vitro in solidified Murashige and Skoog (1962) medium supplied with 3% (w/v) sucrose and incubated at 25 ± 2°C and 50 ± 5% relative humidity and 200 ± 10 μmol m⁻² s⁻¹ light intensity with 16 h photoperiod. After 15 days, the plantlets were transferred to pots containing vermiculite, supplemented with sugar-free MS medium, and cultivated under 80 ± 5 μmol m⁻² s⁻¹ light intensity with 16 h photoperiod. Fresh leaves were harvested on day 7 and stored at −80°C until required for further analysis.

GeLC-MS/MS shotgun proteomics

Total protein was prepared using the modified protocol from Bryant et al. (2016). Protein content was determined by Bradford assay (1976) and 15 μg of total protein was used for gel packing with 12.5% polyacrylamide. The gel pieces were subjected to dehydration in 100% acetonitrile (ACN) and incubated with 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate (AmBic) at 56°C for 1 h. For the alklylation step, 100 mM iodoacetamide in 10 mM AmBic was added to the samples and incubated in the dark at room temperature (RT) for 45 min. Samples were then dehydrated with 100% ACN by shaking at RT for 5 min. Sequencing-grade trypsin (Promega, Germany) was added to the gels and incubated at 37°C overnight for in-gel digestion. Peptide products were extracted from the samples by adding 50% (v/v) ACN in 0.1% (v/v) formic acid, incubated at RT for 10 min, and dried at 45°C for 4 h. Tryptic peptides were protonated with 0.1% (v/v) formic acid before operating LC-MS/MS. The analysis was performed with three experimental replications and all experiments were conducted in duplicate.

Protein identification and bioinformatics

Data were quantified by MaxQuant 1.6.1.12 using the Andromeda search engine to correlate MS/MS spectra to the UniProt/Viridiplantae protein database (Bryant et al. 2016). A unique peptide with at least seven amino acids was required for protein identification and further quantification analysis. Biological relationships between proteomic data obtained from different LED treatments were analyzed using a Venn diagram (http://jvenn.toulouse.inra.fr/app/index.html) (Bardou et al. 2014). The non-overlapping proteins were further subjected to functional identification and classification based on their biological process and molecular function using UniProt identifiers (https://www.uniprot.org). The raw data were deposited in the jPOST repository (Okuda et al. 2016) under reference number PXD027227.

Phytochemical analysis of A. annua leaves by gas chromatography-mass spectrometry (GC-MS)

Fresh leaves of A. annua were ground into a fine powder in liquid nitrogen using a pestle and mortar. The sample powder was transferred to 10 mL glass tubes containing 2 mL of dichloromethane. After mixing, each glass tube containing a sample was sonicated for 15 min and centrifuged at 5000 rpm for 5 min. The supernatant was filtrated with a 0.45 μm syringe filter membrane and stored at −80°C until required for analysis. Phytochemical analysis of the leaf extract was performed by GC-MS under the following condition: the oven was programmed at 100°C for 1 min, then at 5°C/min to 150°C, at 2°C/min to 200°C, at 15°C/min to 300°C, and held for 15 min. Compounds were identified by comparison of retention time and fragments with standards. Mass spectral information was extracted and compared with the Wiley No.7 database to identify unknown compounds. Compounds with at least 80% match quality were relatively quantified against the internal standard (methyl heptadecanoate, C17) and represented as average relative contents (μg mL⁻¹).

Clustering analysis of terpenoid proteins and plant metabolites

To examine the correlation between the abundance of terpenoid compounds and unique terpenoid proteins under a particular light spectrum, hierarchical clustering analysis (HCA) was performed using MultiExperiment Viewer (MeV)
version 4.9.0 (Saeed et al. 2003). Relative contents of terpenoid compounds were obtained from GC-MS data and normalized to the amount of internal standard, while protein levels were obtained from maximum intensity of LC-MS/MS, normalized to log2 values. Grouping results were used to construct a dendrogram, with a heatmap representing the levels of proteins and terpenoid compounds.

