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GENE EXPRESSION OF MICROGRAVITY GERMINATED (*Ocimum sanctum* L.) ON INTERNATIONAL SPACE STATION AND JAPAN EXPERIMENT MODULE

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ABSTRACT

Holy basil or its scientific name *Ocimum sanctum* L. seedlings were grown both in Japanese Experiment module in the International Space Station (ISS) and ground control (earth) for 30 days duration. Plant samples were placed in closed environment incubator under control light, temperature, humidity and CO_2 conditions, before bring back to earth. Pool of first leaf and stem were RNA extracted and sequenced. The sequences were allligned to reference genome *O. basilicum*. We identified differential express genes of holy basil ISS vs ground control with total of 1518 (up regulated)/ 1046 (down regulated) for leaf and 599 (up regulated)/224 (down regulated) for stem. The top 10 upregulated and downregulated genes in leaf/stem ISS vs ground control with more than two fold in gene expression was selected and their possibility of gene function in microgravity environment. The differential express gene were analysed for Gene

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ontology (GO) enrichment. The top 3 prominent GO enrichments for holy basil leaves were cytosol, chloroplast and cytoplasm, while GO enrichment for stems were cytosol, cytoplasm and golgi apparatus. The top 3 differential genes for *Kyoto Encyclopedia of Genes and Genomes* (KEGG) for leaves were metabolic pathway, biosynthesis of secondary metabolites and microbial metabolism in diverse environment, while KEGG enrichment for stems were endocytosis, photosynthesis-antena protein and photosynthesis.

Keywords: *Ocimum sanctum L,* microgravity, RNA sequencing, gene expression and International Space Station (ISS)

1. INTRODUCTION

Plants development are influenced by exogenous and endogenous signal that vary or constant over the time. Gravity is one of parameter that not vary during plant growth [1]. Plant need to maneuver around to their various organ to access resources for surviving and reproduce. Normally root will grow in the direction of the gravity vector, to access water and nutrient, while the shoot negatively gravitropic to acess to the light, moisure, oxigen and carbon dioxide for photosynthesis [2].

Response of gravitional usually involved four steps including (1) sense direction of gravity, (2) converting this biophysical stimulus into a biochemical signal, (3) transmitting the latter signal to the right tissues and (4) the organ changing direction if needed [3]. In root, the growth regulator auxin accumulates to higher levels towards the gravity vector on the lower side of the affected organ [4]. Auxin accumulation results in the localized inhibition of cell expansion and the reorientation of growth towards the gravity vector. In stems, auxin accumulation has the opposite effect and stimulates cell expansion, which causes the growth direction to reorient away from the growth vector.

Creating simulated microgravity environments such as spaceflight studies abroad orbiting spacecraft and through simulated microgravity platform by using clinostat (2D and 3D), rotating wall vessel, Random Positioning Machine (RPM) on the ground [5] has been studied with time limitation of microgravity environment but with space infrastructure such as the International Space Station (ISS), it possible to study the effects of gravitropism on plants over a long period of time [5].

Plant response to various microgravity environment that involved phenotypic, physiological and biochemical, mechano-cellular response for cell wall/cell structure and cell cycle, molecular, genetic, epigenetic and protein level in ISS has been reviewed in detail [5] for *Arabidopsis thalina* [6], [7], [8], [9], [10], [11], [12], *Triticum aestivum* [13], Alaska pea [14], Pea and maize [15], White spruce [16], *Brassica napus* [17], *Eruca sativa* [18] and White spruce [19]. At this moment, no report has been studied on holy basil on microgravity environment. Research on the effects of

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microgravity on germinated *O. sanctum,* gene expression hypotheses related to how microgravity environments influence plant physiology, growth, and secondary metabolite production will be upregulated in microgravity in response to the altered environment.

Holy basil (*O. sanctum*) has been selected among other seed candidates for microgravity enviroment at ISS after going through with Japan Aerospace Exploration Agency (JAXA) because high percentage of seed germination. Furthermor, holy basil is an important Indian traditional medicine that posseses anti-fertility, anti-cancer, anti-diabetic, anti-fungal, anti-microbial, cardioprotective, analgesic, anti-spasmodic and adaptogenic actions [20].

With current next generation sequencing technology that provides low cost and ubiquitous nature of sequencing technology, RNA-seq has become molecular method for comparing gene expression between multiple samples that produce a high-resolution of gene activation at given time. Here we carried out gene expression of holy basil that were germinated in control inviroment at JAXA as ground control (GC) and at ISS (microgravity).

2. MATERIALS AND METHODS

2.1 Growth plant material

Both microgravity and ground control holy basil were conducted in plant chamber. Microgravity holy basil seeds were germinated in plant chambers supplemented with rockwoll medium at ISS (2 boxes) and gravity holy basil as ground control at JAXA (2 boxes) for 30 days.The cultivation period at ISS was from 16/2/2021 until 18/3/2021 and ground control was from 3/8/2021 until 2/9/2021. The growth condition were stricly monitor; initial watering 100 mL(day 0), 1st additional watering 10 mL (day 10) and 2nd additional watering 10 mL (day 21). The average temperature set at 24.5°C, average light intensity 40 µmol/m^2/sec and average CO_2 concentration 2994 ppm respectively. The plant chambers were frozen at -80 °C fridge when return to earth prior RNA extraction.

2.2 Sample harvesting for RNA extraction

Frozen germinated holy basil seedling in growth chamber was send by JAXA and received at Centre for Marker Discovery and Validation laboratory (CMDV) at Biotechnology Research Centre, Malaysian Agriculture Research and Development Institute (MARDI), Selangor, Malaysia. The samples were kept at -80ºC fridge prior harvesting for 1st leaf and stem for RNA extraction.

2.3 RNA extraction

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Holy basil of 1st leaf and stem were harvested from frozen germinated seedling and used for RNA extraction using Qiagen kit (QIAGEN, USA) following manufacture protocol. The quality and quantity of extracted RNA were examined using a 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA, USA). The RNA sample with RNA integrity number > 6 was used for mRNA library costruction.

2.4 mRNA library constructing and sequencing

One μg total RNA of holy basil leaf ISS/GC and holy basil stem ISS/GC were used for library preparation. The poly(A) mRNA isolation was performed using Oligo(dT) beads (Vazyme, China). The mRNA fragmentation was performed using divalent cations and high temperature. Priming was performed using Random Primers. First strand cDNA and second-strand cDNA were synthesized. The purified double-stranded cDNA was then treated to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of Adaptor-ligated DNA was then performed using DNA Clean Beads. Each sample was then amplified by PCR using P5 and P7 primers and the PCR products were validated. Then libraries with different indexes were multiplexed and loaded on an Illumina HiSeq/ Illumina Novaseq/ MGI2000 instrument for sequencing using a 2x150 paired-end (PE) configuration according to manufacturer's instructions.

2.5 Data processing and analysis

Raw reads were cleaned up to remove adapters and reads shorter than 75 reads by cutadapt (version 1.9.1). Quality control was evaluated by FastQC to ensure adapters were removed. Reads were aligned using HiSat2 (v2.0.1) [21] to the *Ocimum basilicum* cv. *Perrie* genome. Short read alignment was performed using Hisat2 (v2.0.1) [22] with default parameters. Read counts per feature were generated using HTseq-Count [23]. Differential gene expressions were determined using DESeq2 [24]. Features with one count or less in any sample were discarded. Differentially expressed genes were filtered on p-values. Analysis of holy basil ISS vs GC with significance cutoff was padj <0.05. Gene Ontology (GO) annotation and enrichment was queried using a GO2 software [25] which is based on an extension of the hypergeometric distribution known as the Wallenius noncentral hyper-geometric distribution that able to account for gene length bias and counts bias when performing GO analysis. Threshold for filtering was over represented_pvalue <= 0.05.

2.6 Light microscopy preparation

The study samples included the combination of fresh leaf specimens and preserved samples. Samples fixation and embedding followed the methods of Johansen [26] and Sass [27] with some modifications. Fresh leaves were fixed in AA (1 Acetic acid:3 Alcohol). Materials were sectioned

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in a range of thickness (15-30µm) using sliding and rotating microtome. A piece of polystyrene was used for support in the clamp. Transverse sections were taken from the middle part of matured leaves. The sections then were cleared using bleaching agent "Clorox", washed and stained in Safranin for 5 minutes and then in Alcian Green for 5 minutes prior dehydrated through an ascending alcohol series. All slides were mounted in Euparal after the final dehydration stages. The slides were then kept in the oven for two weeks at about 60 °C. Photographs of the sections were taken using Olympus SZH40 microscope and digital camera. Images were processed using Image Analysis and ADOBE Photoshop Software.

3. RESULTS AND DISCUSSION

The experiment examined the transcriptome of 30 days holy basil seedling grown aboard at ISS and identical samples were grown on earth (Figure 1). It is important to understand the molecular mechanism that regulate plant adaptation to microgravity that the ISS experiment have examined the transcriptional changes of holy basil. Point of concern is that ISS experiment have employed hardware-matched experimet on earth as control and it is difficult to replicate all the stress that plant face on the ISS and on the ground.

The related photo can be viewed from different angle at Mission-1 growth experiment results and Messages from Astronaut | JAXA Human Spaceflight Technology Directorate. The first leaf and steam of holy basil were collected (Figure 2a) and RNA extracted (Figure 2b).

