Yun *et al. Appl Biol Chem* (2021) 64:26
https://doi.org/10.1186/s13765-021-00598-2

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ARTICLE Open Access

Metabolomics study for exploring metabolic perturbations in soybean adventitious roots by fluorescent light irradiation

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Abstract

Soybeans are the most popular cultivated crops worldwide. They contain abundant functional components. As part of the research to enhance functional components in soybean plants, soybean adventitious roots were cultured under dark and fluorescent light irradiation conditions and difference in their metabolome was explored using ^{1}H NMR-based metabolomics approach. Results revealed that fluorescent light irradiation increased the biomass of soybean adventitious roots and caused considerable metabolic perturbations. In particular, health-beneficial secondary metabolites such as soyasaponin (3.4-fold), isoflavones (3.9-fold), and coumestrol derivatives (1.3-fold) were accumulated more in soybean adventitious roots grown under fluorescent light irradiation than in those grown under a dark condition due to increased photosynthesis that was evidenced by increased levels of glucose. The present study provides useful information on global metabolite compositions of soybean adventitious roots and their quality improvement by controlling growth conditions to enhance functional potentials of soybeans.

Keywords: Metabolomics, Nuclear magnetic resonance (NMR), Soybean adventitious root, Fluorescent light irradiation

Introduction

Soybeans are major nutritious food sources. They are also functional materials. Soybeans contain diverse bioactive compounds, such as flavonoids, isoflavones, and soyasaponins, thus carrying beneficial effects on human health [1]. For example, soybeans may reduce the risk of prostate cancer, colon cancer, and breast cancer, osteoporosis, and other bone health problems. They can also alleviate menopause symptoms [2, 3]. Thanks to soybeans for their high values and functional ingredients for health, many applications of soybean-based products have gained popularity in various industry fields, including functional foods and cosmetics.

In recent years, soybean adventitious root culture has drawn attention of researchers due to its high demand for industrial usages such as food ingredients because soybean roots also contain various valuable phytochemicals such as daidzein, genistein, coumestrol, and their derivatives conjugated with glycosides [4]. In particular, coumestrol and its derivatives have beneficial effects such as anti-cancer [5], anti-obesity [6], and anti-cardiotoxicity [7] effects and for collagen production [8]. The adventitious root culture technique has been used in other plant species for the production of valuable phytochemicals [9]. For example, ginseng adventitious root culture has been used for mass production of ginsenosides [10]. A recent study has also reported that soybean adventitious root culture could be used for the production of coumestrol [8]. Therefore, it is important to develop soybean adventitious root culture techniques for increasing root biomass, improving amounts of phytochemicals, and

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better understanding the metabolism in soybean adventitious roots.

In this respect, a strategy of elicitation has been used to enhance the productivity of adventitious root culture [11]. Among various elicitors, methyl jasmonic acid and salicylic acid have been frequently used during the culture of adventitious or hairy roots of various plants including ginseng [12] and *Scopolia parviflora* [13]. It is well known that light is one of major elicitors that can enhance the production of valuable phytochemicals, such as flavonoids in *Hypericum perforatum* [14] and *Stevia rebaudiana* [15]. However, a comprehensive study on metabolic perturbation of soybean adventitious roots grown under different culture conditions has not been reported yet.

Metabolomics is a study that comprehensively and systematically deals with a series of chemical processes involving various metabolites in biological samples such as cells, tissues, animals, human, and plants [16]. Along with advanced analytical techniques, computer programs, and statistical methods to process massive metabolic data, metabolomics enables researchers to better understand changes of metabolic phenomena by analyzing compositions and concentrations of diverse and comprehensive metabolites in living organisms. It is expected that metabolomics would greatly help us manage crop cultivations and discover hidden mechanisms and potential effects of compounds according to specific physiological conditions [17]. Nuclear magnetic resonance (NMR)-based metabolomics is one of ideal approaches for metabolic analysis. It has substantial advantages, including simple preparation and short analysis time. In addition, it is not destructive or selective. Moreover, it is highly reproducible [18].

Therefore, we cultured soybean adventitious roots under fluorescent light irradiation conditions to enhance the biomass and metabolic productivity of the adventitious roots. Their internal metabolism was then explored by ^{1}H NMR-based metabolomics to comprehensively understand metabolite changes of the roots grown under fluorescent light irradiation conditions.

