Studies on hormonal control of isoprene biosynthesis in tropical tree *Ficus septica*.

（ホルモンによる熱帯樹木オオバイヌビワのイソプレン生合成制御に関する研究）

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A dissertation submitted to the United Graduate School of Agricultural Sciences, Kagoshima University, Japan

In partial fulfillment of the requirements

for the degree of

Doctor of Agriculture

2023

Abstract

Tropical trees emit a huge amount of isoprene into the atmosphere, exerting strong influences on tropospheric chemistry. Meanwhile, isoprene has been considered to protect leaves from many environmental stresses. In this thesis, I addressed the hormonal control of isoprene biosynthesis during short-term and long-term responses using tropical trees *F. septica*:

Plant hormone signaling and circadian clock gene have been implicated in the transcriptional control of isoprene biosynthesis. To get more insight into the hormonal control of isoprene biosynthesis, the first part of present study analyzed the plant hormone concentration of the leaves in our previous two model studies: JA treated leaves in which isoprene biosynthesis was mainly controlled by transcriptional modulation of isoprene synthase (*IspS*) gene; short-term drought stressed leaves in which control of isoprene biosynthesis was mainly by post-transcriptional regulation of *IspS* gene. To explore the hormonal control of isoprene biosynthesis, present study analyzed the correlations between hormone concentration and gene expression of 2-C-methyl-D-erythrytol 4-phosphate (MEP) pathway, hormone signaling and circadian rhythm processes, and metabolite pool sizes of MEP pathway. Results suggested that hormone balance between JA-Ile and IAA plays a central role in the transcriptional regulation of IspS gene through the transcription factor *MYC2* and *SAUR21,* the early auxin responsive gene. Putative cis-acting element for SAUR on *IspS* promoter (TGTCNN and CATATG) in addition to G-box for MYC2 supports above proposal. Whereas, no significant changes were noted in the plant hormone concentration and related signaling pathway gene expression under short-term drought stress suggesting a limited importance of hormone signaling in the post-transcriptional regulation of *IspS*.

To get insight into the role of plant hormone in the long-term control of isoprene emission, next study monitored isoprene emission from *F. septica* leaves, plant hormone concentration and signaling gene expression, MEP pathway metabolite concentration and related enzyme gene expression throughout a year in the field conditions. Isoprene emission largely paralleled with the air temperature and light intensity of the field showing higher emission in summer season (Jul. to Oct) and low or negligible emission rate in winter season (Dec. to Mar.). This seasonality of isoprene emission was mainly driven by temperature and light-dependent changes in substrate availability via the MEP pathway and transcriptional and post-transcriptional regulation of *IspS*. Seasonality of isoprene emission modulated plant hormone profiles due to trade-off between isoprene and hormone biosynthesis. This was most clearly manifested with the profile of cytokinins with decrease in summer and increase in winter season. Among the MEP metabolites, only HMBDP showed a positive correlation with the concentration of cytokinin suggesting that HMBDP and its biosynthesis enzyme *HDS* play a role in the channeling of MEP pathway metabolite to cytokinin biosynthesis. These observations on hormone profile, *IspS* gene expression, MEP pathway metabolite and related gene expression prompted the authors to postulate a putative feed-forward control of isoprene biosynthesis by cytokinins. The hormone balance of IAA/JA-Ile nor respective transcriptional factor *SAUR21/MYC2* suggested to be a factor for short-term transcriptional regulation of *IspS* gene showed no correlation with *IspS* transcript and isoprene emission under long-term natural conditions. This study thus extended our previous study and revealed the difference between short- and long-term hormonal regulation of isoprene biosynthesis and emission from the tropical tree *F. septica*.

Abstract (Japanese)

熱帯植物は、高い外気温とつい強烈な太陽光線によりもたらされる高温ストレスを克服するため、イソプレンという炭化水素を葉から放出することにより葉緑体のチラコイド膜を安定化させ、高温ストレスによって引き起こされる光合成の阻害や他の生理障害から身を守っていると考えられている。イソプレンはこのように植物に対しては有益な保護作用を発揮する一方、大気中においてメタン等の分解を担うヒドロキシラジカルと容易に反応してこれを消費し、温暖化を加速する等の深刻な大気環境化学的な問題を引き起こす可能性が指摘されている。しかしながら、熱帯植物のイソプレン合成・放出の制御機構及びその放出特性については不明な点が多い。本研究は、沖縄に自生する熱帯樹木のオオバイヌビワを対象として、イソプレン合成制御におけるホルモンシグナリングの解明を行うことを目的とした。

まず、これまでの研究によりイソプレン合成制御に関与することが示唆されているストレスホルモンであるジャスモン酸（JA）処理の影響を評価した。IspS遺伝子の上流の制御系の分子機構を明らかにするため、JA処理後のイソプレン放出変化、葉内のホルモン濃度、基質供給に関与するMEP経路およびイソプレン合成酵素（ISPS）遺伝子、ISPSのプロモーター配列、植物ホルモン合成遺伝子及と時計遺伝子関連遺伝子発現および代謝産物の解析を行った。その結果、葉内のジャスモノイルイソロイシン（JA-Ile）とインドール-3-酢酸（IAA）の間のホルモンバランスが、イソプレン合成制御に重要であることが初めて明らかにされた。また、JA-Ileシグナル伝達系下流の転写因子MYC2とIAAシグナル下流のSAUR21の発現の比率がIspS遺伝子の転写調節において中心的な役割を果たしていることも示唆された。IspSプロモーターの転写開始上流

域にMYC2の応答エレメントであるGボックスに加えて、SAUR21のシスエレメントが認められることも上述の制御機構を支持するものと考えられた。他方、短期間の乾燥ストレス下では一過的にIspSタンパク質量とイソプレン合成が増加するが、植物ホルモン濃度および関連するシグナル伝達遺伝子の発現に有意な変化は認められず、ホルモンシグナリングの果たす役割は翻訳レベルのIspS合成制御においては限定的と考えられた。

次に、本研究では季節変動を例として、イソプレン合成の長期的な調節機構におけるホルモンの役割について検討した。イソプレン放出は気温と光強度に依存し、夏場（7-10月）に高く、冬場（12.3-3月）に低かった。この温度あるいは光依存的な季節変動は、MEP経路おけるイソプレン合成酵素（IspS）の基質DMADPの合成、IspSの転写及び翻訳レベルで主に調節されていた。基質DMADPの供給系であるMEP経路はイソプレン合成以外にサイトカイニンやアブシジン酸等のホルモンの合成材料も提供している。このため、イソプレン放出の変動はホルモン濃度にも影響を及ぼし、特にサイトカイニンは夏場に低く冬場に高い顕著な季節変動を示した。MEP経路の中間代謝産物のうち、サイトカイニン合成の直接的な材料となるHMBDPはサイトカイニン濃度と正の相関を示したことから、HMBDPとその合成酵素であるHDSによるサイトカイニン合成への代謝産物の分配を通じてサイトカイニン濃度がイソプレン合成を制御するFeed-forwardループが存在することが示唆された。短期的なイソプレン合成制御において重要な役割を果たしていたIAAとJA-Ile、それらの転写因子SAUR21とMYC2の比率はIspS及びイソプレン放出とは相関せず、長期的なイソプレン合成におけるこれらのホルモンシグナリングの果たす役割は限定的と判断された。

以上これらの成果は、イソプレン合成の転写及び翻訳レベルの調節における短期、長期的なホルモンシグナリングの役割に関する基礎知見を提供するものである。

Acknowledgments

I first want to thank my advisor, Prof. Hirosuke Oku (Center of Molecular Biosciences, University of the Ryukyus) for his helpful criticism and support during the course of my degree. The lab environment that you have provided has been a wonderful place to study. I am grateful for the mistakes that you allowed me to make. Thank you for reigning me in when I was off course. Although it has been a painful process at times, I am better off because of it. A person with an amicable and positive disposition, Prof. Oku has always made himself available to clarify my doubts despite his busy schedule and I consider it a great opportunity to do my doctoral program under his guidance and to learn from his research expertise.