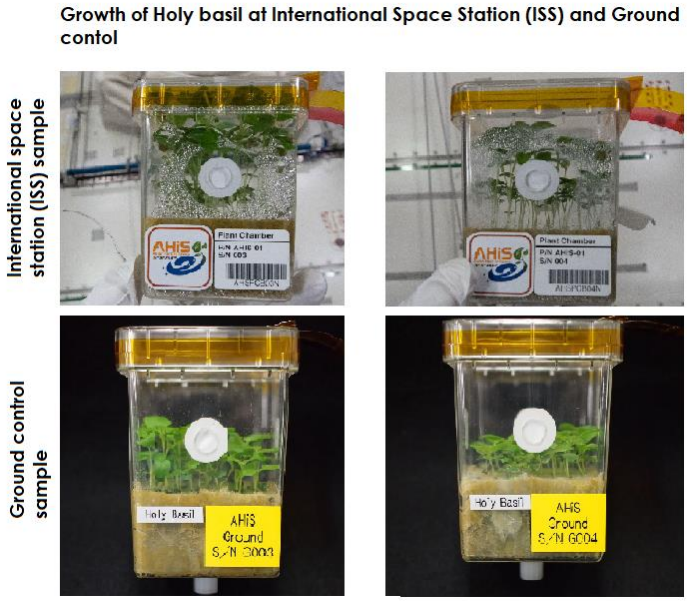

Figure 1: Growth of holy basil in growth chamber at ISS and gGC at JAXA (courtesy of JAXA)

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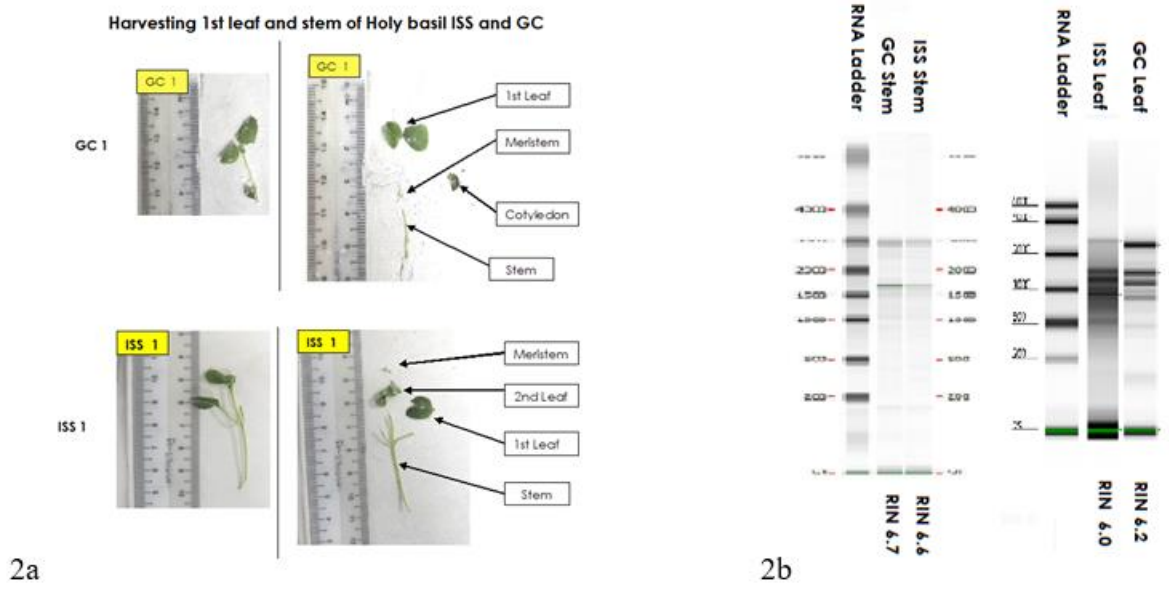

Figure 2: Harvesting 1st leaf and stem of holy basil from growth chamber ISS and GC for RNA extraction (2a) . RNA of 1st leaf and stem of Holy basil with RIN number (2b)

3.1 RNA sequencing

The raw sequencing generated was filtered (Table 1) prior bioinformatics analysis. The sequencing quality score measures the probability that a base is called incorrectly. The sequencing quality score of a given base, Q, is calculated by the Illumina sequencer. Higher Q scores indicate a smaller probability of error. A quality score of 30 (Q30) represents an error rate of 1 in 1000, with a corresponding call accuracy of 99.9%. Normally, pass filtered data contains around 80% of Q30. In these results most of the samples showed 93% 0f Q30 indicated high sequencing quality (Table 1). The filtered sequencing data was aligned to reference genome and annotation files of *Ocimum basilicum* cv. *Perrie*. (genome: https://genomevolution.org/coge/SearchResults.pl?s=59011&p=genome). The genome *O. basilicum* genome was selected due to same genus and only draft mitochondria genome of *O. sanctum* was available at the current study.

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Table 1: Filtered sequence data statistics

Sample	length	Reads	Bases	Q20 (%)	Bases Q30 (%)	GC (%)	N (ppm)
Holy basil-GC-leaf	144.69	57345376	8297456717	97.78	93.66	49.77	11.52
Holy basil-GC-stem	145.46	67549872	9825611267	97.64	93.30	48.02	11.00
Holy basil-ISS-leaf	144.03	68804784	9909743921	97.61	93.27	49.60	11.21
Holy Basil-ISS-stem	145.44	56126730	8162974228	97.56	93.17	49.66	11.31

Table 2: Data sequence alignment statistic

Samples	Total reads	Total mapped	Multiple mapped	Uniquely mapped	Read1	Read2	Reads map to '+'	Reads map to '-'	Non-splice reads	Splice reads	Reads mapped in proper pairs
Holy basil-GC-leaf	57345376	10245248 (17.8659%)	5481534 (9.55881%)	4763714 (8.30706%)	2398432	2365282	2390444	2373270	3512266	1251448	3419168
Holy basil-GC-stem	67549872	10458592 (15.4828%)	5225280 (7.73544%)	5233312 (7.74733%)	2640603	2592709	2642362	2590950	3347759	1885553	3357298
Holy basil-ISS-leaf	68804784	16706830 (24.2815%)	9718196 (14.1243%)	6988634 (10.1572%)	3521661	3466973	3509597	3479037	5323987	1664647	5351894
Holy basil-ISS-stem	56126730	12794944 (22.7965%)	7110552 (12.6687%)	5684392 (10.1278%)	2866629	2817763	2887833	2796559	4138349	1546043	4071010

3.2 Gene expression analysis

The results from Digital Expression Gene sequence (DEGSeq) analysis were further analyzed to determine genes with significant differential expression according to the criteria of fold change greater than 2 and q value (statistical significance) was less than 0.05. A total of 2,564 genes were identified as significantly differently expressed ($P \leq 0.05$) for holy basil leaf. The number of up and downregulated genes for holy basil leaf ISS vs GC were 1518 and 1046 respectively, while a total of 823 genes were significantly differently expressed ($P \leq 0.05$) for holy basil stem. The number of up and downregulated genes for holy basil stem ISS vs GC were 599 and 224 respectively (Figure

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1). List of 10 top upregulated and downregulated genes for leaf and stem ISS vs GC were shown in table 3, 4, 5 and 6 respectively.

3.2.1 Gene upregulated in holy basil leaf ISS vs GC

In top 10 upregulated gene ID in holy basil leaf ISS vs GC, gene ID TCONS_0030245 and TCONS_0022672 were descripted as pectate lyase and showed highest transcript altered. Pectate lyase is an enzyme involved in the degradation of pectin, a complex polysaccharide found in the plant cell walls. Studied of pectate lyase on Arabidopsis showed that pectate lyase was required to coordinate turgor pressure and wall mechanics for proper stomatal function [28]. The upregulated pectate lyase on germinated holy basil on the ISS was important to maintaining the turgor pressure of stomatal due to unique growth patterns and physiological changes in microgravity condition.

Gene ID TCONS_00000094 descripted as ATP synthase gamma chain, chloroplastic-like was highly expressed. ATP synthase is a key enzyme complex found in the inner mitochondrial membrane of eukaryotic and prokaryotic cells. The gamma chain is a crucial component of ATP synthase responsible for transmitting mechanical energy generated during ATP synthesis [29]. In a microgravity environment, changes in fluid dynamics and membrane properties may affect the efficiency of ATP synthesis by ATP synthase of holy basil.

Gene ID TCONS_00041341 descripted as Auxin-responsive protein. Auxin-responsive proteins are involved in the cellular responses to the plant hormone auxin, which plays crucial roles in various aspects of plant growth and development, including cell elongation, tissue differentiation, and tropic responses [30]. Studies have shown that auxin plays a crucial role in leaf initiation in tomato. In tomato vegetative shoot apices treated with the auxin transport inhibitor 1- N-naphthylphthalamic acid (NPA) fails to form leaf primordia [31] and organ formation was blocked in the inflorescence meristems of pin-formed1 (pin1) mutants in *Arabidopsis*, which harbor mutations in the auxin efflux carrier [32], [33] and [34]. In microgravity holy basil, distribution of auxin within the plant may be disrupted due to the lack of gravitational force. This can impact the formation of auxin gradients, which are essential for directed growth and development. Studied on microgravity of *Arabidopsis* in (ISS utilizing the European Modular Cultivation System (EMCS) has shown upregulated auxin related genes in auxin transport and signaling. The auxin group are 2 auxin efflux carriers (PIN3 and PIN4), 6 auxin response factors (ARFs) and 11 small auxins upregulated (SAUR) genes [35].

Gene ID TCONS_00041341 descripted as APETALA 3 (AP3). AP3 is floral homeotic proteins involved in the regulation of flower development in plants. They belong to the class B floral organ identity genes and play crucial roles in specifying petal and stamen identity during floral organogenesis [36]. Studied on microgravity of *Arabidopsis* in ISS utilizing the EMCS has shown

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upregulated APETALA 2/Ethylene response factor superfamily which cytokinin response factor (CRF4 and CRF5) and Aintegumenta (ANT) play established roles in cell proliferation and embryo, cotyledon and leaf development [35].

Gene ID TCONS 00028453 descripted as Stachyose synthetase. Stachyose synthetase is an enzyme involved in the biosynthesis of stachyose, a tetrasaccharide composed of two galactose units, one glucose unit, and one fructose unit [37]. This enzyme plays a crucial role in carbohydrate metabolism in plants, particularly in the synthesis of raffinose family oligosaccharides (RFOs). Two RFOs that play vital roles in stress responses are raffinose and stachyose [38]. In most plants, raffinose accumulates in vegetative tissues during abiotic stress while stachyose accumulates predominantly during seed development/desiccation [39]. The microgravity condition showed high gene expression on Stachyose synthase of holy basil that may have other effects of seed germination during seed development.