Materials and methods

Chemicals

For NMR analysis, methanol-d_4 (CD_3OD, 99.8%) and deuterium oxide (D_2O, 99.9%) were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Soybean adventitious root induction and continuous bioreactor culture

Adventitious root culture was carried out according to Lee et al. [8]. Soybean cultivar used in this study was *Glycine max* Sinhwakong. Adventitious roots were directly induced from in vitro seedling derived from mature seeds. Seeds germinated on full strength Murashige and Skoog medium (MS medium; Duchefa, Haarlem, The Netherlands) supplemented with 30 g/L sucrose and 2.3 g/L Gelrite™ (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) [19]. After each seedling was grown to 10 cm, cotyledons, hypocotyls, and radicles were excised from seedlings and transferred to full strength MS medium supplemented with 4 mg/L of indole-3-butyric acid (IBA; Sigma Aldrich, Merck KGaA, Darmstadt, Germany) to induce adventitious roots. These induced adventitious roots were propagated in full strength MS medium containing 4 mg/L IBA without Gelrite™ in 3-L bulb-type bubble bioreactors. Bioreactor culture was initiated by inoculation with wet adventitious roots at a density of 4.0 g/L. The aeration volume in each bioreactor was adjusted to 0.1 vvm (air volume/culture volume per min) using an air flow meter (RMA series; Dwyer Instruments Inc., Michigan, USA). Adventitious roots were maintained by sub-culturing in wet liquid medium every three weeks in the dark (control group) or fluorescent light irradiation (experimental group) condition at 22 ± 1 °C. The control group was cultured in the dark condition for 28 days. In general, since photosynthesis photon flux density (PPFD) in in vitro plant cell culture system ranges from 20 to 100 μmol/s/m^2, the experimental group was alternately exposed to 60 PPFD via fluorescent light irradiation with fluorescent light lamp (OSRAM FHF32W/865, OSRAM GmbH Co., China). The common light cycle used in the plant cell culture system was employed in the current study, for example, 16 h per day and a dark condition for 8 h per day for a total of 28 days (four weeks). These conditions did not affect the growth of the roots in the preliminary study. After 28 days, adventitious root samples were collected and stored in a deep freezer at − 80 °C. For NMR analysis, adventitious root samples were freeze-dried and ground with a mortar and pestle under liquid nitrogen. These ground samples were transferred into Eppendorf (Ep) tubes using spatulas for extraction.

^{1}H NMR spectroscopic analysis of soybean adventitious root extracts

Metabolite extraction of soybean adventitious root samples for ^{1}H NMR analysis was carried out according to Kim et al. [17]. Each freeze-dried and ground sample (100 mg) was dissolved in a mixture of methanol-d_4 (CD_3OD, 700 μL) and deuterium water (D_2O, 300 μL) in a 2 mL Ep-tube. The mixture was sonicated at room temperature for 20 min to extract metabolites of adventitious roots and then centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant of each sample (550 μL) was transferred into a 5 mm NMR tube. CD_3OD in the

supernatant provided a field frequency lock. Glucose was used as a chemical shift reference (^{1}H, δ 5.23). ^{1}H NMR spectra were acquired on a Bruker Avance 700 spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a frequency of 700.40 MHz ^{1}H and a temperature of 298 K, using a cryogenic triple-resonance probe and a Bruker automatic injector. Signal assignment for representative samples was facilitated by two-dimensional (2D) total correlation spectroscopy (TOCSY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), and spiking experiments.

NMR data processing and multivariate statistical analysis

All NMR spectra were manually corrected for phase and baseline distortions and then converted to ASCII format. Files in ASCII format were imported into MATLAB (R2010b; The Mathworks Inc., Natick, MA, USA). After calibrating using glucose (^{1}H, δ 5.23), spectra were further aligned using the *i*coshift method [20]. Spectra were normalized to a total integral to avoid dilution effects of samples. Full-resolution ^{1}H NMR spectra without spectrum bucketing or binning were used for multivariate statistical analysis, after excluding several regions corresponding to TSP (δ 0–0.5 ppm) and methanol (δ 3.37–3.39 ppm) before normalization and spectrum alignment. Resulting data sets were then imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden), and a mean-centered scaling method was applied for multivariate statistical analysis. Principal component analysis (PCA), an unsupervised pattern recognition method, was initially performed to examine intrinsic variation in the dataset. Orthogonal projection on the latent structure-discriminant analysis (OPLS-DA), a supervised pattern recognition method, was used to extract maximum information on discriminant compounds from the data [21]. OPLS-DA provides a way of removing systematic variation from an input data set X (compounds or metabolites), which is not correlated with the response set Y (discriminant classes) [22]. Hotelling's T^2 regions, shown as an ellipse in the scores plot, defined 95% confidence interval of the modeled variation. OPLS-DA models were validated using a sevenfold cross-validation method and a permutation test with 200 iterations. To facilitate interpretation of results and identify metabolites responsible for metabolic discriminations between two classes, OPLS loading or coefficient plots were generated with a color-coded correlation coefficient for each data point using MATLAB with scripts developed at Imperial College London [23]. The quality of the model was described by R^2X and Q^2 values. R^2X was defined as the proportion of variance in the data explained by the models. It indicated the goodness of fit. Q^2 was defined as the proportion of variance in the data predictable by the model. It indicated predictability. Metabolic network analysis was conducted using MetaboAnalyst (https://www.metaboanalyst.ca) [24].