I am highly indebted to Dr. Masashi Inafuku, Assistant Professor, at the University of the Ryukyus for his advice, guidance and cordial help in completing this endeavor. I am also grateful to him to support my projects and teach me various experimental techniques. I really have to thank him for mentoring me, for organizing the lab and for supporting and accommodating all members despite his very busy schedule.

I want to show my gratitude to Prof. Koji Miyamoto, Department of Biosciences, Teikyo University, and Emi Yumoto, Advanced Instrumental Analysis Center, Teikyo University, Tochigi, Japan, without their cooperation and support this study will almost impossible to finish.

Special thanks to my mentor and teacher Associate prof. Dr. Harun-ur-Rashid and Dr. Shahnaz Parveen for their support throughout the study. I am so much thankful to my dearest friend Dr. Yousuf Hossin, Takuya Higa, Kyta Nakamura, Manami and all my lab members for their support and for making my lab experience a memorable one.

Lastly, I would like to dedicate this dissertation to my beloved family, parents for their invaluable support, unconditional love, and help emotionally throughout my stay in Japan and for bearing all the tough times they went through with me.

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List of abbreviations

ABA	Abscisic acid
ACTB	Actin, beta, cytoplasmic
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BVOCs	Biogenic Volatile Organic Compounds
CDP-ME	4-(cytidine 5`-diphospho)-2-C- methyl-D-erythritol
CDP-MEP	2-phospho-4-(cytidine-5`-di-phospho)-2-C-methyl-D-erythritol
CK	Cytokinin
CL	Light dependence variable in G-93 model
CMK	CDP-ME kinase
CMP	Cytidine monophosphate
CT	Temperature dependence variable in G-93 model
cZOG	cis-zeatin-O-glucoside
cZROG	cis-zeatin riboside-O-glucoside
cZ	cis-zeatin
cZR	cis-zeatin riboside
DMADP	Dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	DXP reductoisomerase
DXS	DXP Synthase
ETR-NAR	Electron transport rate – net carbon assimilation rate

FACE	Free-Air CO_2 and O_3 enrichment
G3P	Glyceraldehyde 3-phosphate
G-93	Guether *et al.* (1993) model
GA	Gibberellic acid
GBF	G-box binding factor
GO	Gene Ontology
HDR	HMBDP reductase
HDS	HMBDP synthase
HMBDP	4-hydroxy-3-methylbut-2-butenyl 4-diphosphate
HSE	Heat shock elements
IAA	Indole-3-acetic acid
Idi	Isopentenyl diphosphate isomerase
IPP	Isopenteyl diphosphate
iP	Isopentyl adenine
iPR	Isopentyl adenosine riboside
IspS	Isoprene synthase
JA	Jasmonic acid
JA-Ile	Jasmonoyl isoleucine
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid Chromatography – tandem mass spectrometry
MCS	MEcDP Synthase
MCT	MEP cytidyltransferase
ME	Module eigenvector

MEcDP	2-C-methyl-D-erythritol 2,4-cyclodiphosphate
MEP	2-C-methyl-D-erythritol 4-phosphate
MSE	Mean Squared Error
MVA	Mevalonate
NMDS	Non-metric multidimensional scaling
O3	Ozone
OH	Hydroxyl radical
OPDA	12-oxo-phytodienoic acid
PAR	Photosynthetically Active Radiation
PPFD	Photosynthetic photon flux density
qRT-PCR	Quantitative real-time PCR
RNA-seq	RNA sequencing
ROS	Reactive Oxygen Species
SA	Salicylic acid
SPE	Solid Phase Extraction
TF	Transcription factor
tZ	trans-zeatin
tZROG	trans-zeatin riboside-O-glucoside
tZR	trans-zeatin riboside
UFLC	Ultra-fast liquid chromatography

CHAPTER I

General Introduction

1.1 Global perspective on isoprene emission

Isoprene is the simplest terpene that is produced by plants profusely. After its synthesis, it volatilized immediately to the atmosphere, as there are no known storage mechanisms inside the plant. (Sanadze 2010). The isoprene synthesis pathway has been elucidated within the past few decades and its regulatory mechanisms have been subject to study in a wide variety of fields (Cordoba et al., 2009; Banerjee and Sharkey, 2014). Its physiological effects in plants are still being cataloged and its modes of action, based on these physiological effects, are still being debated (Sharkey et al., 2008; Velikova et al., 2012). Understanding the molecular mechanism of action and biological function of isoprene is important for several reasons. The study of isoprene regulation and function in plants grew out of a desire to better inform models of future emission rates, and that need is more urgent than ever. According to the projection of Yang Cao et al., 2021, after the 21st century isoprene emission will increase up to 21-57%. Isoprene is a significant contaminant in modern, and generates tropospheric ozone in the presence of high nitric and nitrous oxide (NOx) in atmospheres and one molecule isoprene can lead the production of many ozone molecules (Fehsenfeld et al., 1992; Trainer et al., 1987). Biogenic isoprene expand the life span of greenhouse gas methane (Poisson et al., 2000) by reacting with hydroxyl radicals that functions to oxidize and degrade methane in atmosphere. Isoprene emission is more prevalent in the tropical and subtropical regions (Guenther, 1995; Potter et al., 2001) but it is possible that the tree inventory of temperate forests will shift towards those of isoprene-emitting species in the future and characteristics of overall global climate change including the alteration in CO_2 concentration, temperature, and ozone levels can influence the global isoprene emission rate.

1.2 Biological basis and regulation of isoprene emission

Precursor production

Isoprene is biosynthesized from the precursor dimethylallyl diphosphate (DMADP). Plants possess two distinct and noninteracting pathways for DMADP production: the mevalonic acid pathway in the cytosol and the methyl erythritol phosphate (MEP) pathway in the plastid (Fig. 1.1 MEP pathway). The MEP pathway is more efficient than the MVA pathway in photosynthetic organisms the cost of energy and carbon atoms is less than the MVA pathway. The MEP pathway costs only 6 carbon atoms, 20ATP, and 14NADPH. While in the MVA pathway, it costs 9 carbon atoms, 24 ATP, and 14 NADPH. (Sharkey and Yeh, 2001) In emitting species, isoprene emission accounts for well over 90% of the flux through the MEP pathway (Sharkey, Loreto & Delwiche 1991). The MEP pathway was recognized in the early 1990s (Rohmer M. 1993). The MEP pathway can also produce IPP (McCaskill et al. 1999) that can be isomerized to DMAPP.

Seven stromal enzymes convert photosynthetically derived glyceraldehyde-3-phosphate and pyruvate to DMADP using reducing power and ATP equivalents derived from photosynthesis. (Banerjee and Sharkey, 2014). The first two enzymes, 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS) and DXP reductoisomerase (DXR), are the key regulators of the MEP pathway. Several studies have indicated that MEcDP has potential roles in MEP pathway regulation. MEcDP may connect metabolism in the MEP pathway with other cellular metabolism, independent of its role in making precursors for isoprenoids. (Banerjee, A., & Sharkey, T. D.,

2014). Other enzymes in the pathway are likely regulated in the short-term regulation by phosphorylation and stromal redox status (Li and Sharkey, 2013; Banerjee and Sharkey, 2014).