Gene ID TCONS_00066800 descripted as DNA/RNA-binding (DRB) domain-containing protein. These proteins are a diverse group of proteins that play crucial roles in gene expression regulation, RNA processing, and other nucleic acid-related processes [40]. In microgravity holy basil leaf, these proteins might play a role in modulating the expression of genes involved in the response to microgravity stress. Studied on microgravity of *Arabidopsis* on ISS utilizing the EMCS has shown upregulated DNA-binding bHLH protein [35].

Gene ID TCONS_00000029 descripted as Peroxidase. Peroxidases are enzymes that participate in plant normal metabolism and under oxidative stress [41]. Peroxidase takes part in H_2O_2 detoxcity which is form during dismutation of superoxidase oxygen, in polymerization of cell wall monolignols, in phenols oxidation, and auxin metabolism [42]. Studying on long term simulated microgravity using clinostat on potato minitubers shows intensification of peroxidase activity that connected to strengthened of antioxidant process [43].

Gene ID TCONS_00074290 descripted as Fe-S cluster assembly factor of high chlorophyll fluorescence 101 (HCF101). HCF101 has been shown to be essential for the accumulation of the membrane complex Photosystem I and the soluble ferredoxin-thioredoxin reductases, both containing [4Fe-4S] clusters in ensuring efficient electron transport and energy conversion during photosynthesis [44]. In microgravity holy basil leaf Fe-S cluster of HCF101 may help in maintaining the redox balance within chloroplast for protecting the plant cell from oxidative damage by reactive oxygen species (ROS) generated during photosynthesis.

3.2.2 Gene upregulated in holy basil stem ISS vs GC

In the top 10 upregulated gene ID in stem ISS vs GC, gene IDs TCONS_00049526, TCONS_00001293 and TCONS_TCONS_00050223 were descripted as Heat shock proteins

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(HSPs). These genes were affected in microgravity holy basil stem ISS vs GC, and similar studied on transcriptome (RNASeq) of *Eruca sati*va seed stored on the ISS for six months revealed differential expression of genes associated HSPs, a heat shock factor (HSFA7a-like) [18]. Heat shock proteins are important for stress resistance and adaptation to the environment. They are recognized as stress proteins that protect cells from stress damage and have been involved in re-establishing normal cellular homeostasis in plants [45]. Many of them were known to have diverse roles and to react not only to elevated temperature but also to other extreme conditions such as water, salinity and osmotic, cold and oxidative stress [46], [47], [48], [49].

The transcript of gene ID TCONS_00002328 was descripted as SET domain-containing protein. SET domain-containing proteins are a diverse family of proteins that play critical roles in regulating gene expression through the modification of histones, are proteins around which DNA is wrapped. These proteins possess a SET domain, which is responsible for catalyzing the methylation of lysine residues on histones, influencing chromatin structure and gene expression. A conserved role of SET proteins in plant development was reinforced by the finding that overexpression of the SET domain of the rice CLF homolog, OsSET (SDG711), affects shoot development in transgenic *Arabidopsis* [50]. Studied on microgravity of *Arabidopsis* from ISS has shown upregulated of SET domain group 26 [35]. The microgravity might influence shoot development of SET domain-containing proteins, thereby impacting the holy basil ability to respond to microgravity condition.

Transcript of gene ID TCONS_00026447 descripted as Alcohol dehydrogenase. This result was similar to transgenic *Arabidopsis* plants launched for a 5-day mission on orbiter Columbia (STS-93) that the differential expression of alcohol dehydrogenase (ADH) in root and shoot tissues were differently expressed compared to the ground control plants [51]. ADH was an enzyme involved in the conversion of alcohols to their corresponding aldehydes or ketones, coupled with the reduction of NAD+ to NADH. While ADH was typically associated with alcohol metabolism in plants, it also plays roles in other physiological processes. Similar findings have been shown on microgravity of *Arabidopsis* in ISS that Alcohol dehydrogenase transcription factor Myb/SANT-like family protein was upregulated [35].

Gene ID TCONS_00061426 descripted as Laccase. Laccase was an enzyme that catalyzes the oxidation of a variety of phenolic compounds, often resulting in the formation of polymers known as lignin. Laccases also play crucial roles in various physiological processes in plants in plant growth and development [52]. The altered expression levels of genes encoding laccases in microgravity may lead to changes in enzyme abundance and activity that affect lignin biosynthesis and other physiological processes in plant growth and development.

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Gene ID TCONS_00003876 descripted as SP-RING type domain-containing protein. The SP-RING domain was protein domain found in various organisms, including plants [53]. It typically functions as an E3 ubiquitin ligase, facilitating the transfer of ubiquitin molecules onto target proteins, thereby marking them for degradation via the ubiquitin-proteasome pathway. Microgravity environment on holy basil may influence stress SP-RING proteins that play a role in ubiquitin-proteasome pathways by regulating the degradation of stress-responsive proteins.

Gene ID TCONS_00042887 descripted as Acyl-CoA binding domain (ACB) domain-containing protein. In plants, ACB domain-containing proteins were believed to be involved in various aspects of lipid metabolism, including fatty acid synthesis, lipid trafficking, and storage lipid utilization [54]. They may also play roles in lipid signaling pathways and responses to environmental stress. In microgravity conditions, plants experience altered mechanical stresses and changes in fluid dynamics, which can impact cell structure and function in the stem. Therefore, proteins involved in lipid metabolism, such as ACB domain-containing proteins, may play roles in mediating microgravity holy basil response to these changes.

Gene ID TCONS_00066748 descripted as Plant Homeodomain (PHD) type domain-containing protein. PHD domain-containing proteins were class of proteins found in various organisms, including plants [55]. PHD domain, was involved in recognizing and binding to specific DNA sequences, as well as other protein-protein interaction domains. Studied on microgravity of *Arabidopsis* on ISS utilizing the EMCS has shown upregulated of PHD-type zinc finger protein that function in plant development and abiotic stress responses [35].

Gene ID TCONS_00033188 descripted as F-box domain-containing protein. These proteins were key components of SCF (SKP1-CUL1-F-box protein) E3 ubiquitin ligase complexes, which target specific proteins for ubiquitination and subsequent degradation [56]. This process was essential for regulating various cellular processes, including cell cycle progression, signal transduction, and stress responses. Microgravity in holy basil may affect stability and function of both F-box proteins and their substrate proteins, leading to changes in protein degradation pathways.

3.2.3 Gene downregulated in holy basil leaf ISS vs GC

In top 10 down regulated gene ID in holy basil leaf ISS vs GC, gene ID TCONS_00024301 was descripted as Protein Arabinogalactan Protein-Like Protein 1 (ALP1)-like and showed highest transcript altered. This protein belongs to the family of arabinogalactan proteins (AGPs), that involved in cell wall properties and cell surface signaling in plant [57]. In the microgravity holy basil, plant cellular processes and structural organization of cell wall might undergo cell wall changes to adapt to microgravity.

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Gene ID TCONS_00051411 was descripted as FAD-binding domain containing protein. These proteins were diverse group of flavoproteins that utilized flavin adenine dinucleotide (FAD) as a cofactor and involved in various redox reactions essential for metabolic and cellular processes, such as energy production, oxidative stress responses, and signaling pathways [58]. In microgravity, FAD-binding may induce unique physiological and molecular changes in holy basil.

Gene ID TCONS_00086160 descripted as Ferruginol synthase. Ferruginol synthase was an enzyme that catalyzes the conversion of miltiradiene into ferruginol, a diterpenoid compound. This enzyme plays a critical role in the biosynthesis of abietane diterpenoids biosynthetic pathways for pharmaceutical activities in *Isodon lophanthoides,* a traditional Chinese medicinal herb [59]. Heterologous expression of three CYP76AHs in *Nicotiana benthamiana* resulted in the formation of ferruginol and expressed in the root and leaf. [59]. In microgravity holy basil decrease of Ferruginol synthase activity may induced changes in pharmaceutical activities have to be studied.

Gene ID TCONS_00074857 descripted as WRKY transcription factor. WRKY transcription factors were large family of plant-specific transcriptional regulators that play pivotal roles in plant defense, development, and secondary metabolism [60]. Similar findings were discovered in microgravity of *Arabidopsis* in ISS spaceflight experiments utilizing EMCS [35] where WRKY transcription factor were downregulated. Approximately 200 genes associated with plant defense and response biotic stimuli that in category 24 disease-resistance protein, different classes of receptor-like protein kinases and several WRKY transcription factors.

Gene ID TCONS_00010515 descripted as Mitotic spindle assembly checkpoint. This protein was dynamic structure composed of microtubules and associated proteins that segregate chromosomes during cell division. Proper spindle assembly was crucial for accurate chromosome segregation and cell cycle progression [61]. Microgravity of holy basil leaf may impose unique biophysical challenges that can disrupt cytoskeletal dynamics, including those involved in mitotic spindle formation.

Gene ID TCONS_00010515 descripted as C2H2-type domain. This domain was one of the most common DNA-binding motifs in eukaryotic transcription factors. Proteins containing this domain play essential roles in gene expression, development, stress responses, and environmental adaptation. These domains typically bind to specific DNA sequences to regulate transcription, but they can also interact with RNA and other proteins, contributing to diverse cellular functions [62]. In microgravity, cellular processes such as gene expression, signal transduction, and stress responses were altered in holy basil due to the unique physical environment. Understanding how C2H2-type zinc finger proteins function in microgravity provides insights into the mechanisms of cellular adaptation to space environments.

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Gene ID TCONS_00027021 and TCONS_00079473 descripted as Mitochondrial uncoupling protein. These proteins were integral membrane proteins in the inner mitochondrial membrane that play critical roles in regulating mitochondrial function, including energy production, thermogenesis, and oxidative stress management [63]. In holy basil microgravity, cellular processes, including mitochondrial dynamics, energy metabolism, and reactive oxygen species (ROS) production, were significantly altered. Investigating this protein is crucial for understanding how cells adapt in microgravity.