Results

Differential expression of proteins in leaves, stems and roots of A. annua under different LED light spectra

GeLC-MS/MS shotgun proteomics were performed to examine how A. annua plants responded to LED light qualities and the number of proteins in leaves, stems, and roots of 7-day light-treated plants were investigated. The analysis revealed more than 20,000 proteins in each treatment. Highest total number of proteins was detected under red spectrum (25,987 proteins), followed by blue spectrum (24,556 proteins) and white spectrum (22,863 proteins). Comparative analysis using Venn diagrams revealed that numbers of proteins in leaves, stems and roots under white light condition were different. The highest proportion of protein (54.73%) was recorded in leaves of A. annua grown under white light; however, 3282 proteins were also found in stems and roots (Figure 1(a)). Among the total proteins, 2964, 4414, and 3701 were uniquely detected in leaves, stems, and roots, respectively. Leaves of blue light-treated plants also showed the highest protein response with 66.59% of total proteins detected. Under blue spectrum, 3744 proteins were commonly observed in all organs with 2368, 3522, and 2414 proteins uniquely expressed in leaves, stems, and roots, respectively. Similar to white and blue spectra, 81.53% of total proteins were reported in leaves of red light treatment. A Venn diagram demonstrated that leaves, stems, and roots of A. annua from red light treatment shared 14,462 common proteins with 2644 proteins only detected in leaves. Smaller numbers of organ-specific proteins were found in stems and roots of A. annua.

Functional analysis of uniquely expressed proteins in different organs of A. annua treated with different LED light spectra

Uniquely expressed proteins from each treatment were further characterized for their functional identifications and protein classifications using UniProt identifiers (https://www.uniprot.org). Proteins were classified based on their biological process and molecular function. Results revealed abundances of light-responsive proteins, photosynthetic proteins, and secondary metabolism-related proteins (Figure 1(b)). Light-responsive proteins comprised those with light stimulus-response [GO:0009416, GO:0071482], photosynthesis [GO:0015979], light reaction of photosynthesis [GO:0019684], red or far-red light signaling pathway [GO:0010017], blue light signaling pathway [GO:0009785], UV-B response [GO:0010224], and light intensity response [GO:0071484]. The highest number of proteins in this group was reported in stems of A. annua grown under white and blue spectra. By contrast, leaves showed the highest number of proteins when exposed to red spectrum. Photosynthetic proteins are the largest subdivision of light-responsive proteins, and light has been widely recognized for its central role in the photosynthesis process. Proteins involved in photosynthesis [GO:0015979], dark reaction [GO:0019685], light reaction [GO:0019684], light harvesting in photosystem I [GO:0009786], and the light-independent chlorophyl biosynthetic process [GO:0036068] were assembled in this group. Results revealed the presence of 100–200 photosynthetic proteins in leaves, stems, and roots of A. annua in each treatment. Expression patterns were similar to the spectral response of light-responsive proteins, with the highest numbers found in stems of A. annua under white and blue treatments but not in red treatment. Moreover, proteins related to oxidative stress response [GO:0006979] such as catalase (EC 1.11.1.6), glutathione S-transferase (EC 2.5.1.18), peroxidase (EC 1.11.1.7), superoxide dismutase (EC 1.15.1.1), and other scavenging enzymes were also detected, especially in stems of A. annua.

Proteins involved in plant metabolite production were also explored. This group included sterol [GO:0016126], alkaloid [GO:0009821], flavonoid [GO:0009813], and terpenoid [GO:0016114] biosynthetic proteins. Responses differed among the organs and light spectra. Under white light, leaves showed the highest number of non-overlapping proteins, while stems and roots expressed a similar number. Under blue spectrum, secondary metabolic proteins were highly expressed in stems followed by roots and leaves, respectively. In red light treatment, the highest number was found in leaves, with fewer proteins in stems and roots. Based on biological process and molecular function in UniProt protein identifiers, a total of 502 proteins found in all organs under all light conditions were classified as terpenoid proteins, consisting of 50 proteins in the MVA and MEP pathways, an isoprene synthase, 62 monoterpene synthases, 149 sesquiterpene synthases, 75 diterpene synthases, 74 triterpene synthases, 74 tetrasporene synthases, and 17 other terpene synthases (Table S1). Leaves of A. annua showed 45 unique proteins under white light treatment, while the other organs gave reduced numbers. Expression patterns of terpenoid proteins under blue spectrum were similar to those of secondary metabolic proteins, with the highest number of non-overlaps (49 proteins) found in stems, while red light showed the highest number of terpenoid proteins in leaves.

Accumulation of terpenoid-related proteins in leaves of A. annua under different light spectra