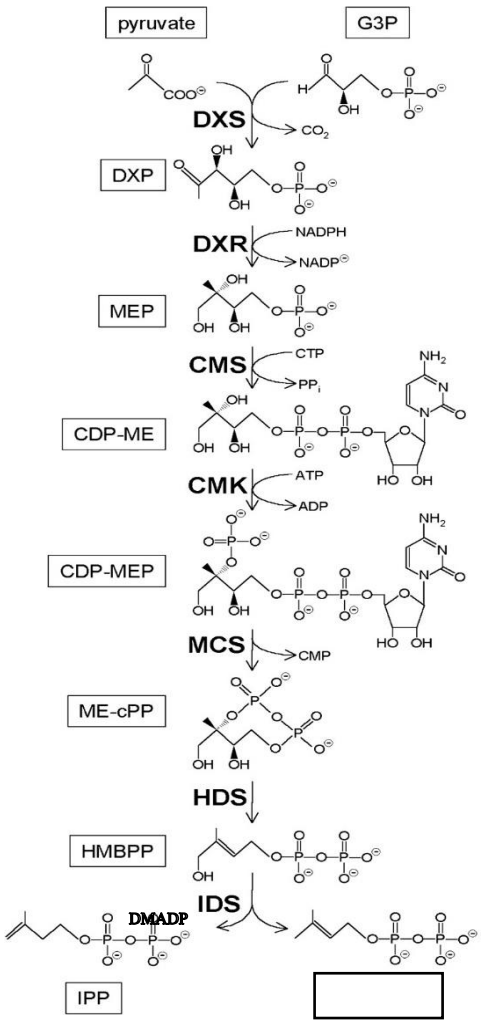

Figure 1.2-1. The MEP (2-C-methyl-D-erythritol 4-phosphate) pathway is showing the formation of isoprene. This pathway uses pyruvate and glyceraldehyde 3-phosphate (G3P) to synthesize substrates for isoprene biosynthesis in seven steps. Intermediate metabolites are: DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP–ME, 4-(cytidine 5`-diphospho)-2-C- methyl-D-erythritol; CDP-MEP, 2-phospho-4-(cytidine-5`-di-phospho)-2-C-methyl-D-erythritol; MEcDP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBDP, 4-

hydroxy-3-methylbut-2-butenyl 4-diphosphate; IPP, isopentenyl diphosphate and DMADP, dimethylallyl diphosphate. CMP in the structures denotes cytidine monophosphate. Enzymes are *DXS*, DXP synthase; *DXR*, DXP reductoisomerase; *MCT*, MEP cytidyltransferase; *CMK*, CDP-ME kinase; *MCS*, MEcDP Synthase; *HDS*, HMBDP synthase; *HDR*, HMBDP reductase and *IspS*, isoprene synthase.

Isoprene synthase

After the production of DMADP by the MEP pathway, it is converted to isoprene by isoprene synthase (*IspS*). Several studies have indicated that extractable *IspS* activity correlates with isoprene emission (Monson et al., 1992; Kuzma and Fall, 1993; Schnitzler et al., 1997, 2005; Lehning et al., 1999; Bru¨ggemann and Schnitzler, 2002). The enzyme has a low K_m of millimolar range as well as a low turnover number, which has led to the conclusion that it is inefficient relative to synthases of some higher isoprenoids (Silver and Fall, 1995; Sharkey et al., 2005). Not all plants contain isoprene synthase, and recent molecular studies have shown that introduction of an *IspS* gene into Arabidopsis is sufficient to cause the plant to emit isoprene (Sharkey et al., 2005; Sasaki et al., 2007). Studies reveals that *IspS* can be modulated by phytohormones via signal transduction, such as jasmonic acid (JA), cytokinin (CK), auxin, ethylene and salicylic acid (SA) signalling that were demonstrated to modulate genes of MEP, monoterpene and sesquiterpene pathways (Ginis *et al*. 2012; Martin *et al*. 2003; Pateraki & Kanellis 2010). Study of the upstream promoters of IspS genes provide clues that temperature and circadian regulation of *IspS* gene expression through heat shock elements (HSEs), circadian motifs and various heat and light responsive elements present in those promoters (Cinege *et al*. 2009; Loivamäki *et al*. 2007; Schnitzler *et al*. 2010; Sharkey *et al*. 2008; Wiberley *et al*. 2009). Isoprene synthase

protein levels are developmentally and environmentally regulated, but fine temporal control of isoprene emission rate appears to be controlled by changes in DMADP availability, rather than by abundance or activity of the synthase (Vickers et al., 2010). Isoprene emission and IspS transcript levels show strong circadian rhythms (Wilkinson et al., 2006; Loivama¨ki et al., 2007b). Arabidopsis LHY transcription factor was later revealed to bind to promoter region of poplar *IspS*, providing molecular evidence of transcriptional regulation of *IspS* (Loivamäki *et al*. 2007). When grown at high temperatures, leaves begin to emit isoprene at least 1 week after the acquisition of photosynthetic competence, and IspS mRNA and protein begin to accumulate at the same developmental stage (Sharkey et. al., 2008).

Environmental regulation of isoprene emission rate

In the case of isoprene emission, the effect of environmental factors on the overall emission rate is quite well documented. Isoprene emissions are influenced by the leaf developmental stage, growth conditions, nitrogen nutrition, and by seasonal and diurnal variations (Niinemets et al. 2010); circadian regulation of isoprene emission and isoprene synthase transcript has also been demonstrated (Loivamäki et al. 2006; Wiberley et al. 2009). Isoprene emission has been recognized as light-dependent since the first observation (Sanadze, 1969; Sanadze and Kalandaze, 1966). Without light, flux through the MEP pathway ceases, as the pathway uses carbon skeletons, reducing power, and ATP equivalents from the photosynthetic electron transport chain and Calvin cycle (Rasulov et al., 2009). Temperature, as well as air temperature, affects the rate of isoprene emission. Interestingly, this effect cannot be explained by increasing the availability of reducing power from the MEP pathway, as the stroma becomes oxidized at high temperatures (Schrader et al., 2007). Above 30 ^{o}C isoprene emission rise with temperature as photosynthesis is constant or declined. This could be due to the 2% of the carbon fixed by

photosynthesis is emitted as isoprene at 30°C but at 40°C 15% is emitted as isoprene (Sharkey et al. 1996). An MEP pathway metabolite profiling of previous study suggests that the rapid increase was explained by increases in the rate of isoprene synthase rather than substrate availability (Li et al., 2011). This is in agreement with kinetic studies of isoprene synthase that exhibited its temperature optimum at 45-50 °C (Monson et al., 1992; Lehning et al., 1999). Over extended periods of heat stress damage the photosynthetic apparatus thus reduces the rate of photosynthesis, DMADP synthesis, and hence isoprene emission.

1.3 Effects and modes of action of isoprene emission

Atmospheric chemistry of isoprene

The global annual production of isoprene has been estimated at ~500 Tg (Guenther et al., 1995; Guenther et al., 2012), or slightly less than a cubic kilometer in the liquid phase. This is a significant contribution to the atmosphere and has been the impetus for many studies of the gas phase chemistry of isoprene. Isoprene primarily undergoes UV-catalyzed reactions with hydroxyl radicals and ozone. Various intermediate peroxides are formed whose ultimate breakdown is affected by the concentration of NOx (Fehsenfeld et al., 1992; Harley et al., 1999). At low NOx concentrations isoprene effectively reduces atmospheric ozone, while at high concentrations it catalyzes ozone formation and can be considered a pollutant. Isoprene also reacts with NOx to generate organic nitrates, which affects nitrate deposition rates (Horowitz et al., 1998; Zhang et al., 2012).

Physiological effects of isoprene emission

Under the drought or temperature stress conditions, native emitters can emit a quarter to one-half of their recently fixed carbon as isoprene (Sharkey and Loreto, 1993; Way et al., 2011).

As a significant loss of energy happened in the emitter plants, therefore, likely provides some benefit in return. One process that could provide a large enough return on investment is protection of photosynthetic apparatus. This view is supported by the fact that isoprene emission is maximal at 38-45 °C, temperatures at which photosynthesis is damaged. (Loreto and Sharkey, 1990; Monson et.al., 1992) This observation led to the discovery of isoprene-induced thermotolerance of photosynthesis in the mid-1990s (Sharkey and Singsaas, 1995; Singsaas et al., 1997).

The protective effect of isoprene on photosynthesis has been shown in non-emitters by fumigation as well as by transformation with isoprene synthase (Loivamäki et al., 2007; Ryan et al., 2014). In native emitters, studies have prevented endogenous isoprene production through the use of RNAi knockdown of isoprene synthase or the use of MEP pathway inhibitors (Sharkey et al., 2001; Behnke et al., 2007; Behnke et al., 2010). Isoprene is also known to prevent damage caused by reactive oxygen species, specifically ozone (Loreto et al., 2001; Loreto and Velikova, 2001) and singlet oxygen (Affek and Yakir, 2002). This observation may be explained by the known chemistry of isoprene in the atmosphere, and studies from many different laboratory confirmed this phenomenon (Loreto et al., 2001; Loreto and Velikova, 2001). This has led some researchers to propose that ROS quenching is the basis of all of the abiotic stress-mitigating properties of isoprene, including the enhanced thermo protection of photosynthesis (Vickers et al., 2009).