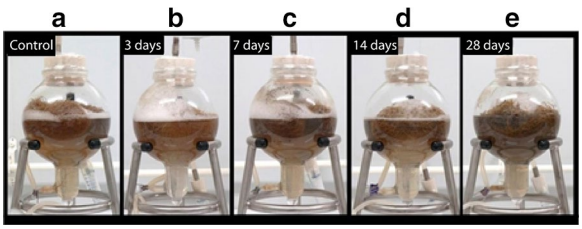

Fig. 1 Soybean adventitious roots cultured under a dark condition for 28 days (**a**, Control) or under a fluorescent light irradiation condition for 28 days (**b**–**e**)

Table 1 Biomass of soybean adventitious root

Treatments	Wet weight (g/L)	Dry weight (g/L)	Fold change
Dark	47.9 ± 3.44	4.2 ± 0.31	–
Fluorescent light	59.2 ± 3.48*	5.8 ± 0.55*	1.24

Asterisk represents significant difference between soybean adventitious roots grown under a dark and a fluorescent light irradiation condition determined by Student's *t*-test at $P < 0.05$

Statistical analysis

All results are expressed as means ± standard deviations (SDs) with ten independent measurements. Significant difference was calculated by SPSS one-way ANOVA followed by Duncan's test or Student's *t*-test; *P* values < 0.05 were considered to be significant. Paired Student's *t*-test was used to determine the significance of metabolite differences observed in OPLS coefficient or loading plot for pairwise comparison, in which the relative quantification of metabolites in ^{1}H NMR spectra was calculated with the integral area of each peak corresponding to the metabolite.

Results and discussion

Adventitious root growth

Bioreactor culture is widely used to increase productivity or biomass in plant cell cultures. Figure 1 shows soybean adventitious roots cultured in bioreactors for 28 days. Fluorescent light affected the biomass of adventitious roots. As shown in Table 1, wet and dry biomasses of soybean adventitious roots cultured under a dark condition were 47.9 ± 3.44 and 4.2 ± 0.31 g/L, respectively. Wet and dry biomasses of soybean adventitious roots cultured under a fluorescent light irradiation condition were 59.2 ± 3.48 and 5.8 ± 0.55 g/L, respectively.

Soybean adventitious roots cultured under a fluorescent light irradiation condition were significantly heavier than those cultured under a dark condition for both wet and dry weights. Similar to the present result, it has been reported that the growth of cultured hairy roots of *Artemisia annua* L. is increased when they grow under a fluorescent light [25]. On the contrary, fluorescent light had an inhibitory effect on the growth of ginseng hairy roots [10] and *Hypericum perforatum* adventitious roots [14]. In the case of *Scutellaria lateriflora,* the effect of fluorescent light on biomasses of its cultured hairy roots varied according to compositions of the culture medium [26]. These results suggest that fluorescent light has positive effects on the growth of soybean adventitious roots, although effects of fluorescent light irradiation on the growth may depend on plant species.

Metabolites in cultured soybean adventitious roots

Figure 2 shows representative ^{1}H NMR spectra of soybean adventitious roots. A wide range of metabolites, such as alanine (Ala), acetate (Ac), allantoin (All), allantoate (AllA), asparagine (Asn), choline (Chol), coumestrol derivatives (CMT1 and CMT2), daidzein derivatives (DD1 and DD2), ethanol (EtOH), ethanolamine (EA), fatty acids (FAs), fructose (Fru), γ-aminobutyric acid (GABA), glycine betaine (GB), genistein derivative (GeD), α- and β-glucose (α- and β-Glc), glutamine (Gln), isoleucine (Ile), propylene glycol (PG), purines (Pur), putrescine (Put), sterols (S1 and S2), soyasaponine (SS), sucrose (Suc), threonine (Thr), trigonelline (Tri), valine (Val), and hydroxycinnamic acids (HA) were identified. These metabolites were confirmed by 1D ^{1}H NMR and 2D NMR experiments, such as ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC (Additional file 1: Table S1). Visual inspection of ^{1}H NMR spectra revealed clear metabolic differences between soybean adventitious roots cultured under a dark condition and a fluorescent light irradiation condition (Fig. 2). Briefly, peaks in the low-field region beyond 5.5 ppm of the ^{1}H NMR spectrum with region consisting of secondary metabolites, typically flavonoids, were