Different light qualities induced different numbers of terpenoid proteins in A. annua, and effects of light qualities were highlighted in leaves where terpenoid compounds were mainly produced. Analysis revealed a total of 422 terpenoid-related proteins. Leaves of A. annua exhibited high sensitivity to red LED light and 335 proteins (79.38% of total terpenoid proteins) were detected. The smallest number (260 proteins) was observed under blue LED light, approximately 61.6% of the total terpenoid proteins in leaves. A Venn diagram revealed 144 common proteins in all light treatments (Figure 1(c)), with 20, 31, and 53 proteins explicitly expressed under white, blue, and red spectra, respectively. Under white LED condition, 20 unique proteins were reported (Table 1), with a high proportion of MVA and MEP pathway-related proteins (25.0%) followed by sesquiterpene synthases (20.0%) and monoterpene synthases.
Out of five proteins in the MVA and MEP pathways, three related to the cytosolic MVA pathway and one was localized in the plastidic MEP pathway, while the rest were involved in the exchange of the common precursor between both pathways. Among these, diphosphomevalonate decarboxylase (Acc. No. O23722), reported in *Arabidopsis thaliana* (Cordier et al. 1999), presented at the highest level (Table 2). This protein catalyzes the first committed step in isopentenyl diphosphate (IPP) biosynthesis, leading to biosynthesis of isoprene-containing compounds such as sterols and terpenoids (Henry et al. 2015). The MEP-related protein, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (Acc. No. Q6EPN6) involved in the formation of isoprenoid intermediates, IPP and dimethylallyl diphosphate (DMAPP) via the MEP pathway (Huang et al. 2018), was also detected with relative expression value of 14.01. The highest level of isopentenyl diphosphate (IPP) biosynthesis, leading to biosynthesis of isoprene-containing compounds such as sterols and terpenoids (Henry et al. 2015). The MEP-related protein, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (Acc. No. Q6EPN6) involved in the formation of isoprenoid intermediates, IPP and dimethylallyl diphosphate (DMAPP) via the MEP pathway (Huang et al. 2018), was also detected with relative expression value of 14.01. The highest level of
terpenoid synthase was vetispiradiene synthase 2 (Acc. No. Q39979) that showed relative expression value of 19.19.

Light spectrum interfered with the numbers of unique terpenoid proteins, suggesting the role of this spectrum in regulating terpenoid proteins. Among several groups of terpenoid proteins, isoprene synthase (Acc. No. Q8S4Y1) was only detected under this spectrum. This protein is responsible for the conversion of DMAPP to isoprene, a building block of the MVA biosynthetic pathway. High numbers of functional proteins were involved in the biosynthesis of sesquiterpenes and tetraterpenes. Alpha-bisabolol synthase (Acc. No. E3W206) showed the highest protein level among sesquiterpene synthases. This protein was cloned and characterized from Santalum spicatum and showed its role in the formation of α-, β-, α-bisabolol, α-bisabolene and also farnesene isomers from FPP intermediate (Jones et al. 2011). A high proportion of tetraterpene synthases including phytoene synthase, 15-cis-phytoene desaturase, lycopene beta cyclase, beta-carotene 3-hydroxylase, and zeta-carotene desaturase were also detected in this condition.

**Table 1. Numbers and proportions of unique terpenoid proteins detected in leaves of A. annua grown under white (W), blue (B) and red (R) light spectra.**

| Protein group                  | W (445, 554 nm) | B (445 nm) | R (660 nm) |
|-------------------------------|----------------|------------|------------|
| MVA and MEP pathways          | 5 (25.0%)      | 7 (22.6%)  | 8 (15.1%)  |
| Isoprene synthases            | -              | -          | 1 (1.9%)   |
| Monoterpene synthases         | 3 (15.0%)      | 3 (9.7%)   | 5 (9.4%)   |
| Sesquiterpene synthases       | 4 (20.0%)      | 6 (19.4%)  | 11 (20.8%) |
| Diterpene synthases           | 2 (10.0%)      | 4 (12.9%)  | 7 (13.2%)  |
| Triterpene synthases          | 2 (10.0%)      | 4 (12.9%)  | 7 (13.2%)  |
| Tetraterpene synthases        | 2 (10.0%)      | 4 (12.9%)  | 10 (18.9%) |
| Other terpene synthases       | 2 (10.0%)      | 3 (9.7%)   | 4 (7.5%)   |
| Total proteins                | 20 (100%)      | 31 (100%)  | 53 (100%)  |

Light spectrum interfered with the numbers of terpenoid proteins as well as the production of terpenoid compounds. Variations in proteins and terpenoids were observed among different light conditions. Therefore, we presumed that the light spectrum regulated the terpenoid biosynthetic pathway through the activation of particular proteins. To clarify the effect of light spectrum on alteration of terpenoid biosynthesis, localizations of functional proteins were illustrated in the MVA, MEP, and terpenoid biosynthetic pathways of A. annua plants under a particular light spectrum (Figures 2–4).