Isoprene emission is a sensitive indicator of wound signals that can travel through plants (Loreto and Sharkey, 1993). Wounding was inflicted by puncturing, smashing, cutting, and, burning leaves. Isoprene also affects insects feeding behavior and tritrophic interactions (Laothawornkitkul et al. 2008; Loivamäki et al. 2008). During study, In *F. septica* we find that wounding the leaves declined or cease the isoprene emission.

The use of isoprene by plants to confer stress tolerance contrasts with the use of mono- and diterpenes by plants for biological defense (Trapp and Croteau, 2001). Isoprene also reduces the formation of nitric oxide in the mesophyll, thereby indirectly modulating the signaling of defense-induced biosynthetic pathways (Velikova et al. 2005). Isoprene has been also reported to enhance the drought resistance of many species. In all cases, isoprene-emitting lines showed reduced depression of photosynthesis, and less oxidative damage than non-emitting lines, when exposed to drought (Ryan et al. 2014; Tattini et al. 2014).

1.4: Phytohormones and Isoprene emission

Plant hormones constitute the fundamental signal transduction system of living plant cells and control development, stress response and senescence. Recently a lot of work has been progressing on isoprene emissions but there is scarce information on the hormonal regulation of isoprene emission. The DMADP biosynthesized through the MEP pathway also produces longer-chain isoprenoids, such as carotenoids and abscisic acid (ABA). (Giovanni Marino et,al, 2017). It has been reported that isoprene emission modulates the plant hormone profile and influences the growth and stress response of plant cells. A study reported that exogenous treatment of poplar leaves with JA increased isoprene emission without any transcriptional regulation (Ferrieri *et al*. 2005). An earlier study on *F. septica* for short-term cold treatment shows that several putative binding motifs for transcription factors have been predicted on the promoter of IspS. The composite phytohormone-mediated signal transduction may be a possible mechanism controlling isoprene biosynthesis (Mutanda et al. 2016a). Similarly, the multitasking role of isoprene as a signaling molecule has been proposed (Zuo et al., 2019). A very recent study also showed that isoprene emission enhanced the abundance of CKs by > 200%, significantly upregulated genes

coding for CK synthesis, CK-signaling and CK-degradation in unstressed leaves. (Dani et. al., 2022). This clearly indicates that isoprene emission indirectly or the isoprene molecule itself modulates the plant hormone profile and controls the biological event of plant cells.

1.5 *F. septica* Burm. F., a tropical plant with an impact

Tropical forests are the largest source of biogenic isoprene emissions (Rasmussen and Khalil, 1988), especially tropical broadleaf trees (Arneth et al. 2008; Guenther et al. 2006; Guenther et al. 2012). However, many studies on isoprene emission response to environmental drivers and efforts to understand its regulation and biological significance were conducted on temperate plant species such as poplars and oaks. Tropical trees are estimated to be representing the one third (70%) of global BVOC emissions (Karl et al., 2007; Jones et al., 2011). Therefore, we used a broadleaf tree species, *F. septica* (Rosales: Moraceae), also known as "Oobainubiwa" (オオバイヌビワ) to better simulate the isoprene emission from tropical forest. This plant is very common and well distributed in East Asia - southern Japan, Taiwan, Malaysia, Indonesia and Philippines, New Guinea, northern Australia, Solomon Islands. Besides, this plant is studied by Oku et al., 2014 to understand the isoprene emission capacity in response to fluctuating ambient temperature.

Figure 1.5-1. A tropical plant, *F. septica Burm. F*

1.6 Objectives of the studies

The main goal was to advance the knowledge of regulatory mechanism of isoprene emission from tropical tree. Focus was placed on the plant hormone profile and transcriptional control of isoprene biosynthesis. This thesis thus studied the relationship between plant hormone profile and control of isoprene biosynthesis or emission in both exogenous JA hormone treated and drought stressed leaves to explore the short-term mechanism. Meanwhile, third chapter

monitored the relationship throughout the year to understand the long-term hormonal control of isoprene biosynthesis and emission.

CHAPTER II

Plant hormone profile and control over isoprene biosynthesis in a tropical tree *Ficus septica*.

2.1 Introduction

Isoprene is the largest biogenic volatile organic compound emitted from terrestrial vegetation and plays important role in the tropospheric chemistry (Arneth et al. 2008; Guenther 1995; Guenther et al. 2006; Potter et al. 2001). Biosynthesis and emission of isoprene are considered to confer some advantages to plant including better adaptation to high-temperature and high-light episodes, O_3 scavenging and stabilization of thylakoid membrane (Loreto and Velikova 2001; Sharkey and Monson 2014; Sharkey and Yeh 2001; Singsaas et al. 1997; Velikova 2008).Tropical and subtropical regions account for half of the global isoprene emissions because of year-round high temperature and light intensity (Guenther 1995; Potter et al. 2001). Despite this major role in the global isoprene dynamism, studies on isoprene emissions and its regulation have been conducted with temperate tree species.

Isoprene is synthesized from the substrate dimethylallyl diphosphate (DMADP) supplied by the plastidal 2-C-methyl-D-erythritol 4-phopsphte (MEP) pathway using newly fixed carbon and reducing power via the photosynthesis (Lichtenthaler, Rohmer, and Schwender 1997; Zeidler et al. 1997). Control of isoprene emission is complex and explained at several regulatory levels: (1) isoprene synthase kinetics and enzyme activation:(2) gene expression of isoprene synthase (IspS) and its protein level:(3) substrate DMDP availability depending on the activity of MEP pathway.

Our previous study unveiled the transcriptional regulation of *IspS* gene by cold temperature through circadian rhythm and plant hormone signaling (Mutanda, Inafuku, et al. 2016). The isoprene emission at a time scale of hours to few days are regulated by phytohormone mediated transcriptional modulation of *IspS* gene. The cold temperature perceived by upstream sensor is likely to activate ethylene, jasmonic acid (JA) and abscisic acid (ABA) signal transduction pathway whilst suppressing auxin signaling through activation of IAA (Mutanda, Inafuku, et al. 2016). This finding has been extended to exogenous application of the plant hormone to modulate the isoprene emission (Parveen et al. 2019). Among five phytohormones tested, JA was found to have most potent activity to modulate isoprene emission. It is likely that JA modulates IspS gene through the basic helix helix-loop-helix transcription factor MYC2 that directly binds to G-box cis acting element on IspS promotor. Our previous data also supported the hormone signaling cross talk between JA and auxin under synchronization by circadian clock. These data pointed that there was a substantial change in hormone concentration on exogenous application of plant hormone to modulate the isoprene emission. However, there was no information on the relationship between plant hormone profile and molecular regulatory mechanism of isoprene biosynthesis and emission.

In this study, we analyzed the plant hormone concentration in JA treated leaves to explore the relationship between plant hormone profile and transcriptional control of isoprene biosynthesis. Plant hormone signaling network plays an important role in the protection of the plant from drought stress (Ullah et al. 2018). Our another study investigated the molecular regulatory mechanism of isoprene emission under short-term drought stress and found post transcriptional regulation of isoprene biosynthesis in coordination with the gene expression of antioxidation enzymes (Parveen et al. 2018). By analogy with the JA treatment experiment, the

authors analyzed the hormone signaling gene expression and hormone concentration of drought stressed leaves to understand the relevance of plant hormone profile with the post transcriptional regulation of isoprene biosynthesis. Interpretation and integration of these two results provide a view guiding future study on molecular regulatory mechanism of isoprene biosynthesis.

2.2 Materials and Method

2.2.1 Plant and treatment

F. septica plants were prepared from the cutting branch of mature trees grown on the campus of University of the Ryukyus (26°15′N, 127°46′E), Okinawa, Japan as described in our previous study (Parveen et al. 2019). Saplings of about 1 m in height were used for both JA treatment and drought stress experiment.