Gene ID TCONS_00088868 descripted as Myb proto-oncogene protein. This protein was transcription factor involved in regulating cell proliferation, differentiation, and survival. It plays critical roles in hematopoiesis, development, and stress responses. Myb proteins bind to specific DNA sequences to activate or repress target gene expression, often influencing pathways related to cell cycle regulation and apoptosis [64]. In microgravity holy basil decrease of Myb proto-oncogene protein have to be studied.

Gene ID TCONS_00084040 descripted as Peptide/histidine transporter. These proteins were integral membrane proteins that belong to the peptide transporter (PTR) family and involved in the uptake of di- and tri-peptides, as well as histidine and other small molecules, across cellular membranes. They play crucial roles in nutrient absorption, nitrogen metabolism, and cellular signaling [65]. In microgravity holy basil leaf, cellular processes such as nutrient transport, metabolism, and signaling are significantly altered, making it essential to understand how peptide/histidine transporters function in microgravity environment.

3.2.4 Gene downregulated in holy basil stem ISS vs GC

In top 10 downregulated gene ID in holy basil stem ISS vs GC. Gene ID TCONS_00015134 descripted as Major facilitator superfamily (MFS). This protein was one of the largest diverse families of membrane transport proteins. MFS transporters facilitate the movement of a wide range of substrates, including ions, sugars, amino acids, peptides, and metabolites, across cellular membranes. They play crucial roles in maintaining cellular homeostasis, nutrient uptake, and waste removal [66]. In a microgravity holy basil environment, cellular processes such as transport, signaling, and metabolism are altered, making the study of MFS transporters in microgravity essential for understanding cellular adaptation and survival mechanisms.

Gene ID TCONS_00044485 descripted as copper chaperone. These proteins were specialized proteins that facilitate the safe transport and delivery of copper ions within cells. Copper was an essential trace element that acts as a cofactor for many enzymes involved in critical processes such as energy production, oxidative stress management, and connective tissue formation and plant

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immunity [67]. In microgravity of holy basil stem, understanding the role of copper chaperones in microgravity is essential for addressing physiological challenges.

Gene ID TCONS_00020437 descripted as Synaptotagmin-5 isoform X1. This protein was a member of the synaptotagmin protein family, which plays a key role in membrane trafficking and environmental stress [68]. In microgravity holy basil, understanding the behavior of synaptotagmin-5 isoform X1 in microgravity environment is crucial for comprehending how microgravity impacts cellular communication and vesicular transport systems.

Gene ID TCONS_00046841 descripted as Nascent Polypeptide-Associated Complex subunit beta (NAC). This protein was a multi-subunit complex involved in protein synthesis and quality control. NAC was composed of two main subunits: NACα (NAC alpha) and NACβ (NAC beta). Studies have shown the knock down of individual NAC subunit(s) led usually to a higher sensitivity to stress [69]. In *A. thaliana* genome, five genes encoding NACα subunit, and two genes encoding NACβ. Double homozygous mutant in both genes coding for NACβ showed a delayed development compared to the wild type, had abnormal number of flower organs, shorter siliques and greatly reduced seed set [69]. Understanding how NACβ functions in microgravity is important for elucidating how protein synthesis and quality control mechanisms adapt or malfunction under space conditions.

Gene ID TCONS_00088737 descripted as DELLA proteins. These proteins were family of growth-repressing transcriptional regulators that play a key role in plant growth and development. They were part of the Gibberellin (GA) signaling pathway, where they act as negative regulators of growth. DELLA proteins were characterized by the presence of a conserved DELLA domain, which was essential for their function in repressing growth in the presence of gibberellins. In the absence of gibberellins, DELLA proteins accumulate and bind to other transcription factors, inhibiting the expression of growth-promoting genes. When gibberellins were present, they bind to DELLA proteins, leading to their degradation and the promotion of growth [70]. In microgravity, plants undergo various changes in growth patterns, including altered gene expression, changes in cell wall structure, and modifications in hormone signaling pathways. Since DELLA proteins are central to the regulation of growth and development, understanding how they function in microgravity is important for comprehending how gravity influences plant growth and how plants adapt to space environments.

Gene ID TCONS_00032601 descripted as Photosystem I (PSI). This protein was a critical component of the photosynthetic machinery in plants. It was responsible for the light-driven reduction of NADP+ to NADPH, a key step in the light reactions of photosynthesis. The PSI reaction center is a complex of proteins and pigments that work together to capture light energy and convert it into chemical energy. One of the subunits of the PSI reaction center is PSI-F, a

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membrane-bound protein that plays an essential role in stabilizing the PSI complex and facilitating electron transfer during photosynthesis [71]. Studied of PSI-F in *Arabidopsis* plants transformed with an antisense construct of the psaF cDNA showed several plant lines with reduced amounts of the PSI-F subunit were died, indicate that PSI-F subunit was required for survival of transgenic plants [63].In microgravity holy basil, cellular processes such as protein synthesis, energy production, and electron transport was altered. The impact of microgravity holy basil on PSI-F and its role in the photosystem could provide valuable insights into how photosynthesis is affected in space environments.

Gene ID TCONS_00036187 descripted as Prenylated Rab acceptor 1 (PRA1) family protein. This protein was a group of proteins characterized by the presence of the PRA1 domain, which is involved in various cellular processes, including membrane trafficking and stress responses [72]. Studied gene silencing of *GhPRA1.B1-1A* in *Verticillium dahliae.* showed reduce in resistance, reactive oxygen species accumulation (H_2O_2) salicylic acid, and jasmonic acid contents [73]. In holy basil microgravity, cellular processes such as protein folding, trafficking, and stress responses were altered. Given the role of PRA1 family proteins in these processes, understanding how they behave in microgravity could provide insights into how cells adapt to microgravity.

Gene IDTCONS_00051991 descripted as 60S ribosomal protein L11. This protein was a key component of the large subunit of the ribosome in eukaryotic cells. Ribosomes are essential for protein synthesis, translating messenger RNA (mRNA) into polypeptides. The 60S ribosomal subunit was involved in the elongation phase of protein synthesis, where amino acids were added to the growing polypeptide chain. Studied of microgravity *Arabidopsis* [74] showed 11 ribosomal protein transcripts downregulated for a structural component. This suggested that microgravity caused change in ribosome biosynthesis for an uncoupling of cellular growth and cellular proliferation [75].

Gene ID TCONS_00083087 descripted as Carnosine N-methyltransferase (CNMT). This protein was an enzyme that catalyzes the methylation of carnosine, a dipeptide composed of β-alanine and histidine. While carnosine metabolism was well-studied in animals [76] and its role in plants is less understood.

Gene ID TCONS_0004343 descripted as 4-Coumarate-CoA ligase (4CL). This protein was an enzyme involved in the biosynthesis of lignin, flavonoids, and other phenolic compounds [77]. It catalyzes the activation of 4-coumaric acid by converting into 4-coumaroyl-CoA, which was a key intermediate in the biosynthetic pathways of lignin and other secondary metabolites in plants [78]. Studied on transgenic tobacco with *Dryopteris fragrans* 4CL (*Df*4CL2) showed thicker tissue and had an earlier flowering period than wild type tobacco [78]. Studying the behavior of 4-coumarate-

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CoA ligase in microgravity is essential to understand how microgravity environments influence and the synthesis of key compounds like lignin and flavonoids.

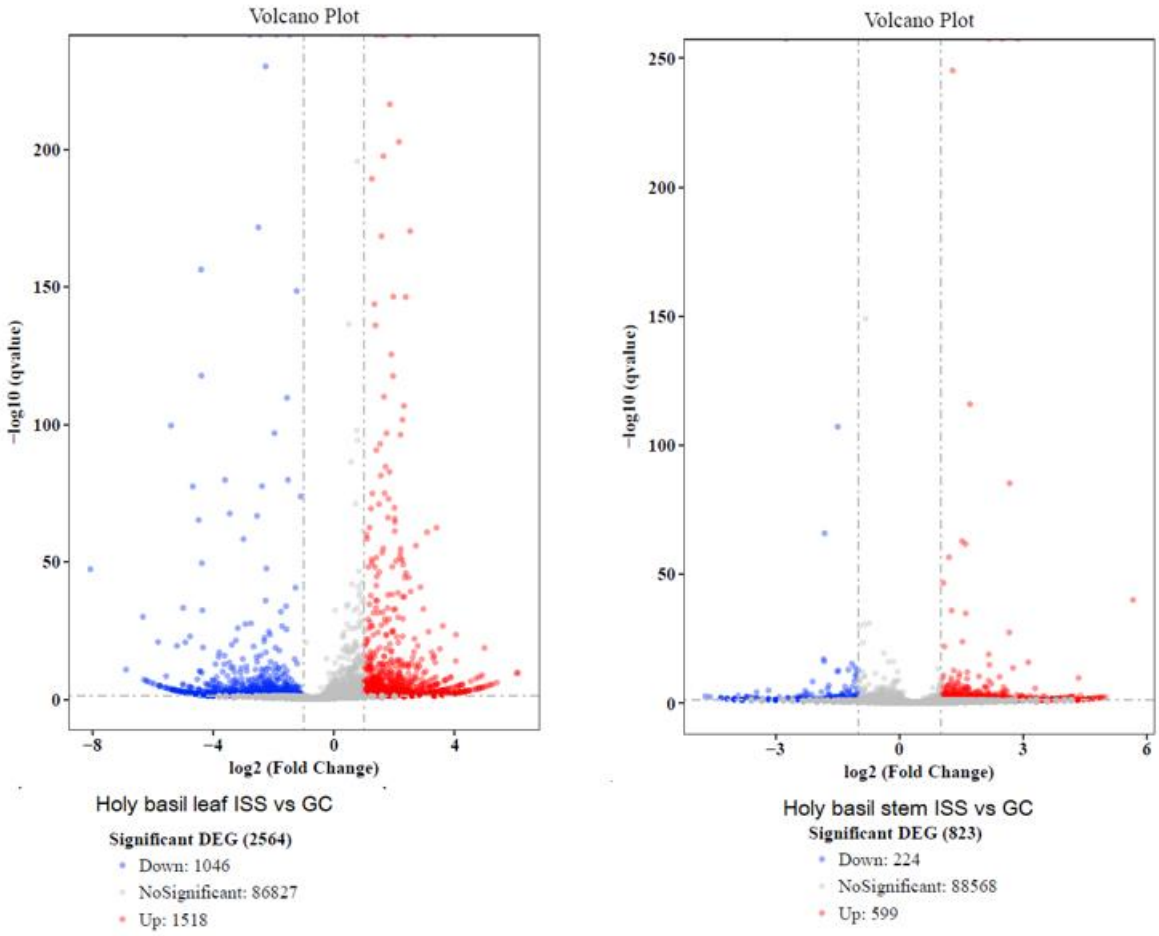

Figure 3: Differential expression volcano plot, red dots represent genes that are significantly up regulated and blue dots represent those that are significantly down regulated. X axis: log2 fold change of gene expression. Y axis: statistical significance of the differential expression in log10(qvalue(fdr,padj))