**LED spectra affected plant metabolite production in leaves of A. annua**

Light qualities have shown their important role in regulating plant metabolite production in several plant species. Numbers of metabolic proteins were reported to be influenced by light spectra. Here, we determined how plant metabolite production responded to light signals. The presence of mono-, sesqui-, di- and triterpenoids was analyzed by GC-MS. Phytochemical profiles differed depending on light compositions. Analysis results revealed 28 volatile terpenoid compounds with more than 80% match quality to the Wiley 7 No.1 Library, comprising 8 monoterpenes, 11 sesquiterpenes, 2 diterpenes and 7 triterpenes (Table 3).

Similar numbers of monoterpenoid compounds were observed in all light spectra; however, camphor content was high (23.5 µg mL⁻¹ relative content), especially under blue spectrum. Maximum total content of sesquiterpenes was obtained from blue light treatment. Germacrene-D was the most abundant compound with 24.5 µg mL⁻¹ relative content, approximately 2 times the lowest content under red LED treatment. Variations were observed among the C15 compounds as the artemisinin derivative deoxyqinghaosu only found under blue spectrum irradiation. Two diterpenes, neop孵化dene and phyto, also showed highest contents under this spectrum. Similar to other terpenoids, highest content of triterpenoids was found in blue light treatment. Among seven triterpenes reported in this study, beta-amesone was found with greatest abundance under blue light followed by white and red spectra, respectively. Correlation between the abundance of volatile terpenoids and light spectrum was assessed by hierarchical clustering analysis (HCA) with Pearson correlation (Figure 1(d)). The heatmap showed that terpenoid profiles of A. annua grown under white and blue spectra were strongly clustered, while the red spectrum profile was separately clustered.

**Distribution of functional proteins in artemisinin biosynthetic pathway of A. annua**

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Table 2. Identification of uniquely expressed terpenoid proteins and their expression levels in leaves of A. annua grown under different light spectra.