In the case of JA treatment, plants were sprayed with 50 μM JA from day 2 to day5, kept further up to day 10 without JA treatment under controlled room temperature (34 and 27 ℃ for day and night time, respectively) and light intensity (300 μmol m^{-2} s^{-1}) in a phytotron. Three leaves were sampled from 3 trees (one leaf from each tree) on day 1, 2, 3, 5, 7 and 10 for analysis (Fig 2.2-1 A).

Similar protocol was applied for drought stress experiment. Plants were not supplied with water from day 2 to 5, followed by rewatering from day 6 to 7 for recovery. Sampling of leaves were made on day 1, 2, 4, 5, 6 and 7.(Fig 2.2-1 B).

A. JA treated Experimental layout

DAY	D 1	D 2	D3	D 5	D 7	D 10
Temperature	34℃ and 27 ℃ for day and night time, respectively					
Light intensiy	Day= 300 µmol m^{-2} $s^{-1,}$, Night= Off					
Photoperiod	Day= 12 hrs, Night=12 hrs					
JA Treatment	No spray	JA Sprayed (50 µM JA)			Recovery Day	
						

B. Short Term drought stress treated Experimental layout

DAY	D 1	D 2	D3	D 5	D 6	D 7
Temperature	30℃ and 25 ℃ for day and night time, respectively					
Light intensiy	Day= 300 µmol m^{-2} $s^{-1,}$, Night= Off					
Photoperiod	Day= 12 hrs, Night=12 hrs					
Drought stress	No spray	No watering			Rewatering	
						

Fig 2.2-1. Experimental layout to study plant hormone profiles of *F. septica* leaves. A. JA treated experimental layout and B. Short term drought stress treated experimental layout.

2.2.2 Hormone analyses

Plant hormone was extracted by the method described by Miyamoto et al., 2016, with slight modifications. Briefly, 50 mg leaf was homogenized for 30 sec with Pro200 Bio Gen series homogenizer (Pro Scientific Inc. Oxford CT, USA) in 2 mL 80 % (v/v) methanol. To this homogenate, was added the stable isotope labeled internal standard: 20 ng of IAA-d5 and ABA-d6, 10 ng of JA-d6 and [^{13}C6]-JA-Ile. Then the suspension was centrifuged at 10,000×g for 5 min

at 4°C. The supernatant was loaded onto a Sep-Pak C18 cartridge (500 mg, 3 mL; Waters Corp., Milford, MA, USA), which had been pre-equilibrated with 2 mL 80 % (v/v) methanol after sequential washing with 2 mL of methanol and water. Plant hormones eluted with 2 mL 80 % (v/v) methanol were collected and concentrated to dryness in a vacuum concentrator. Plant hormones were re-dissolved in 500µL 50 % (v/v) methanol and analyzed on ODS column (2 mm x150 mm; Senshu Scientific Co. Ltd, Tokyo, Japan) by liquid chromatography–electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS) composed of 1200 HPLC instrument (Agilent Technologies) and quadrupole tandem mass spectrometer with an electrospray ion source. The solvents used for the column were water containing 0.1 % (v/v) acetic acid (A) and acetonitrile containing 0.1 % (v/v) acetic acid (B). The concentrations of plant hormones were calculated based on the relative proportions of peak area against internal standards or peak area of known concentration of authentic standard run under same conditions.

2.2.3 Isoprene emission

Isoprene emission rate was measured by the method described previously (Oku et al., 2014) using a leaf cuvette (PLC-4C, ADC BioScientific, Hertfordshire, UK) and a real-time isoprene analyzer (KFCL-500, Anatec Yanaco, Kyoto, Japan). Stable isoprene emission of an individual leaf was measured for 20 min under standard light and temperature conditions (1,000 $\mu molm^{-2}\ s^{-1}$ PPFD, 30°C). After measurements, the leaves were harvested and snap frozen in liquid nitrogen and then stored at −80°C for further analyses.

2.2.4 MEP pathway metabolite

Ultrafast liquid chromatography–tandem mass spectroscopy was performed for quantification of MEP metabolite pools as described by Mutanda et al. 2016 with slight modifications. Metabolites were extracted with methanol: 50 mM ammonium acetate, (2:1:1), pH 10 and centrifuged for 10 min. The pH of the supernatant was adjusted to 6.8, filtered and the filtrate was loaded to the conditioned LC-NH_2 solid-phase extraction cartridge (Sigma-Aldrich, Mo). The metabolites were eluted with a mixture of methanol: Milli-Q (1:1 v/v) at pH 11.6 and dried by a vacuum speed evaporator at 4° C. Samples were re-extracted in 50 µl ethanol, mixed with an equal amount of acetonitrile and analyzed on a Shimadzu prominence ultrafast liquid chromatography XR system (LC-20ADXR) and Waters Quattro mass spectrometer (Waters Corporation, Milford, MA). Metabolites were passed through the ZIC-pHILIC PEEK HPLC column (5 µm, 50 × 2.1 mm, Merck SeQuant, Umea, Sweden) with a HILIC guard column (5 µm, 20 × 2.1 mm) and using two gradient solvents system as described previously (Parveen et al. 2018). The metabolites were quantified by comparing to peak area of authentic standards (Echelon Biosciences, Logan, UT).

2.2.5 Western blotting

Leaf tissue (40 mg) was homogenized in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5; 5 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM dithiothreitol), and the homogenate was centrifuged for 5 min. Nine (9)µg of proteins were separated on a 10% sodium dodecyl sulphate–polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride membrane followed by blocking at room temperature for 1 hr. The membrane was incubated with an anti-*F. septica* IspS polyclonal primary antibody. After washing five times in TBS-T buffer,

the membranes were incubated for 1 hr (room temperature) with a secondary antibody (anti-Guinea pig IgG, GeneTex, Irvine, CA). ECL Advance Western Blotting Detection System (GE Healthcare) was used to detect the protein bands, and the image was visualized by an ImageQuant™ LAS4000 imaging system (GE Healthcare) with 120 s exposure time. Band intensity was quantified using ImageJ software (NIH, Bethesda, MD)

2.2.6 Gene expression

Total RNA was extracted by TRIzol reagent and purified by the RNeasy Plant Mini Kit as described previously with slight modifications. cDNA was synthesized from 2 μg RNA using High-Capacity RNA-to-cDNA Kit (Applied Biosystems, CA). qRT-PCR was conducted using Power Up™ SYBR® Green Master Mix (Applied Biosystems) and run on an Applied Biosystems StepOne system (Thermo Fisher Scientific). mRNA levels were normalized by actin2 as the housekeeping gene. The primer pairs designed in our previous study (Mutanda, Inafuku, et al. 2016; Mutanda, Saitoh, et al. 2016) were used to analyze gene expressions of the MEP pathway, hormone signaling pathway, and circadian rhythm. Seven primers for SAUR genes were newly designed and SAUR21 primer was used same as mentioned in Mutanda, Inafuku et.al. 2016 for qRT-PCR in this study.

2.2.7 Statistical analysis

Statistical significance of the differences in gene expression levels and hormones were performed using the Tukey honestly significant difference (HSD) test by XLSTAT (Addinsoft) software. Nonmetric multi-dimensional scaling (NMDS) was performed by BellCurve for Excel based on the distance of 1- | r |.

2.3 Results

2.3.1 Plant hormone profile in JA treated leaves

The leaves of *F. septica* were sprayed with 50μM JA from day 2 to 5 and left without treatment from day 6 to 10. Isoprene emission was decreased by JA, and re-increased but not full recovery to the initial rate after relief from exogenous JA application. Gene expression and protein level of IspS largely showed similar changes with isoprene emission (Fig. 2.3-2 A).