Table 3: Top 10 up regulated gene ID in leaf ISS vs GC

No	Gene ID	Description	E-Value UniProt	log2FoldChange	P value	Statistical Significance	Regulation
1	TCONS_00030245	Pectate lyase (*Scarlet sage*)	0	6.087462841	9.23E-12	1.44E-10	Ups
2	TCONS_00022672	Pectate lyase (*Sesamum orientale*)	0	6.044394119	1.77E-11	2.69E-10	Ups
3	TCONS_00000094	ATP synthase gamma chain, chloroplastic-like (*Sesamum orientale*)	0	5.392317423	5.87E-08	6.03E-07	Ups
4	TCONS_00041341	Auxin-responsive protein (*Scarlet sage*)	6.00E-162	5.247927513	2.43E-07	2.30E-06	Ups
5	TCONS_00026744	Uncharacterized protein LOC105177291 (*Sesamum orientale*)	4.00E-147	5.169925001	5.00E-07	4.40E-06	Ups
6	TCONS_00013494	APETALA 3 (*Sesamum orientale*)	6.00E-134	5.087462841	1.03E-06	8.60E-06	Ups
7	TCONS_00028453	Stachyose synthetase (*Scarlet sage*)	0	5	2.15E-06	1.69E-05	Ups
8	TCONS_00066800	DNA/RNA-binding domain-containing protein (*Penicillium antarcticum*)	8.5	4.977279923	5.35E-21	1.49E-19	Ups
9	TCONS_00000029	Peroxidase (*Sesamum orientale*)	0	4.906890596	4.52E-06	3.33E-05	Ups
10	TCONS_00074290	Fe-S cluster assembly factor HCF101, chloroplastic (*Sesamum orientale*)	0	4.906890596	4.52E-06	3.33E-05	Ups

Table 4: Top 10 up regulated gene ID in stem ISS vs GC

No	Gene ID	Description	E-value UniProt	log2FoldChange	P value	Statistical Significance	Regulation
1	TCONS_00049526	Molecular chaperone (HSP90 family) (*Handroanthus impetiginosus*)	0	5.665335917	1.82E-43	8.86E-41	Ups
2	TCONS_00001293	Heat shock protein 83 (*Sesamum orientale*)	0	5	0.000142317	0.003488927	Ups
3	TCONS_00002328	SET domain-containing protein (*Scarlet sage*)	0	4.906890596	0.000245105	0.005246682	Ups
4	TCONS_00026447	Alcohol dehydrogenase, class III, 1.2.1.76 (*Handroanthus impetiginosus*)	0	4.906890596	0.000245105	0.005246682	Ups
5	TCONS_00061426	Laccase (*Scarlet sage*)	0	4.906890596	0.000245105	0.005246682	Ups
6	TCONS_00003876	SP-RING-type domain-containing protein (*Mimulus guttatus*)	0	4.807354922	0.000423708	0.007861815	Ups
7	TCONS_00042887	Acyl-CoA binding domain (ACB) domain-containing protein (*Scarlet sage*)	1.00E-76	4.807354922	0.000423708	0.007861815	Ups
8	TCONS_00066748	Plant Homeodomain (PHD)-type domain-containing protein (*Scarlet sage*)	0	4.807354922	0.000423708	0.007861815	Ups
9	TCONS_00033188	F-box domain-containing protein (*Scarlet sage*)	8.00E-41	4.700439718	0.000735306	0.01187082	Ups
10	TCONS_00050223	Molecular chaperone (HSP90 family) (*Handroanthus impetiginosus*)	0	4.700439718	0.000735306	0.01187082	Ups

Table 5: Top 10 downregulated gene ID in leaf ISS vs GC

No	GeneID	Gene Description	E-value UniProt	log2FoldChange	Pvalue	Statistical significance	Regulation
1	TCONS_00024301	Protein ALP1-like (Sesamum indicum (*Oriental sesame*)	2.00E-137	8.06608919	5.81E-50	3.70E-48	down
2	TCONS_00051411	FAD-binding domain-containing protein (*Salvia splendens*)	0	6.882643049	6.46E-13	1.10E-11	down
3	TCONS_00086160	Ferruginol synthase (*Rabdosia rubescens*)	0	6.321928095	1.67E-32	7.09E-31	down
4	TCONS_00074857	WRKY domain-containing protein (*Scarlet sage*)	3.00E-167	6.285402219	4.63E-09	5.52E-08	down
5	TCONS_00036057	Mitotic spindle assembly checkpoint (*Salvia splendens*)	0	6.209453366	1.16E-08	1.31E-07	down
6	TCONS_00010515	C2H2-type domain (*Salvia splendens*)	3.00E-92	6.169925001	1.85E-08	2.01E-07	down
7	TCONS_00027021	Mitochondrial uncoupling protein (*Oriental sesame*)	1.00E-92	6.044394119	7.49E-08	7.62E-07	down
8	TCONS_00088868	Myb proto-oncogene protein, plant (*Scarlet sage*)	4.00E-156	5.95419631	1.92E-07	1.84E-06	down
9	TCONS_00079473	Mitochondrial uncoupling protein 5-like isoform X1 (*Oriental sesame*)	0	5.820178962	3.54E-23	1.10E-21	down
10	TCONS_00084040	Peptide/histidine transporter (Salvia splendens (*Scarlet sage*)	0	5.807354922	8.03E-07	6.74E-06	down

Table 6: Top 10 downregulated gene ID in stem ISS vs GC

No	GeneID	Gene Description	E-value UniProt	log2FoldChange	P value	Statistical Significance	Regulation
1	TCONS_00015134	Major facilitator superfamily (*Handroanthus impetiginosus*)	0	4.700439718	5.87E-05	0.00181529	Down
2	TCONS_00044485	Copper chaperone (*Handroanthus impetiginosus*)	5.00E-151	4.584962501	0.0001185	0.00315186	Down
3	TCONS_00020437	Synaptotagmin-5 isoform X1 (*Sesamum orientale*)	0	4.321928095	0.0004941	0.00877194	Down
4	TCONS_00046841	Nascent polypeptide-associated complex subunit beta (*Scarlet sage*)	1.00E-92	4.321928095	0.0004941	0.00877194	Down
5	TCONS_00088737	DELLA protein (*Scarlet sage*)	0	4.321928095	0.0004941	0.00877194	Down
6	TCONS_00032601	Photosystem I reaction center subunit III, PSI-F (*Handroanthus impetiginosus*)	3.00E-140	4.169925001	0.0010223	0.01507966	Down
7	TCONS_00036187	PRA1 family protein (*Scarlet sage*)	2.00E-138	4.169925001	0.0010223	0.01507966	Down
8	TCONS_00051991	60S ribosomal protein L11 (*Handroanthus impetiginosus*)	1.00E-123	4.169925001	0.0010223	0.01507966	Down
9	TCONS_00083087	Carnosine N-methyltransferase (*Sesamum orientale*)	0	4.169925001	0.0010223	0.01507966	Down
10	TCONS_00004343	4-coumarate--CoA ligase (*Scarlet sage*)	0	4	0.002136	0.02506236	Down

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3.3 Differential Gene Ontology (GO) enrichment analysis

To determine the global effect of microgravity condition in ISS, an enrichment analysis of GO was performed in holy basil. The number differentially expressed genes in each GO term was shown in figure 4 with the specification of the relevant biological process, cellular component and molecular function that affected by microgravity. The top 30 most prominent GO categories for leaf and stem ISS vs GC were shown in figure 4. In leaf/stem ISS vs GC, the highest up regulated genes in molecular functions were mRNA binding (leaf) and RNA binding (stem). Genes that identified in top 10 mRNA/RNA binding were involved in pectate lyase (involved in pectin catabolic process in cell well), copper chaperone activity (involved in cellular copper ion homeostasis metal ion binding), ATP binding (involved in NAD biosynthetic process, cellular response to heat shock, protein folding, protein stabilization, cellular response to hypoxia and defense response to stress), methylthranferase activity (involved in choline biosynthetic process, phosphatidylcholine biosynthetic process, pollen development and post-embryonic root development), myo-inositol:proton symporter activity (involved in myo-inositol transport), inorganic diphosphatase activity (involved phosphate-containing compound metabolic process and response to cadmium ion), histidine phosphotransfer kinase activity (involved in cytokinin-activated signaling pathway), nuclear export signal receptor activity (involved in flower development, meristem initiation and tRNA export from nucleus), L-tyrosine:2-oxoglutarate aminotransferase activity (involved in tyrosine catabolic process, vitamin E biosynthetic process) and phosphoenolpyruvate carboxylase activity (involved in carbon fixation, cellular response to phosphate starvation, leaf development, photosynthesis, protein tetramerization and tricarboxylic acid cycle). This result showed some of GO enrichment in microgravity holy basil have similar response in microgravity *Arabidopsis* transcriptomic revealed GO enrichment in responses in oxidative stress, heat shock and changes in cell wall dynamics [79].

In GO enrichment p value, the top 30 significantly enriched GO for leaf/stem ISS vs GC was shown (figure 5). The chloroplast thylakoid membrane was the highest for the leaf ISS vs GC and photosystem 1 was the highest for the stem ISS vs GC.