| Protein type | Accession number | Protein name | 445, 554 nm | 445 nm | 660 nm | Previous report of identified protein found in plants |
|--------------|-----------------|--------------|-------------|-------|--------|-----------------------------------------------------|
| MEP and MVA pathways | O64967 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 | 13.91 | – | – | Gossypium hirsutum* |
| | P48020 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 | 15.00 | – | – | Arabidopsis thaliana (Cordier et al. 1999) |
| | Q23722 | Diphosphomevalonate decarboxylase | 18.22 | – | – | Arabidopsis thaliana (Choi et al. 1992) |
| | Q6EPN6 | 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase | – | 14.01 | – | Oriza sativa subsp. japonica (International Rice Genome Sequencing Project and Sasaki 2005) |
| | Q39471 | Isopentenyl-diphosphate Delta-isomerase II | – | 14.31 | – | Clarkia breweri* |
| Isoprene synthases | P46086 | Mevalonate kinase | – | 14.43 | – | Arabidopsis thaliana (Riou et al. 1994) |
| | Q24594 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase | – | 14.46 | – | Zea mays* |
| | Q41437 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 | – | 15.30 | – | Solanum tuberosum (Korth et al. 1997) |
| | Q01559 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase | – | 15.71 | – | Nicotiana sylvestris (Genschik et al. 1992) |
| Monoterpene synthases | Q854Y1 | Acetyl-CoA acetyltransferase | – | 15.99 | – | Arabidopsis thaliana (Lluch et al. 2000) |
| | Q39664 | Isopentenyl-diphosphate Delta-isomerase II | – | 11.82 | – | Clarkia xantiana* |
| | Q8W250 | 1-deoxy-D-xylulose 5-phosphate reductoisomerase | – | 12.20 | – | Oriza sativa subsp. japonica (Yu et al. 2005) |
| Sesquiterpene synthases | F4JCU3 | Diphosphomevalonate decarboxylase | – | – | 13.12 | Arabidopsis thaliana (Henry et al. 2015) |
| | P54873 | Hydroxymethylglutaryl-CoA synthase | – | – | 13.57 | Arabidopsis thaliana (Montant et al. 1995) |
| | Q00583 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase 3 | – | – | 14.57 | Hevea brasiliensis (Chye et al. 2001) |
| | Q41438 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase 3 | – | – | 16.69 | Solanum tuberosum (Korth et al. 1997) |
| | P56848 | 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase | – | – | 16.77 | Mentha piperita (Lange and Croteau 1999) |
| | Q48965 | Isopentenyl-diphosphate Delta-isomerase II | – | – | 15.97 | Camptotheca acuminata* |
| | P93841 | 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase | – | – | 16.39 | Solanum lycopersicum (Rohdich et al. 2000) |
| | Q6AVG6 | 4-hydroxy-3-methylbut-2-enyl diphosphate reductase | – | – | 18.30 | Oriza sativa subsp. japonica (Okada et al. 2007) |
| Diterpene synthases | Q50L36 | Isoprene synthase | – | – | 16.99 | Populus alba (Sasaki et al. 2005) |
| | Q947B7 | Menthofuran synthase | 13.84 | – | – | Mentha piperita (Bertia et al. 2001) |
| | Q5UB07 | Tricyclene synthase TP54 | 15.51 | – | – | Medicago truncatula (Gomez et al. 2005) |
| | Q5SSP2 | Endo-fenchol synthase | 16.27 | – | – | Ocimum basilicum (Iijima et al. 2004) |
| | Q6WQJ0 | Pulegone reductase | – | 15.39 | – | Mentha piperita (Ringer et al. 2003) |
| | Q4A757 | Pinene synthase | – | 15.74 | – | Abies grandis (Bolhmann et al. 1997) |
| | P0C565 | Chrysanthemol synthase | – | 16.36 | – | Tanacetum cinerariifolium (Rivera et al. 2001) |
| | F1C06 | Carene synthase 1 | – | – | 14.10 | Picea sitchensis (Hall et al. 2011) |
| | F1C09 | Carene synthase 3 | – | – | 14.98 | Picea sitchensis (Hall et al. 2011) |
| | Q8AKL3 | Pinene synthase | – | – | 15.18 | Pinus taeda (Phillips et al. 2003) |
| | Q8AN49 | Tricyclene synthase 1e20 | – | – | 16.47 | Anarthria magnus (Dudareva et al. 2003) |
| | Q5SSP8 | Terpinolene synthase | – | – | 17.26 | Ocimum basilicum (Iijima et al. 2004) |
| | C75W0 | beta-farnesene synthase | 13.33 | – | – | Zea perennis (Kollner et al. 2009) |
| | DS9U8 | Germacrene A hydroxylase | 14.55 | – | – | Lactuca sativa (Nguyen et al. 2010) |
| | Q5GJ60 | 5(-)-beta-macrocarpene synthase | 16.91 | – | – | Zea mays (Kollner et al. 2008) |
| | Q3P979 | Vetispiradiene synthase 2 | 19.19 | – | – | Hyoscyamus muticus (Back and Chappell 1995) |
| | I6QPS3 | Germacrene D synthase | – | 13.23 | – | Matricaria chamomilla var. recutita (Linnisch et al. 2012) |
| | GSCV46 | Viridiflorene synthase | – | 13.72 | – | Solanum lycopersicum (Bleecker et al. 2011) |
| | B7PQ6 | Beta-cubebene synthase | – | 13.82 | – | Magnolia grandiflora (Lee and Chappell 2008) |
| | O48261 | Germacrene C synthase | – | 13.91 | – | Solanum lycopersicum (Colby et al. 1998) |
| | P49352 | Farnesy1 pyrophosphate synthase 2 | – | 15.32 | – | Lupinus albus (Attucci et al. 1995) |
| | Q4LF0 | 5-epi-alocholesterol synthase 2 | – | 15.54 | – | Nicotiana attenuate (Bolhmann et al. 2002) |
| | Q67NN7 | Farnesol kinase | – | 13.41 | – | Arabidopsis thaliana (Fitzpatrick et al. 2011) |
| | D8RNZ9 | (3S,6E)-nerolidol synthase 1 | – | 14.97 | – | Selaginella moellendorffii (Li et al. 2012) |
| | Q67L0 | Longifolene synthase | – | – | 15.10 | Picea abies (Martin et al. 2004) |
| | F6M8H4 | Probable sesquiterpene synthase | – | 15.33 | – | Santalum album (Jones et al. 2011) |
| | JTLH11 | (+-e)-alpha-bisabolyl synthase | – | 15.33 | – | Phyla dulcis (Atta et al. 2012) |
| | Q95PS6 | Germacrene D synthase 2 | – | 15.68 | – | Pogostemon cablin (Deguerry et al. 2006) |
| | Q3P978 | Vetispiradiene synthase 1 | – | 16.00 | – | Syzygium muticu (Back and Chappell 1995) |
| | F6M8H7 | Probable sesquiterpene synthase | – | – | 17.05 | Santalum murrayanum (Jones et al. 2011) |
| | B6SCF6 | Germacrene A synthase | – | 17.51 | – | Humulus lupulus (Wang et al. 2008) |
| | P0CV96 | (3S,6E)-nerolidol synthase 1 | – | – | 17.57 | Fragaria vesca (Aharoni et al. 2004) |
| | E3W206 | Alpha-bisabolyl synthase | – | – | 18.09 | Santalum spicatum (Jones et al. 2011) |
| | A4KAG8 | Epi-isokaur-15-ene synthase | – | 12.42 | – | Oriza sativa subsp. japonica (Xu et al. 2007) |
| | Q69SX8 | Geranylgeranyl pyrophosphate synthase synthase 11 | – | 15.37 | – | Arabidopsis thaliana (Cheng et al. 2017) |

(Continued)
induced by red spectrum. Furthermore, 10 proteins involved in carotenoid biosynthesis were explicitly expressed in this condition. Proteins in the MVA and MEP pathways and di- and triterpene biosynthesis were also induced under this particular wavelength.