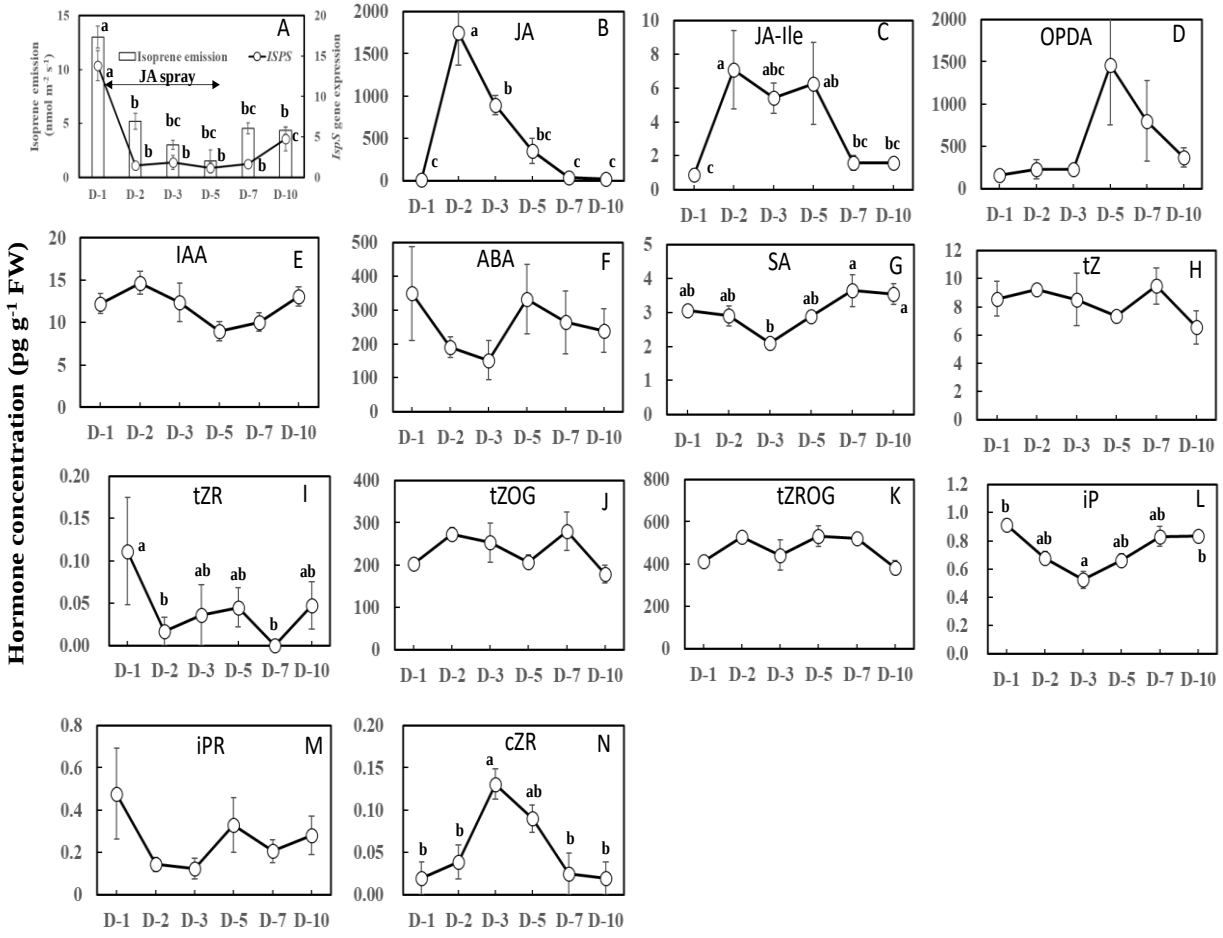

Fig. 2.3-2. Plant hormone profiles in JA sprayed *F. septica* leaves. Plants were sprayed with 50 μM JA from day 2 to day 5, kept further up to day 10 without JA treatment under controlled room temperature and light intensity. A, isoprene emission, IspS expression and IspS protein level; B, jasmonic acid (JA); C, jasmonoyl isoleucine (JA-Ile); D, 12-oxo-phytodienoic acid (OPDA);E, indole-3-acetic acid (IAA); F, abscisic acid (ABA); G, salicylic acid (SA); H, trans-zeatin (tZ); I, trans-zeatin riboside (tZR); J, trans-Zeatin-d5 O-glucoside (tZOG); K, trans-Zeatin-d5 O-glucoside Riboside (tZROG); L, N6 -(D2 -isopentenyl)adenine (iP); M, isopentenyl adenosine riboside (iPR); N, cis-zeatin riboside (cZR); Data are mean±SE of three leaves. Different letters on the data show statistically significant difference ($p < 0.05$ by Tukey test).

Figure 2.3-2 depicts the changes in plant hormone concentrations of JA treated leaves of *F. septica*. JA concentration peaked on day 2 and decreased gradually toward day 10 while jasmonoyl isoleucine (JA-Ile), the effector molecule of JA, increased on day 2 and this elevated level persisted up to day 5 and returned to the initial level on and after day 7. 12-Oxo-phytodienoic acid (OPDA) showed similar pattern as JA but with peak on day 5. Concentration of 3-indole acetic acid (IAA) decreased with JA treatment, bottomed on day 5 and re-increased toward day 10. No statistically significant change was noted with the concentration of abscisic acid (ABA) and salicylic acid (SA). Of the cytokinins, cis-zeatin riboside (cZR) showed significant changes with peaking on day 3.

The concentration of JA-Ile showed the negative and statistically significant correlation with both isoprene emission and IspS gene expression. tZR highly and positively correlated with IspS gene expression whilst cZR correlated negatively with isoprene emission (Table 2.3-1). To get more insight into the signaling crosstalk, the correlations between the ratio of plant hormones to JA-Ile and isoprene emission, IspS gene expression and IspS protein level were examined. The ratios of IAA, ABA, tZR iPR to JA-Ile were highly correlated with isoprene emission, IspS gene expression and IspS protein level.

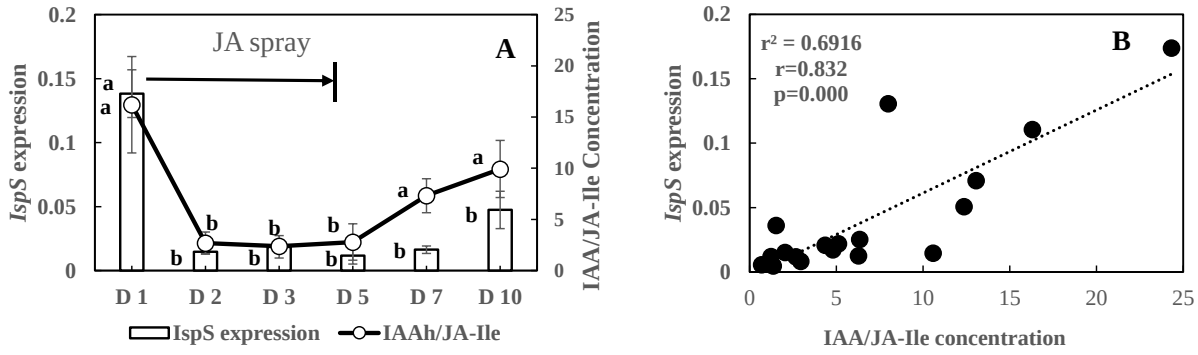

Fig. 2.3-3. Daily variation of *IspS* gene expression and the ratio between IAA and JA-Ile concentration (A) and relationship between these two parameters (B) in JA sprayed *F. septica* leaves. Different letters on the data show statistically significant difference ($p < 0.05$ by Tukey test). Statistical significance for correlation coefficient was evaluated by Student's t-test.

Table 2.3-1. Correlation coefficient analysis between plant hormone concentration, isoprene emission, *IspS* transcripts, IspS protein and other parameters in JA sprayed *F. septica* leaves.