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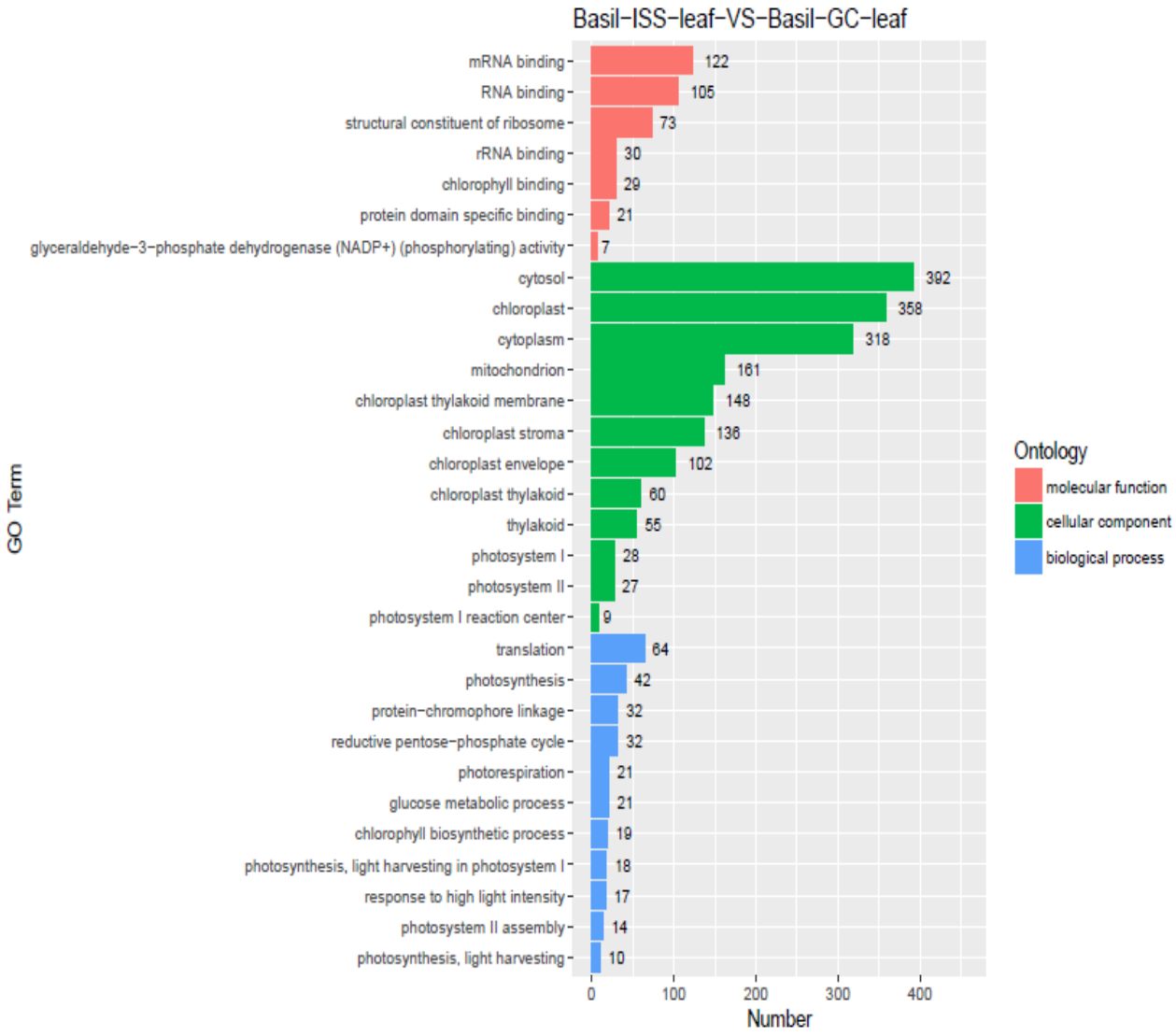

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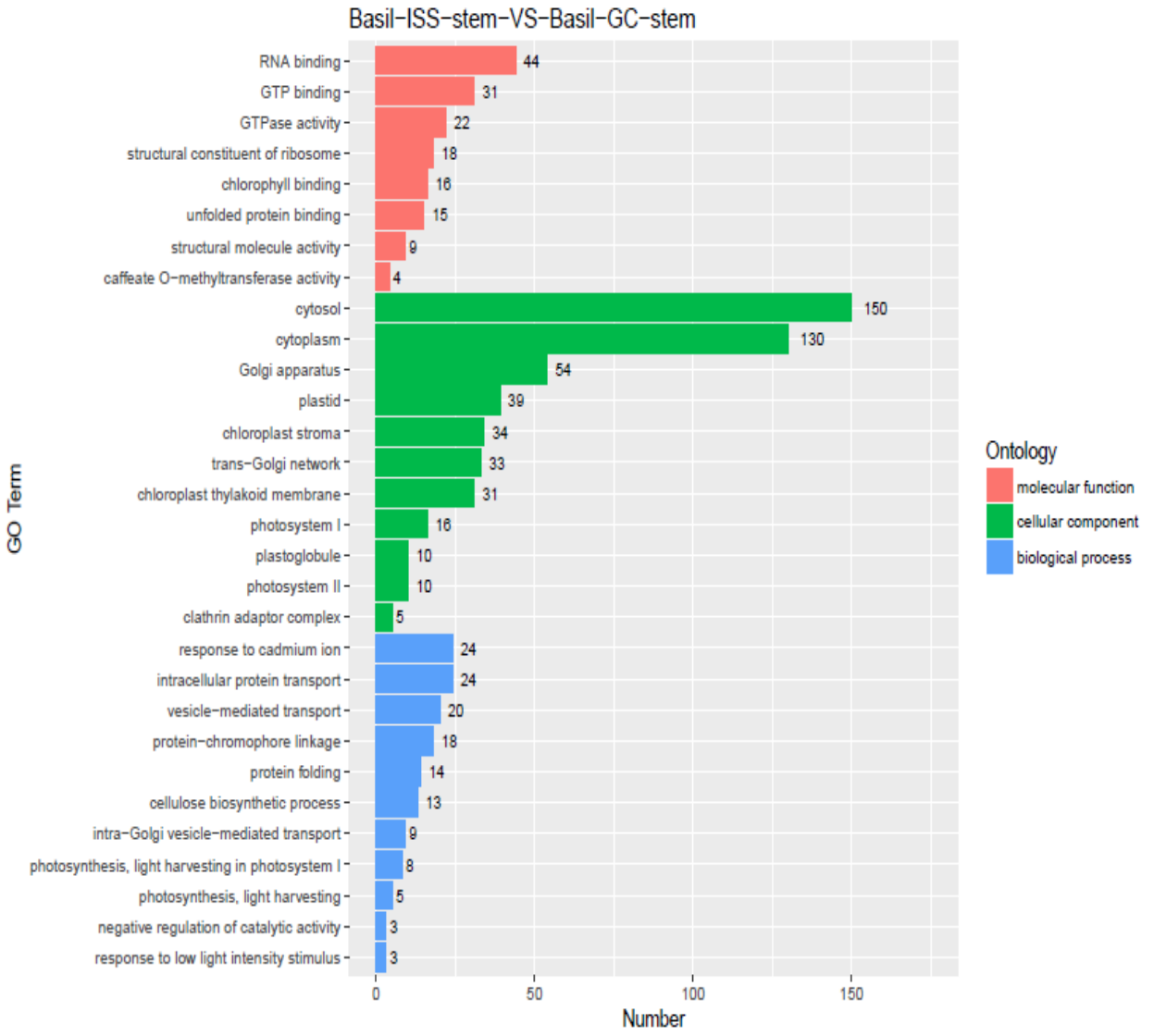

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Figure 4: GO enrichment histogram. X axis: number of differentially expressed gene in this GO category. Color code is to distinguish the categories - biological processes cellular components and molecular functions . A. Holy basil ISS leaf vs GC leaf. B. Holy basil ISS stem vs GC stem

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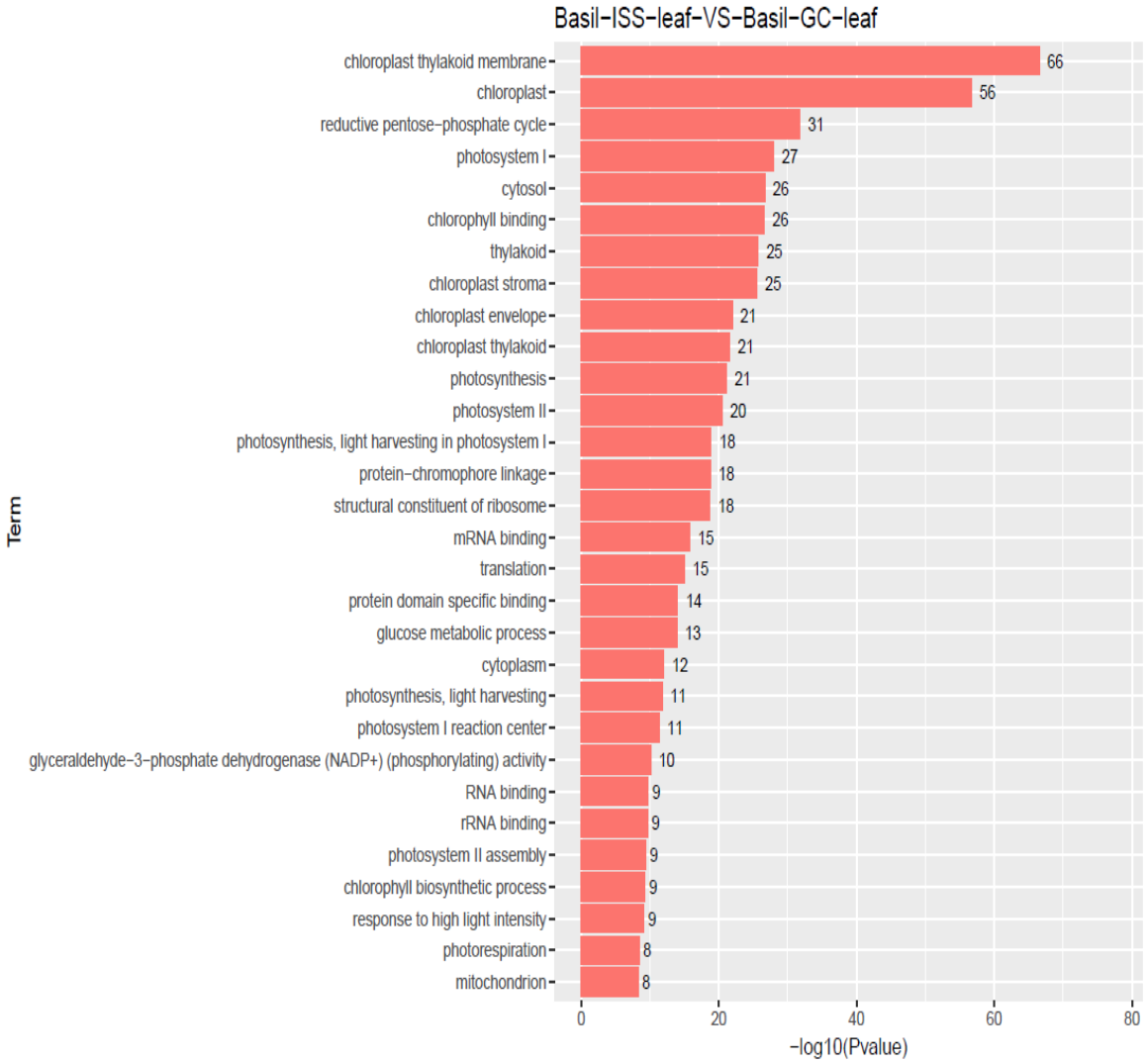