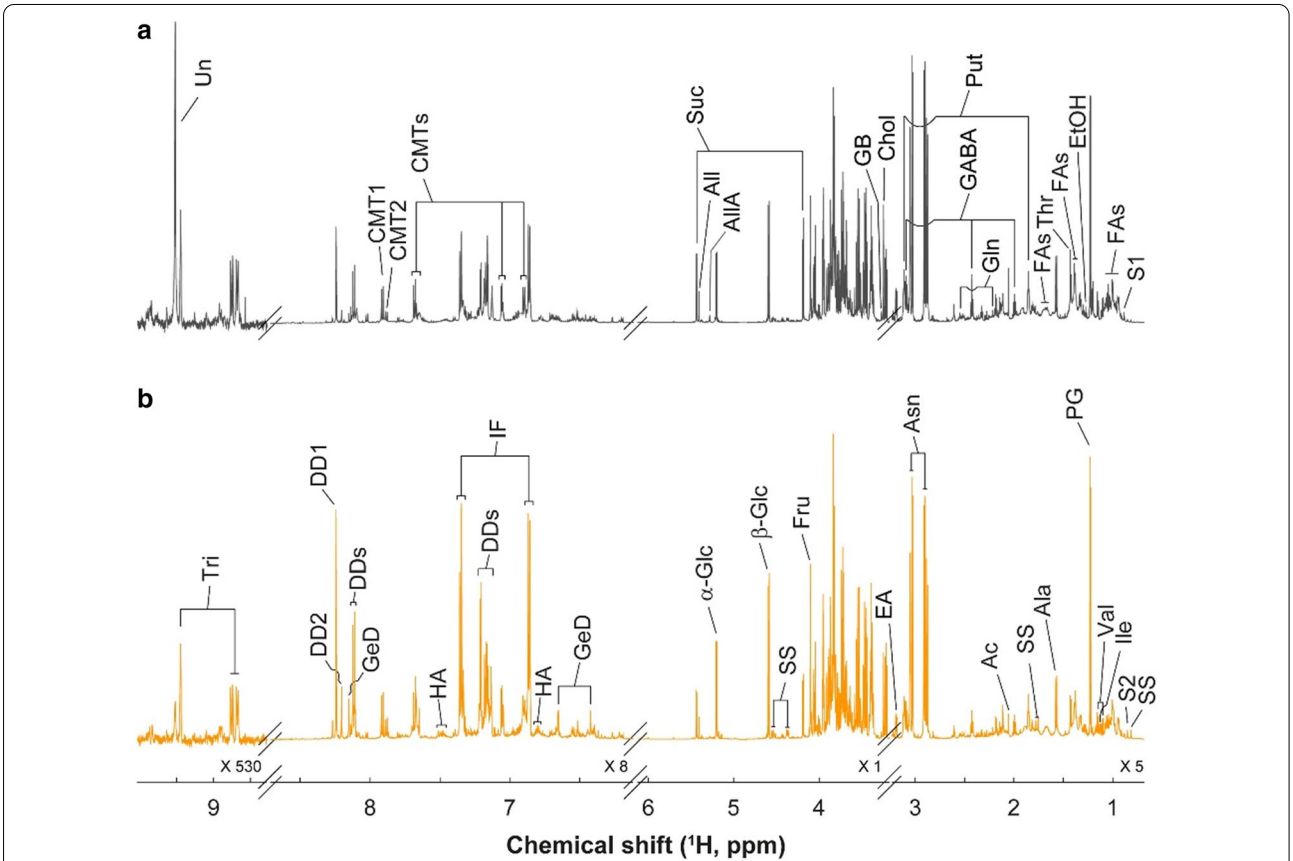

Fig. 2 Representative ^{1}H NMR spectra of soybean adventitious roots cultured under a dark (**a**) or fluorescent light irradiation condition (**b**). *Ala* Alanine, *Ac* Acetate; *All* Allantoin, *AllA* Allantoate, *Asn* Asparagine, *Chol* Choline, *CMT1* Coumestrol derivative 1, *CMT2* Coumestrol derivative 2, *DD1* Daidzein derivative 1, *DD2* Daidzein derivative 2, *EtOH* Ethanol, *EA* Ethanolamine, *FAs* Fatty acids, *Fru* Fructose, *GABA* γ-aminobutyric acid, *GB* Glycine betaine, *GeD* Genistein derivative, *α-Glc* α-glucose, *β-Glc* β-glucose, *Gln* Glutamine, *IF* Isoflavones, *Ile* Isoleucine, *PG* Propylene glycol, *Put* Putrescine, *S1* Sterol 1, *S2* Sterol 2, *SS* Soyasaponines, *Suc* Sucrose, *Thr* Threonine, *Tri* Trigonelline, *Val* Valine, *HA* Hydroxycinnamic acids, *Un* Unknown compound

higher in soybean adventitious roots cultured under a fluorescent light irradiation condition than in those cultured under a dark condition. These results implied that fluorescent light irradiation influenced not only biomass, but also metabolic compositions of soybean adventitious roots.

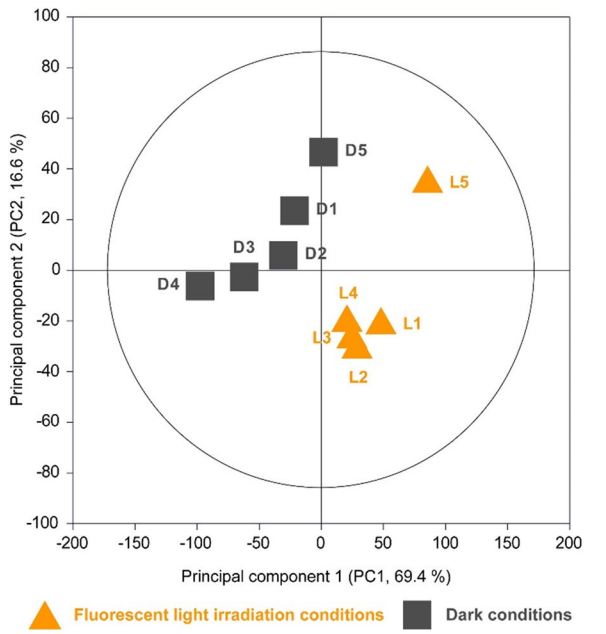

Fig. 3 PCA score plot generated from ^{1}H NMR spectra of soybean adventitious roots cultured under a fluorescent light irradiation or a dark condition

Multivariate statistical analysis of ^{1}H NMR spectra of soybean adventitious roots

The dataset of ^{1}H NMR spectra of cultured soybean adventitious roots was applied to multivariate statistical analysis, such as principal component analysis (PCA) and orthogonal partial least squares discriminate analysis (OPLS-DA), to obtain further information on metabolic differentiation between soybean adventitious roots cultured under the two different growth conditions. In PCA score plot, a noticeable discriminatory pattern was observed, indicating metabolic differentiation between the two groups (Fig. 3). This result implied that fluorescent light irradiation induced considerable metabolic perturbations in soybean adventitious roots. An OPLS-DA model was generated from the dataset of ^{1}H NMR spectra of soybean adventitious roots as X variables and group categorization as Y variables to analyze two groups of samples (Fig. 4). Results revealed clear metabolic differences between soybean adventitious roots cultured under dark and fluorescent light conditions in OPLS-DA score plot (Fig. 4a). OPLS-DA loading plot provided metabolites closely associated with metabolic perturbations in soybean adventitious roots under fluorescent light irradiation conditions (Fig. 4b). In OPLS-DA