	Isoprene emisson	***IspS* Transcript**	**IspS protein**
Isoprene emisson	1.00	-	-
IspS	**0.90*** **	1.00	-
IspS protein	**0.89*** **	**0.86*** **	1.00
Phytohormones			
IAA	0.27	0.19	0.33
ABA	0.24	0.38	0.31
JA	-0.24	-0.40	-0.10
JA-Ile	**-0.51***	**-0.52***	-0.28
OPDA	-0.39	-0.32	-0.29
tZR	0.46	**0.63****	0.45
cZR	**-0.49***	-0.32	-0.36
iPR	0.32	0.44	**0.47***
SA	0.19	0.17	-0.05
IAA/JA-Ile	**0.77*** **	**0.83*** **	**0.63****
ABA/JA-Ile	**0.65****	**0.77*** **	**0.58***
OPDA/JA-Ile	-0.07	-0.01	-0.24
SA/JA-Ile	**0.69****	**0.73*** **	**0.50***
Hormone signaling genes			
IAA	**-0.58***	**-0.53***	-0.42
TIR1	**0.63****	**0.49***	0.43
GH3	**0.53***	**0.55***	**0.60****
SAUR	**0.84*** **	**0.67****	**0.68****
ARF1	-0.38	-0.26	-0.40
MYC2	**-0.68****	**-0.52***	**-0.49***
PR1	-0.25	-0.19	-0.42
TGA1	-0.41	-0.24	-0.28
NPR1	0.11	0.24	-0.07
WRKY1	-0.06	0.08	-0.04
WRKY2	0.38	0.33	0.11
ARR-A	**0.56***	0.45	0.37
AHP	**0.82*** **	**0.82*** **	**0.72*** **
AHK2_3_4	-0.39	-0.29	-0.48*
GID1	0.01	0.13	-0.18
MYB	-0.04	-0.03	-0.16
BZR1/2	-0.16	-0.13	-0.23
ERF1	-0.35	-0.17	-0.42
Circadian clock genes			
PIF3	-0.19	-0.01	-0.20
PIF4	**-0.49***	-0.34	-0.43
PhyA	-0.31	-0.24	-0.45
PRR3	-0.12	-0.11	-0.23
PRR5	**0.52***	**0.55***	0.42
PRR7	0.44	**0.60****	0.33
ELF3	-0.22	-0.08	-0.27
LHY	-0.21	-0.03	-0.08
HY5	-0.50*	-0.25	-0.27
MEP pathway Genes			
DXS	**-0.68****	**-0.64****	**-0.63****
DXR	0.19	0.25	0.00
MCT	**-0.47***	-0.43	**-0.51***
CMK	**-0.53***	**-0.49***	**-0.53***
MCS	0.07	0.12	-0.12
HDS	**0.63****	**0.52***	**0.49***
HDR	-0.28	-0.19	-0.36

Letters in bold face indicates statistical significance by Student's t-test (P < 0.05): ***P < 0.001; **P < 0.01; *P < 0.05

Table 2.3-2. Correlation coefficient analysis between hormone concentration, isoprene emission, *IspS* transcripts, IspS protein and other parameters in drought stressed *F. septica* leaves.

	Isoprene emisson	***IspS* Transcript**	**Isps protein**
Isoprene emisson	1.00	-	-
IspS	0.36	1.00	-
IspS protein	**0.88*** **	0.26	1.00
Phytohormones			
IAA	0.35	-0.21	0.24
ABA	0.23	0.01	0.31
JA Iie	0.15	0.18	0.18
OPDA	0.12	0.16	0.24
SA	-0.20	-0.26	-0.11
tZR	-0.08	-0.31	-0.08
iPR	-0.02	**-0.57***	-0.08
IAA/JA-Ile	-0.16	-0.19	-0.19
ABA/JA-Ile	0.25	0.03	0.33
SA/JA-Ile	-0.18	-0.26	-0.09
OPDA/JA-ILE	0.08	0.16	0.21
Hormone signaling genes			
IAA	0.27	**0.55***	0.30
SAUR	**0.44***	**0.59***	0.42
ARF1	0.10	**0.49***	0.05
TIR1	0.15	**0.45***	0.19
GH3	0.39	**0.69***	0.34
MYC2	0.13	0.20	0.26
GID1	0.18	0.05	0.24
NPRI	-0.13	0.12	-0.12
PRI	-0.14	-0.02	-0.12
TGA	-0.05	0.08	-0.04
WRKY1	0.34	0.32	0.35
ERF1	**0.53***	0.15	**0.67****
BZRI	0.11	0.05	0.10
AHP	0.14	0.02	0.16
AKH234	0.31	0.12	0.34
MYB	-0.43	0.04	-0.32
ABF 1	0.33	0.05	0.43
Circadian clock genes			
PIF 4	-0.13	0.26	-0.26
PIF 3	0.07	0.36	-0.07
ELF 3	0.09	0.22	-0.05
LHY	-0.40	-0.01	-0.36
PHY A	-0.30	0.01	-0.36
PRR3	-0.01	0.08	-0.02
PRR5	-0.21	0.00	-0.25
PRR 7	-0.12	0.06	-0.18
HY5	0.30	0.17	0.23
MEP pathway genes			
DXS	0.29	-0.15	**0.43***
DXR	0.41	0.23	**0.49***
MCT	**0.44***	-0.03	**0.62****
CMK	0.01	**0.51***	0.01
MCS	**0.61****	0.38	**0.70****
HDS	**0.50***	0.38	**0.44***
HDR	**0.56***	**0.67****	0.41

Letters in bold face indicates statistical significance by Student's t-test ($P < 0.05$): ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Our previous study suggested that the JA and IAA signaling plays a pivotal role in the transcriptional regulation of IspS under synchronization by the circadian clock (Mutanda, Saitoh, et al. 2016; Parveen et al. 2018) . Figure 2.3-3 shows the daily variation of IAA/JA-Ile, isoprene emission, and IspS protein level in JA treated leaves of *F. septica*. It was shown that 59.4%, 69.2% and 40.0% of the total variations in isoprene emission, IspS gene expression and IspS protein were explained by IAA/JA-Ile, respectively.