**Discussion**

Light signals play important roles in regulating terpenoid production of *A. annua*, which could be monitored in terms of transcriptomic expressions correlated with target terpenoid compounds (Zhang et al. 2018; Lopes et al. 2020), however, there has no obvious report on enzymes (proteins) involved in the metabolic flux of cytosolic MVA and plastidic MEP pathways of terpenoid biosynthesis. Zhang et al. (2018) previously described the effect of LED spectra at 470 and 670 nm on promoting artemisinin and artemisinic acid contents over those illuminated with 545, 445, and 660 nm respectively. Previous report of identified protein found in plants (Swaminathan et al. 2009) and (Cheng et al. 2017).

**Table 2. Continued.**

| Protein type | Accession number | Protein name | Relative expression (log2) 445, 554 nm | Relative expression (log2) 445 nm | Relative expression (log2) 660 nm | Previous report of identified protein found in plants |
|--------------|------------------|--------------|----------------------------------------|-----------------------------------|-----------------------------------|------------------------------------------------------|
| Triterpene synthases | Q9FH66 | Cytochrome P450 71A16 (Marneral oxidase) | – | 15.86 | – | 15.82 |
| | F8WQD0 | Shionone synthase | 15.91 | – | – | – |
| | A0A125XN3 | Cycloartenol synthase LCA | – | 12.14 | – | – |
| | O65727 | Squalene monoxygenase 1,1 | – | 12.87 | – | – |
| | E2UJ7 | Gliotanol synthase | – | 13.47 | – | – |
| | AR9C80 | Geranial synthase | – | 15.22 | – | – |
| | G0Y286 | Precalane diphenol synthase | – | 13.60 | – | – |
| | Q9FJ0 | Marneral synthase | – | 14.53 | – | – |
| | Q9FZ02 | Lupenol synthase 5 | – | 15.20 | – | – |
| | Q9LHR7 | Mixed-amyrin synthase | – | 15.44 | – | – |
| | A0A125XN11 | Pre-alpha-tocorin synthase LLC | – | 16.55 | – | – |
| | Q95LP9 | Cycloartenol synthase | – | 17.09 | – | – |
| | Q6BE25 | Cycloartenol synthase | – | 17.66 | – | – |
| Tetraterpene synthases | P49293 | Phytene synthase | 14.65 | – | – | – |
| | Q52Q93 | Prolycopene isomerase 1 | 18.32 | – | – | – |
| | P37273 | Phytene synthase 2 | 13.77 | – | – | – |
| | Q40424 | Lycopene beta cyclase | 14.48 | – | – | – |
| | P37271 | Phytene synthase | 14.51 | – | – | – |
| | Q39982 | Beta-carotene ketolase | 15.50 | – | – | – |
| | D9L23 | Lycopene beta cyclase | 11.03 | – | – | – |
| | Q5S3M3 | Zeta-carotene desaturase | 14.05 | – | – | – |
| | P80003 | 15-cis-phytoene desaturase | 14.71 | – | – | – |
| | Q40406 | 15-cis-phytoene desaturase | 14.72 | – | – | – |
| Other synthases | B9DFU2 | Cytochrome P450 71A1 | 15.45 | – | – | – |
| | B9PR03 | Probable terpen synthase 9 | 18.22 | – | – | – |
| | B9RZ03 | Probable terpen synthase 12 | 15.52 | – | – | – |
| | Q9LH31 | Terpenoid synthase 30 | 16.13 | – | – | – |
| | Q9LUE2 | Terpenoid synthase 18 | 17.15 | – | – | – |
| | B9SIN2 | Probable terpen synthase 8 | 13.51 | – | – | – |
| | Q9LVY7 | Cytochrome P450 71A1 | – | 14.93 | – | – |
| | Q9C748 | Terpenoid synthase 28 | – | 15.16 | – | – |
| | Q9FF52 | Terpenoid synthase 2 | – | 18.26 | – | – |