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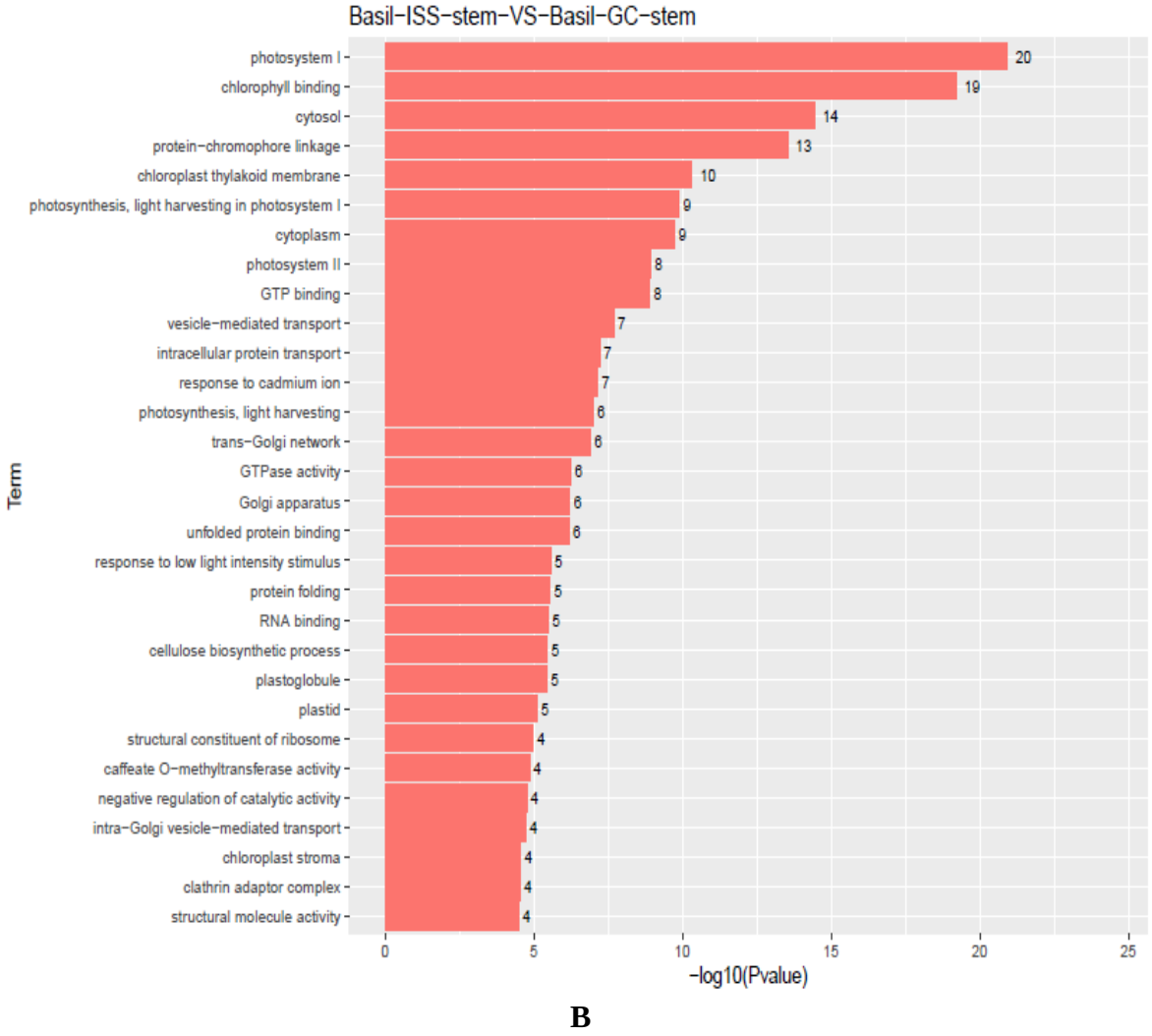

B

Figure 5: GO enrichment pvalue histogram. X axis: -log10(p-value) of each term. Y axis: significant enriched GO term. A. Holy basil ISS leaf vs GC leaf. B. Holy basil ISS stem vs GC stem

3.4 Plots of differential gene KEGG enrichment

To study physiological activities between genes with various functions, a pathway functional enrichment was carried out to differentially expressed genes involved in holy basil of leaf/stem ISS vs GC using *Kyoto Encyclopedia of Genes and Genomes* (KEGG) (Figure 6). In leaf ISS vs GC, the most genes were in metabolic pathway and in genetic information processing was spliceosome. In stem the most genes in metabolism were in photosynthesis-antenna protein, in environmental information processing were ABC transporters and cellular process were in

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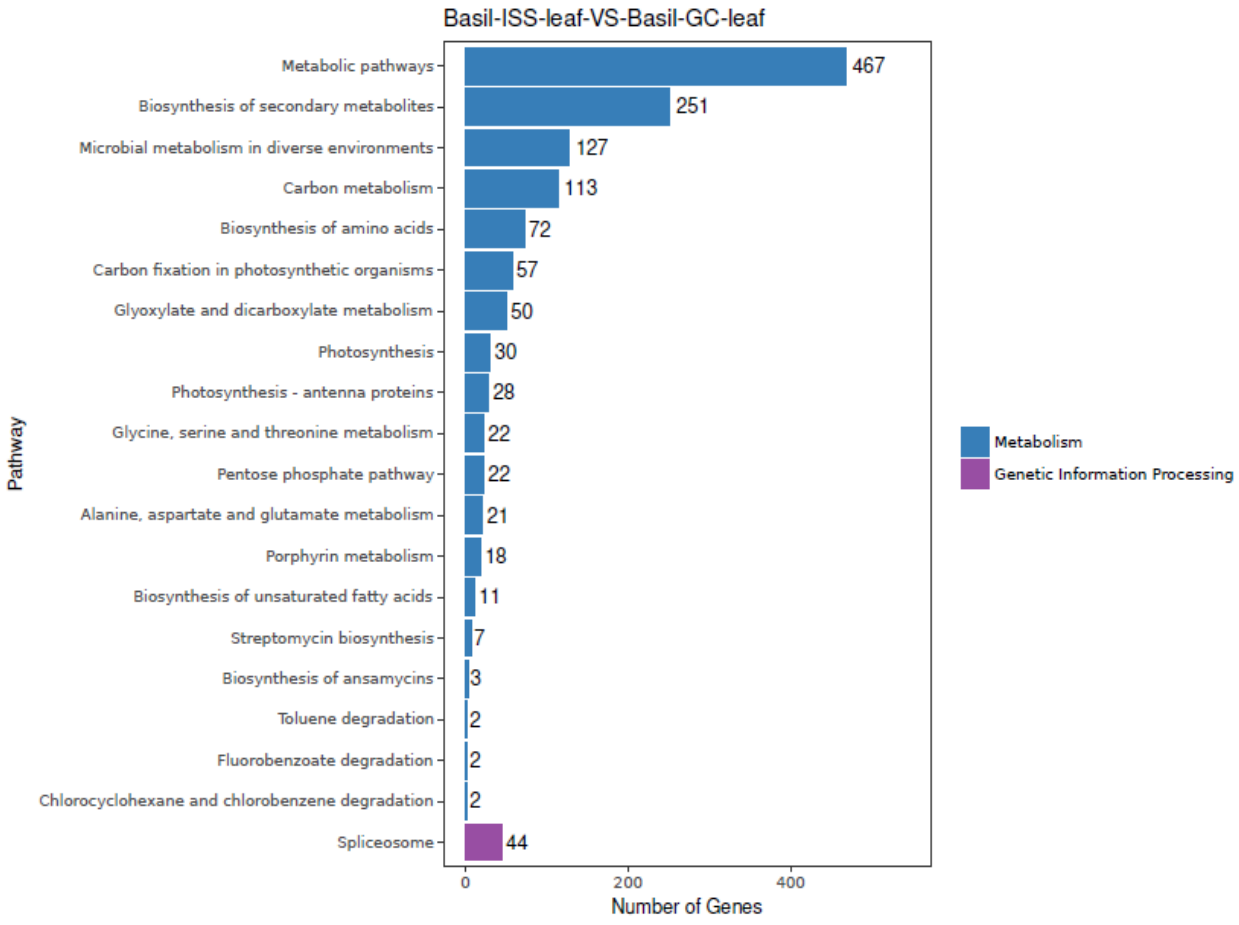
endocytosis. Studied microgravity response in *Arabidopsis* transcriptomic for enriched networks interactions in a response network showed associated with ion transport, chaperone activity and protein ubiquitinylation [79] that has been identified in holy basil microgravity and suggested spaceflight may have triggered alterations in proteasome activity, possibly related to stress-induced protein turnover. Heat shock protein was upregulated in holy basil stem and similar in microgravity *Arabidopsis* [79]. Heat Shock Proteins are molecular chaperones associated with protecting and refolding proteins in response to cellular damage consistent with the enriched to photosynthesis. Heat shock protein suggested may play an important role in ameliorating chloroplastic proteotoxic stress possibly resulting from spaceflight-induced production of reactive oxygen species (ROS) in the plastid and have even been linked to tolerance to the proteotoxic damage caused by hypoxia [79].