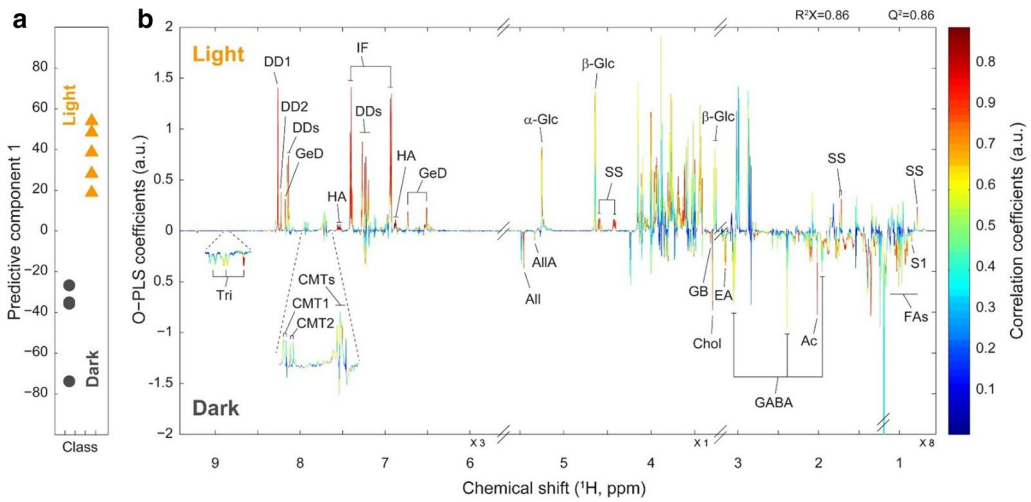

Fig. 4 OPLS-DA score (**a**) and coefficient or loading (**b**) plots derived from ^{1}H NMR spectra of soybean adventitious roots for metabolic differentiation between soybean adventitious roots cultured under a dark condition (Dark) or under a fluorescent light irradiation condition (Light). OPLS-DA models were derived from ^{1}H NMR spectra of soybean adventitious root extracts, showing identifications of metabolites responsible for metabolic differences. The upper section in panel B represents higher levels of metabolites in soybean adventitious roots cultured under a light condition than in soybean adventitious roots cultured under a dark condition, whereas the lower section represents lower levels of metabolites in soybean adventitious roots cultured under a light condition. The color code in the loading plot corresponds to the correlation among variables. All OPLS-DA models were generated with one predictive component and one orthogonal component. Their reliabilities and predictabilities were indicated by R^2X and Q^2, respectively. These models were validated by permutation tests (Additional file 1: Fig. S1). Abbreviations of assigned metabolites are the same as shown in legends of Fig. 2

loading plot with pairwise comparisons, the upper section displayed higher levels of metabolites in the roots cultured under a fluorescent light irradiation condition than in those cultured under a dark condition, whereas the lower section showed lower levels of metabolites in soybean adventitious roots cultured under a fluorescent light irradiation condition. Determination coefficients of the OPLS-DA model in the present study showed highly significant values (R^2X: 0.86; Q^2: 0.86), indicating high fitness and predictability of the model. The OPLS-DA model was also validated by 200 times of permutation tests in the corresponding PLS-DA model with the same number of predictive components used in the OPLS-DA model (Additional file 1: Figs. S1 and S2). The OPLS-DA loading plot given in Fig. 4b showed that soybean adventitious roots cultured under a fluorescent light irradiation condition contained higher levels of soyasaponins (SS), α- and β-glucose (α-Glc and β-Glc), genistein derivative (GeD), daidzein derivatives 1 and 2 (DD1 and DD2), hydroxycinnamic acids (HA) and coumestrol derivatives 1 and 2 (CMT1 and CMT2), but lower levels of sterol 1 (S1), acetate (Ac), γ-aminobutyrate (GABA), ethanolamine (EA), glycine betaine (GB), choline (Chol), allantoin (All), allantoate (AllA), and trigonelline (Tri) than in soybean adventitious roots cultured under a dark condition. Metabolic network and interaction were investigated with relative quantifications estimated from integral areas of ^{1}H NMR peaks corresponding to individual metabolites, together with paired statistical comparisons between the two groups (Fig. 5).

In general, plant roots grow underground with a dark environment. However, as shown in the present study, fluorescent light irradiation markedly influenced not only the growth of soybean adventitious roots, but also their metabolites' compositions during culture. As indicated in Fig. 5, glucose was significantly accumulated in