Note: Protein identification and annotation were analyzed by UniProt identifiers (https://www.uniprot.org). * Protein was registered in UniProt protein identifiers but not published in PubMed.
Note: The metabolites detected from 7-day-treated leaves were determined by GC-MS and quantitatively analyzed by comparison with the internal standard (methyl heptadecanoate, C17) and represented as average relative contents (µg mL⁻¹). Only compounds with more than 80% match to the Wiley No.7 database were shown. W; leaf extract from PFAL with white spectrum (445, 554 nm), B; leaf extract from PFAL with blue spectrum (445 nm), R; leaf extract from PFAL with red spectrum (660 nm).

| Terpene types | Compound name | Light spectrum of LED lamp |
|---------------|---------------|---------------------------|
| Monoterpene (C10) | α-pinene | 0.8 ± 0.0 | 0.4 ± 0.1 | 1.0 ± 0.1 |
| Camphene | 2.9 ± 0.1 | 2.1 ± 0.1 | 2.2 ± 0.2 |
| 2-β-pinene | 1.5 ± 0.2 | 0.7 ± 0.1 | 1.2 ± 0.2 |
| Cymene | 1.5 ± 0.1 | 0.6 ± 0.2 | 1.3 ± 0.1 |
| Eucalyptol | 2.0 ± 0.3 | 2.2 ± 0.1 | 1.2 ± 0.2 |
| Camphor | 17.0 ± 0.7 | 23.5 ± 1.8 | 18.5 ± 0.7 |
| Bornol | 2.3 ± 0.2 | 3.8 ± 0.1 | 1.2 ± 0.1 |
| Santolina triene | 1.5 ± 0.2 | 1.3 ± 0.2 | 1.0 ± 0.1 |
| β-Caryophyllene | 1.1 ± 0.2 | 1.0 ± 0.2 | 1.1 ± 0.1 |
| Caryophyllene | 10.0 ± 0.8 | 7.2 ± 0.7 | 10.4 ± 1.4 |
| Sesquiterpene (C15) | Germacrene D (I) | 1.8 ± 0.3 | 1.9 ± 0.3 | 1.2 ± 0.3 |
| Germacrene D (II) | 0.8 ± 0.0 | 0.6 ± 0.1 | 0.6 ± 0.1 |
| Germacrene B | 3.2 ± 0.2 | 3.7 ± 0.9 | 1.8 ± 0.4 |
| β-Caryophyllene | 1.7 ± 0.0 | 1.7 ± 0.2 | 1.0 ± 0.1 |
| Trans-β-Farnesene | 11.3 ± 0.7 | 16.3 ± 2.0 | 9.6 ± 0.9 |
| Beta-caryophyllene | 1.5 ± 0.1 | - | - |
| Germacrene D (III) | 21.6 ± 1.5 | 24.5 ± 1.9 | 11.9 ± 1.3 |
| Aromadendrene | 0.9 ± 0.1 | 0.5 ± 0.1 | 0.7 ± 0.0 |
| Germacrene B | 3.2 ± 0.2 | 3.7 ± 0.9 | 1.8 ± 0.4 |
| o-Cedrol | - | - | 1.0 ± 0.1 |
| Deoxyginghaosus | - | 5.8 ± 0.8 | - |
| Diterpene (C20) | Neophytadiene | 4.7 ± 0.5 | 6.3 ± 0.1 | 2.2 ± 0.1 |
| Phytol | 4.1 ± 0.5 | 3.7 ± 0.9 | 3.4 ± 0.2 |
| Triterpene (C30) | Squalene | 3.3 ± 0.3 | 2.7 ± 0.7 | 2.3 ± 0.4 |
| Ergosterol | 0.7 ± 0.1 | 0.9 ± 0.2 | 1.0 ± 0.1 |
| Stigmastanol | 4.7 ± 0.5 | 5.1 ± 0.6 | 4.8 ± 0.4 |
| o-Amyrene | 11.4 ± 0.6 | 14.5 ± 0.8 | 12.0 ± 1.2 |
| β-Amyrene | 21.2 ± 1.7 | 23.4 ± 2.0 | 18.2 ± 1.3 |
| o-Amyrone | 1.1 ± 0.3 | 0.8 ± 0.3 | 1.4 ± 0.2 |
| Lupeol | 1.1 ± 0.2 | 0.3 ± 0.0 | 1.1 ± 0.3 |

Table 3. Phytochemical proles obtained from leaf extracts of A. annua grown under white (W), blue (B) and red (R) LED spectra.