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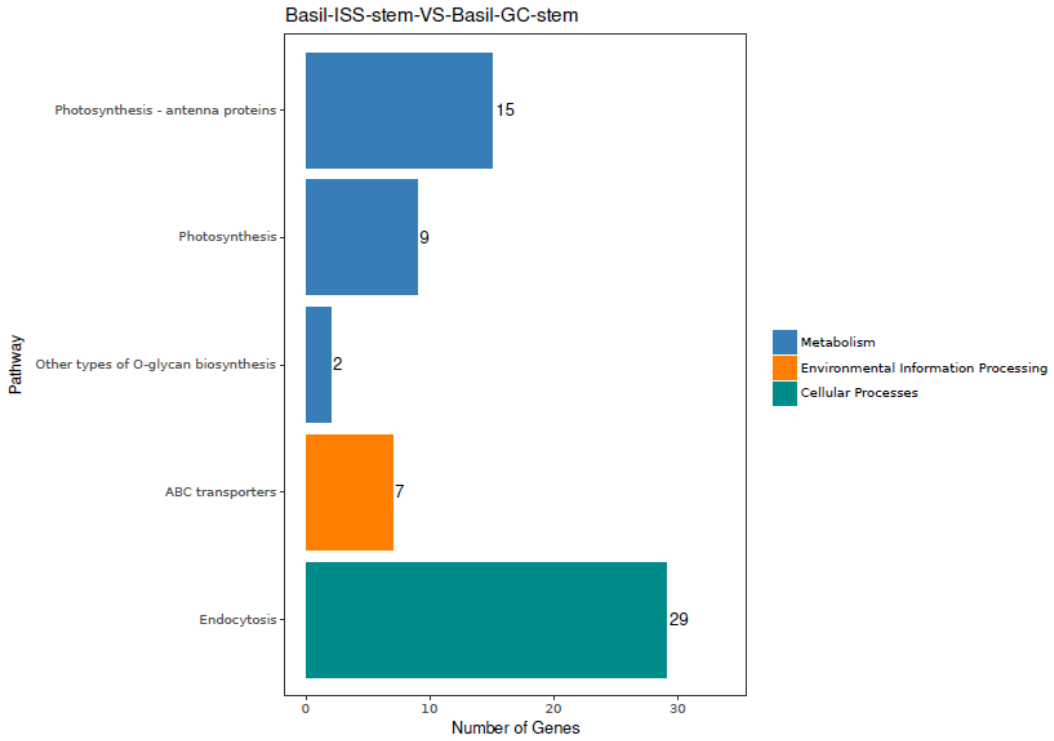

Figure 6: Holy basil ISS leaf vs holy basil ISS leaf. a. KEGG enrichment histogram. X axis: gene number. Y axis: pathway term

3.5 Anatomical cell structure

There was no difference in the leaf transverse thickness and cell elongation for the holy basil under microgravity effects (Figure 7A and 7B) and earth (Figure 7C and 7D). The leaf petiole consists of adaxial concavity and abaxial prominent petiole with the lamina projected towards upper side (Figure 7A), with no difference in the leaf transverse thickness. Only slight linear decrease observed in petiole size with 1.5 mm wide and 60 um thickness, due to sample selection. The ground tissue was parenchymatous and the cells were still polygonal and elongated (Figure 7C, 7D). For petiole, the vascular strand was single, wide and wider oval shaped, while the vascular strand consists of short three or four cells angular narrow xylem with wide parenchymatous. The phloem elements located along the lower end of xylem strand and the phloem was also seen in small discrete masses.

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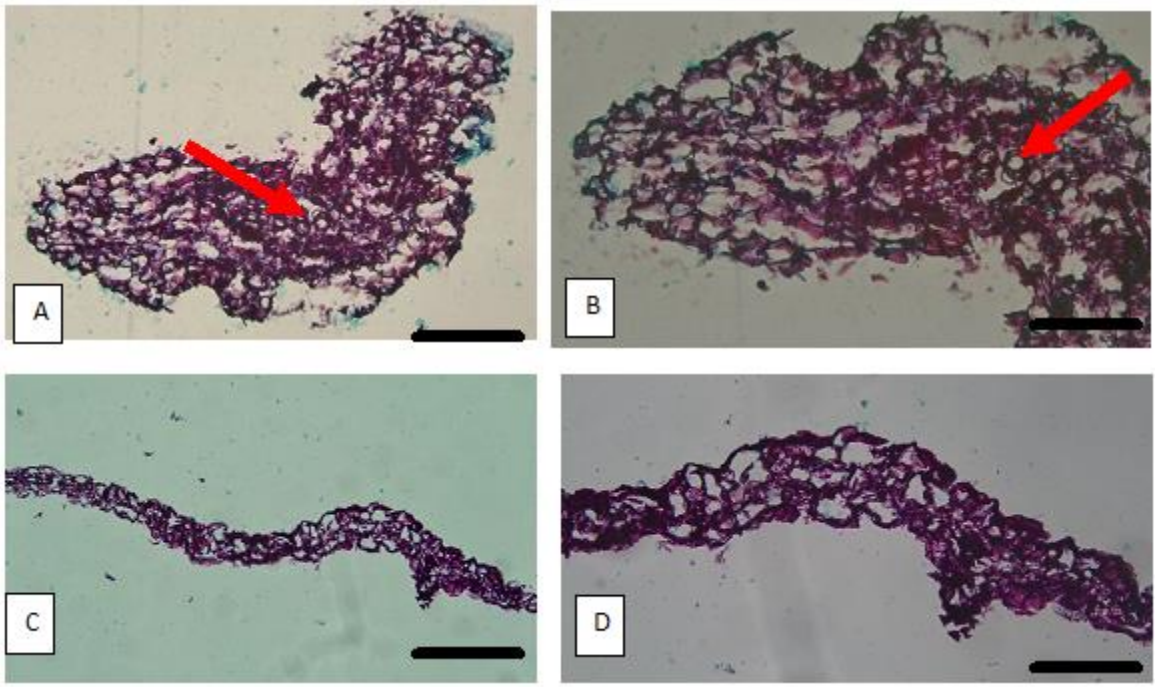

Figure 7: A and B (microgravity), petiole transverse section (100µm, 50µm), C and D (earth), lamina transverse section (100µm, 50µm) of holy basil

CONCLUSION

We have completed gene expression analysis of microgravity holy basil leaf/stem ISS (microgravity) vs GC (earth). We have identified differential express genes of holy basil ISS (microgravity) vs GC (earth) with total of 1518 (downregulated)/ 1046 (upregulated) for leaf and 599 (downregulated) / 224 (upregulted) for stem. The top 10 upregulated genes in leaf were Pectate lyase, ATP synthase gamma chain, Auxin-responsive protein, uncharacterized protein, APETALA 3, Stachyose synthetase, DNA/RNA-binding domain-containing protein, Peroxidase and Fe-S cluster assembly factor HCF101, while top 10 upregulated genes in stem were Heat shock protein, SET domain-containing protein, Alcohol dehydrogenase, Laccase, SP-RING-type domain-containing protein, SP-RING-type domain-containing protein, PHD-type domain-containing and F-box domain-containing protein. The top downregulated genes in leaf were Protein ALP1-like, FAD-binding domain, Ferruginol synthase, WRKY domain, Mitotic spindle assembly, C2H2-type domain, Mitochondrial uncoupling, Myb proto-oncogene and Peptide/histidine transporter, while in stem were Major facilitator superfamily, Copper chaperone, Synaptotagmin-5 isoform X1, Nascent polypeptide-associated complex subunit beta, DELLA protein, Photosystem I reaction center subunit III, PSI-F, PRA1 family protein, 60S ribosomal protein L11, Carnosine N-methyltransferase and 4-coumarate--CoA ligase. Differential GO

enrichment in leaf/stem ISS vs GC, showed the highest up regulated genes in molecular function were mRNA binding(leaf/stem); in cellular component was cytosol (leaf/stem) and in biological process was translation (leaf)/ response to cadmium ion and intracellular protein transport (stem). A pathway functional enrichment to differentially expressed genes in holy basil leaf/stem ISS vs GC using KEGG showed the most genes in metabolism were in metabolic pathway and in genetic information processing was spliceosome for leaf, while in metabolism were in photosynthesis-antenna protein, in environmental information processing were ABC transporters and cellular process were in endocytosis for stem. In anatomical cell structure of holy basil ISS vs GC was no difference in the leaf transverse thickness and cell elongation. The gene expressions studied of microgravity holy basil were focused on a narrow window of life cycle due to arrangement that supported short duration experiments. Therefore, we cannot assume that the transcriptional response to microgravity seen in these experiments would be universally applicable to long duration plant experiments on the ISS. The seed in plant chamber was controlled in optimal environment for seedling growth with sufficient lighting, moisture, water and humidity. Long term experiments where plants were grown from seed to maturity were needed to characterize critical stages in plant development. These transcriptional studies were valuable in identify key transcriptional regulators and offer the first signs of genes in holy basil which may be altered in microgravity environment. These finding will provide information of engineer plants for optimal growth during long-term spaceflight missions in the future.

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