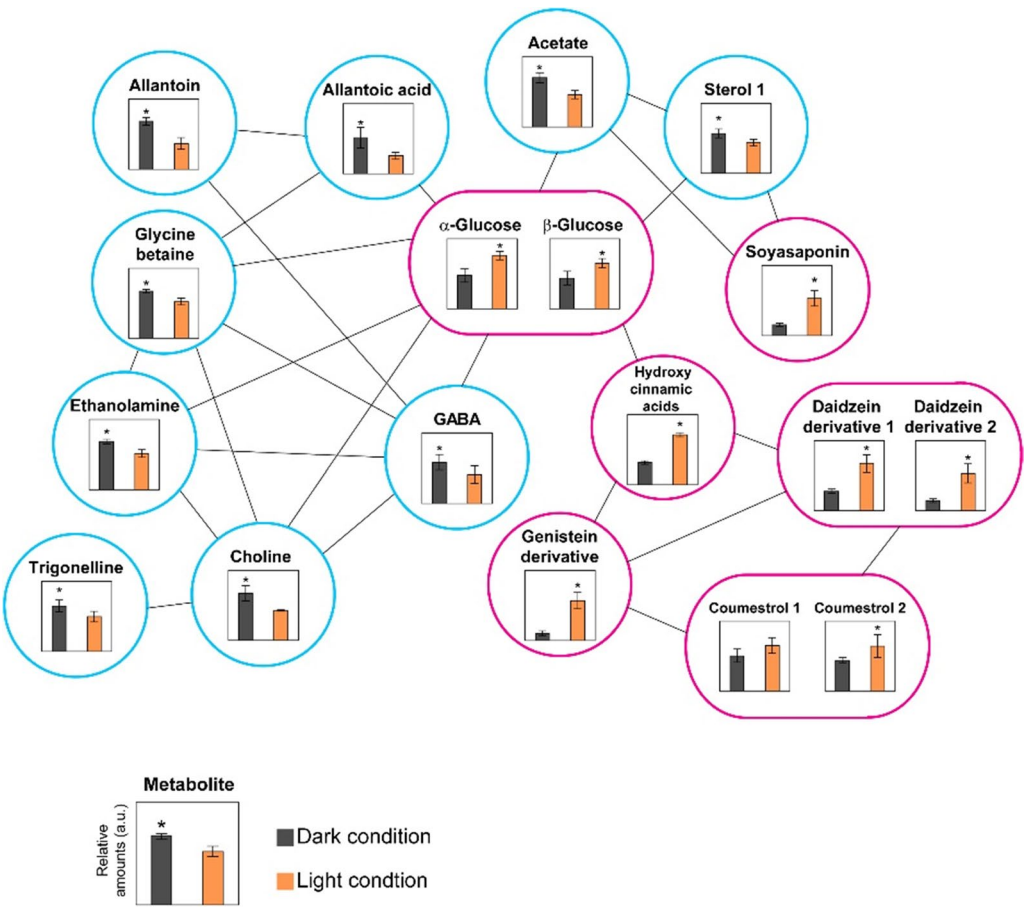

Fig. 5 Schematic metabolite-metabolite interaction network in soybean adventitious roots cultured under dark and fluorescent light irradiation conditions. Gray and orange color of bars in graph represent relatvie quantitative value of metabolites in the adventitious roots culured under dark and fluorsecnt light irradiation conditions, respectively. Asterisk represents significant difference between soybean adventitious roots grown under a dark and a fluorescent light irradiation condition at $P < 0.05$. Blue and red circles indicate decreased and increased amounts of metabolites according to fluorescent light irradiation, respectively

soybean adventitious roots cultured under a fluorescent light irradiation condition. Such accumulation of glucose in the adventitious roots could be due to photosynthesis caused by fluorescent light irradiation. Previous studies have reported that when roots are exposed to light, chloroplasts or their homologs can be formed in roots. Thus, roots can partially facilitate photosynthesis for carbon utilization [27–29]. Accumulation of glucose could also be associated with increased biomass of soybean adventitious roots cultured under a fluorescent light irradiation condition, than in those cultured under a dark condition, because glucose could be used as carbon backbone of plant cell wall, leading to increased growth of roots. Moreover, reduction of acetate level in soybean adventitious roots cultured under a fluorescent light irradiation condition might be associated with decreased anaerobic respiration in the bioreactor due to photosynthesis by fluorescence light irradiation [30].

It has been known that GABA as a non-protein amino acid is related to carbon/nitrogen metabolism and can function as a signaling molecule in plants [31]. Recent studies have revealed that GABA can negatively regulate the development of wheat root and poplar adventitious root [32, 33]. Lower GABA levels in soybean adventitious roots cultured a under fluorescent light irradiation condition than in those cultured under a dark condition provided metabolic evidence for facilitating development of soybean adventitious roots by using light irradiation in the current study. Lower levels of allantoin and allantoate in soybean adventitious roots cultured under a fluorescent light irradiation condition could be also associated with root growth. Allantoin is a purine derivative that is often accumulated in stressed plants [34]. Allantoin can activate jasmonate signaling that induces anthocyanin production, wound response, suppression of root growth and pathogen defense [34]. Therefore, reductions of GABA, allantoin and allantoate levels in soybean adventitious roots cultured under a fluorescent light irradiation condition might reflect metabolic status related to reduced environmental stress compared to soybean adventitious roots grown under a dark condition.