Our study firstly demonstrated the evidence of enzymes (proteins) involved in the metabolic flux of cytosolic MVA and plastidic MEP pathways of terpenoid biosynthesis. The different numbers of proteins were detected in leaves, stems, and roots of A. annua at 7 days after LED light exposure (Figure 1(a)). As expected, variations in proteomic profiles were observed among different organs of A. annua treated with different light spectra. Fully exposed leaves exhibited the highest total number of proteins under LED light spectra irradiation, suggesting leaves as the most responsive organ in response to light exposure. Our results demonstrated that proteins involved in the molecular mechanism of light-sensing and light response such as photoreceptors and photosynthesis were mainly expressed. Although light is an important factor for plant growth and development, the adverse effect of light as an oxidative stress-inducing factor was also reported (Barber and Anderson 1992; Li et al. 2009; Dinakar et al. 2012). The high presence of detoxification enzymes in all light conditions suggested the role of LED spectra as stress-inducing factors. However, oxidative stress that occurred in this study did not interfere with physiological processes since proteins involved in photosynthesis were also noticed.

Since the leaves were considered as the main sources of terpenoid production, the effect of the LED light spectrum on the number of terpenoid proteins in leaves of A. annua was highlighted. A total of 289, 260, and 335 terpenoid proteins were reported in leaves of A. annua treated with white, blue, and red spectra, respectively. Most terpenoid proteins...
responded to a particular light spectrum in a similar manner but differential proteins were noticeable for 20, 31, and 53 proteins uniquely expressed in white, blue, and red light treatments, respectively (Figure 1(c)). Interestingly, high numbers of MVA and MEP pathways-related proteins and sesquiterpene synthases were found under white and blue light treatments (Figures 2–3). The red spectrum influenced sesquiterpene and tetraterpene (carotenoid) biosynthesis (Figure 4). Our study demonstrated that the specific wavelength of 445 nm present in white and blue spectra exhibited similar terpenoid profiles, possibly due to the activity of photoreceptors in the explicit region of the light spectrum (Kong and Okajima 2016; Mawphlang and Kharshing 2017). Influences of LED light signals on expression patterns of biosynthetic proteins and terpenoid compounds were demonstrated. Among all LED spectra, the red spectrum was the most effective condition for regulating terpenoid proteins. HCA revealed that sesquiterpenes were
highly responsible for induction of terpenoid proteins in each light treatment. A bicyclic sesquiterpene, β-chamigrene, showed strong correlation with the expression level of terpenoid proteins under white spectrum (Figure 2(a,c)). This compound exhibited promising biological properties such as antibacterial activities and cytotoxic activities against HeLa and HEP-2 cancer cell lines (Dias et al. 2005; Antonsen et al. 2014). Deoxyxignhaosu positively correlated with the expression level of unique terpenoid proteins under blue spectrum (Figure 3(a,c)). Although it is an inactive form of artemisinin and has no antimalarial activity against Plasmodium berghei (Li 2012), our previous study revealed a strong correlation between deoxyxignhaosu and increased antimalarial activity against P. falciparum NF54 (unpublished data). Alpha cedrol was only detected in red light condition (Figure 4(a,c)). This compound has been found to exert antioxidant, anti-inflammatory, antimicrobial, and antifungal activities along with cytotoxic effects against some cancer cells (Loizzo et al. 2008; Khoury et al. 2014; Wang et al. 2019; Mishra et al. 2021). Accordingly, specific induction of terpenoid proteins and correlated sesquiterpenes were conceivably caused by LED spectra. These will provide more understanding of light signals on metabolic proteins in regulating the overall pathway of terpenoid biosynthesis under particular light signals. To support and further validate our results, a transcriptomic study of biosynthetic enzymes is required.

Conclusions
This study demonstrated the integration of metabolomic and proteomic analyses to investigate the responses of A. annua plants under different light spectra. An LC-MS/MS platform combined with functional analysis revealed differential inductions of light-responsive proteins, oxidative stress-related proteins, and metabolite biosynthetic enzymes in all tested organs. Among these, 422 terpenoid-related proteins were detected in leaves, with some unique proteins for each treatment. Twenty proteins were explicitly expressed under white spectrum, while 31 proteins were only found in blue spectrum treatment. Unexpectedly, red light had more extensive effects on terpenoid protein inductions, especially the protein involved in sesquiterpene and tetraterpene biosynthesis. Integrated metabolomics and proteomics revealed possible roles of the light spectrum in regulating production of sesquiterpenoid compounds. HCA revealed specific correlations between terpenoid proteins and sesquiterpenes under a particular light spectrum. Our results suggested light quality as an important factor for creating proteomic variations, leading to differences in terpenoid production. This information can be applied for the functional study of light-inducing proteins in terpenoid biosynthesis to provide improvement of important compounds for pharmaceutical use.

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Data availability statement
All data generated in this study were deposited in the jPOST repository at https://repository.jpostdb.org/entry/JPST001250, reference number PXD027227.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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