Fluorescent light irradiation also led to decreased levels of amine-related metabolites such as choline, ethanolamine, and glycine betaine in soybean adventitious roots during culture. These amines are known to be accumulated in response to osmotic stresses in many plants [35–37]. In other words, elevations of these amine metabolites in soybean adventitious roots cultured under a dark condition indicate that a dark condition can caus stress for the growth of roots. These metabolic statuses of amines were biologically correlated with GABA with respect to the growth of roots and environmental stress.

Light could trigger complex defense systems in plants to prevent or repair damage from oxidant stress induced by light. Thus, various secondary metabolites functioning as antioxidant agents could be synthesized [38]. Diverse secondary metabolites such as daidzein, genistein, coumestrol, hydroxycinnamic acid derivatives, and soyasaponins were identified in soybean adventitious roots (Fig. 2). Interestingly, we could observe that fluorescent light irradiation provoked significant accumulations of these compounds (Fig. 6). Similar phenomena in many plants have been reported, indicating that light irradiation could amplify the production of secondary metabolites in hairy or adventitious roots [9]. For example, fluorescent light can lead to accumulation of ginsenosides such as Rg1 and Rg2 in ginseng hairy roots [10]. Accumulations of secondary metabolites, including hypericin, flavonoids, and phenolics, in adventitious roots of *Hypericum perforatum* [14] and *Stevia rebaudiana* [15] have also been reported. Higher levels of hydroxycinnamic acids in roots grown under a fluorescent light condition might be associated with root biomass. Hydroxycinnamic acids such as ferulic acid and *p*-coumaric acid can function as intermediates in the biosynthesis of lignin, a component of cell wall and biomass. It has been reported that hydroxycinnamic acids are deposited in cell walls during lignification [39]. Therefore, accumulations of these secondary metabolites in soybean adventitious roots grown under a fluorescent

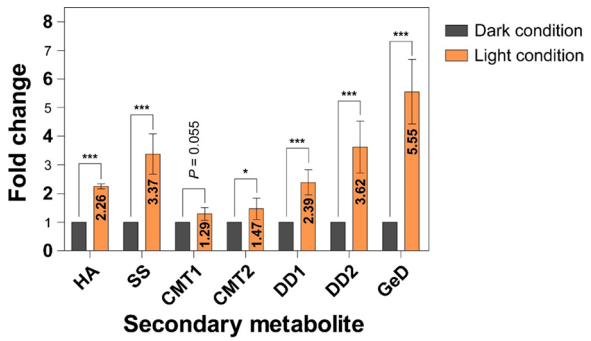

Fig. 6 Fold change of secondary metabolites in soybean adventitious roots grown under a fluorescent light irradiation condition, compared to a dark condition. Gray and orange color of bars in graph represent fold change value of metabolites in the adventitious roots culured under dark and fluorsecnt light irradiation conditions, respectively. HA, hydroxycinnamic acids; SS, soyasaponin; CMT, coumestrol derivative; DD, daidzein derivative; GeD, genistein derivative. *, **, and ***Represent significant difference by Student's *t*-test at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively

light condition could be due to an active growth of roots rather than of responses to environmental stress.

The present study revealed that fluorescent light irradiation caused increased biomass along with considerable metabolic perturbations in soybean adventitious roots. In particular, marked increases in secondary metabolites such as hydroxycinnamic acids, coumestrol derivatives, isoflavones, and soyasaponins in soybean adventitious roots grown under fluorescent light conditions demonstrated the potential of using fluorescent light to enhance the biological function of root materials for human health. Results of this study suggest that metabolomics can assist in biological interpretation to provide useful information and that a fluorescent light irradiation can be utilized during culture of soybean adventitious root as an effective elicitor for the production of valuable compounds.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-021-00598-2.

Additional file 1. Table S1. Assigned metabolites in soybean adventitious roots. **Figure S1.** Permutation test in the PLS-DA model. **Figure S2.** Schematic metabolic flux in soybean adventitious roots cultured under a dark and a fluorescent light irradiation condition.

Acknowledgements
We would like to thank the Korea Basic Science Institute (KBSI) for providing excellent technical assistance with 700 MHz and 900 MHz NMR experiments under a KBSI R&D program (Project No. D39701).

Author's contributions
YDY, KYG and HYS performed experiments and wrote the manuscript. LEJ, KD and KEH collected samples and data. All authors read and approved the final manuscript.

Competing interests
The authors have no conflicts of interests relevant to this study to disclose.

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Received: 17 December 2020 Accepted: 19 February 2021
Published online: 02 March 2021

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