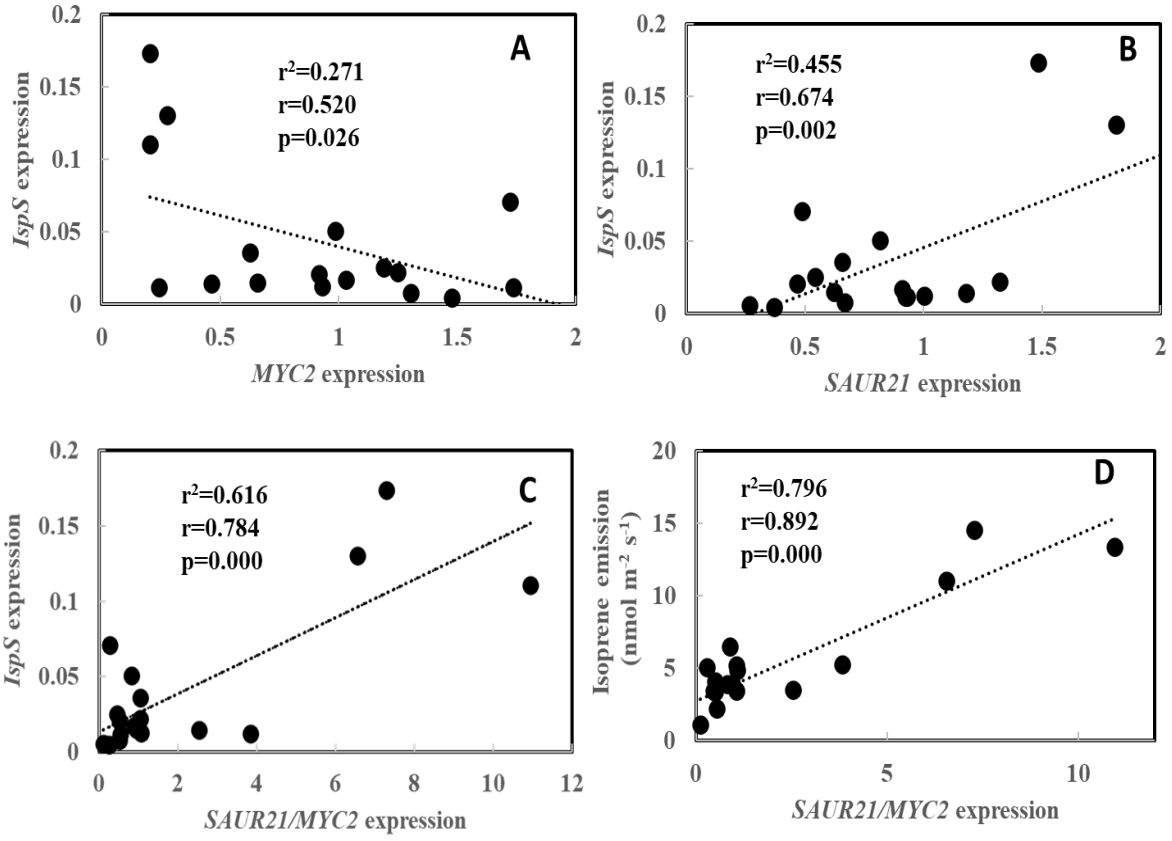

Fig. 2.3-4. Relationship between *IspS*, *SAUR21* and *MYC2* gene expression. A, *IspS* vs. *MYC2*; B, *IspS* vs. *SAUR21*; C, *IspS* vs. ratio of *SAUR21/MYC2*; D, isoprene emission vs. ratio of *SAUR21/MYC2*. Statistical significance was evaluated by Student's t-test.

I postulated that there is an interaction between JA and IAA signaling pathway to control IspS gene expression and IAA signaling is converged into modulation of MYC2 transcription factor in the JA signaling pathway. However, there was a weak negative correlation between *MYC2* expression and IAA/JA-Ile accounting for only 17.8% of total variation of this transcription factor (Fig. 2.3-4 A). This observation suggested the occurrence of another IAA responsive transcription factor and sis-acting element on *IspS* promoter to control IspS gene expression. In plant, most early IAA responsive genes are classified into three groups: *AUX/IAA*s, Gretchen Hagen3s (*GH3*s) and *SAUR*s (Hagen and Guilfoyle 2002). Of these genes, *AUX/IAA* locates upstream of the signaling pathway suppressing auxin response factor (ARF) at low auxin concentration whereas *GH3* is acyl-amido synthase of Gretchen Hagen3 and catalyzes the conjugation reaction of IAA with amino acids to maintain auxin homeostasis (Peat et al. 2012; Staswick et al. 2005). Thus, it is unlikely that *AUX/IAA* and *GH3* directly interact with the cis-acting element on promoter of IspS genes, alternatively implying that SAURs are involved in the process of signal transduction as transcription factor. In support of this postulation, we found an enrichment of auxin responsive cis-acting element, AuxRE and CATATGGMSAUR, in the promoter of IspS gene (Fig. 2.4-8). To test the involvement of *SAUR*s in the transcription, we examined the relationship between SAUR21 gene expression and IAA/JA-Ile, the mixing ratio of plant hormones in the leaves was studied. IAA/JA-Ile, the mixing ratio of plant hormones in the leaves, positively correlated with SAUR21 gene expression, accounting for 34% of total variation (Fig. 2.3-4 B). Furthermore, 80% and 61% of total variation in isoprene emission and IspS gene

expressions respectively were explained by the gene expression ratio between *SAUR21* and *MYC2* Fig. 2.3-4 C, D). Our previous study also suggested the transcriptional regulation of IspS gene under synchronization with the circadian clock gene LHY (Mutanda, Saitoh, et al. 2016; Parveen et al. 2019). However, IAA/JA-Ile only accounted for negligible proportion of total variation in LHY ($R^2=10^{-5}$, data not shown). Therefore, the expression of this gene as a control factor was not taken into consideration in present analysis.

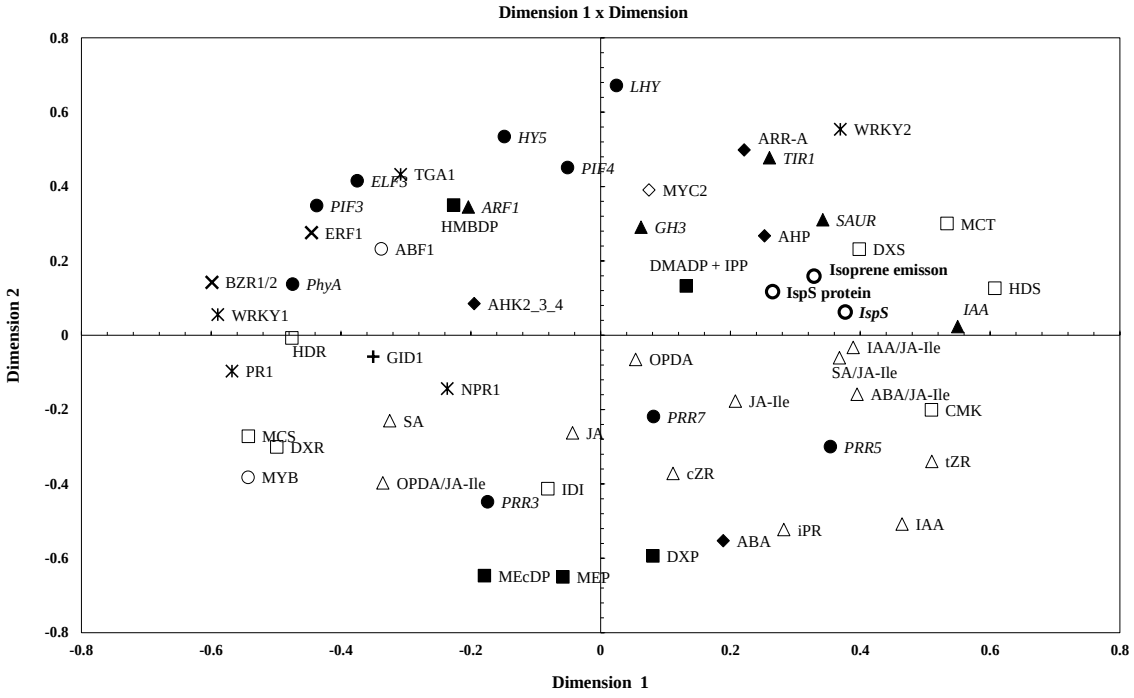

Fig. 2.3-5. Nonmetric multi-dimensional scaling (NMDS) is illustrated using a bidimensional plot in which the points show different variables of JA treated *F. septica* leaves. Isoprene emission, IspS protein level and *IspS* transcript (⓿); different hormone concentration and their ratio (Δ); ABA signaling genes (○) ; Auxin signaling genes (▲); JA Signaling genes (◊); Giberrellic acid signaling genes (+); Ethylene signaling gene (–); Brassinosteroid signaling genes (×); Cytokinin Signaling genes (♦), SA signaling genes (*); Circadian Rhythm signaling

genes (●); MEP pathway metabolites (■); and MEP pathway enzymes (□) are presented. Gene transcription levels are denoted by italic letter. The Kruskal's stress value was 0.269 and 1- | r | was input distance data for NMDS analysis.

The correlations between parameters of isoprene biosynthesis and emission, gene expression of hormone signaling pathway, MEP pathway metabolism, are summarized by non-metric multidimensional scaling (MDS) as displayed in two dimensions (Fig. 2.3-5). The close relationship between dots in the plot denote a higher correlation between parameters. The mixing ratio between each plant hormones and JA-Ile rather than their sole concentration located close to isoprene emission and IspS gene expression therefore indicating their higher correlation with the isoprene metabolism. MEP, MEcDP were found rather near to sole concentration of plant hormones, JA, cZR, iPR and ABA.

2.3.2 Plant hormone profile in the leaves under short-term drought stress

Plants confer abiotic stress tolerance such as dehyration stress by complex regulatory systems involving hormone signaling and metabolism (Bari and Jones 2009; Urano et al. 2017). Our previous paper described molecular regulatory mechanism of isoprene emission from *F. septica* under short term drought stress (Parveen et al. 2018). This experiment also provides a good example to explore the precise hormonal control of isoprene biosynthesis and emission. The authors therefor analyzed the gene expression of hormone signaling and hormone concentration of this sample to explore the precise control mechanism of isoprene biosynthesis.

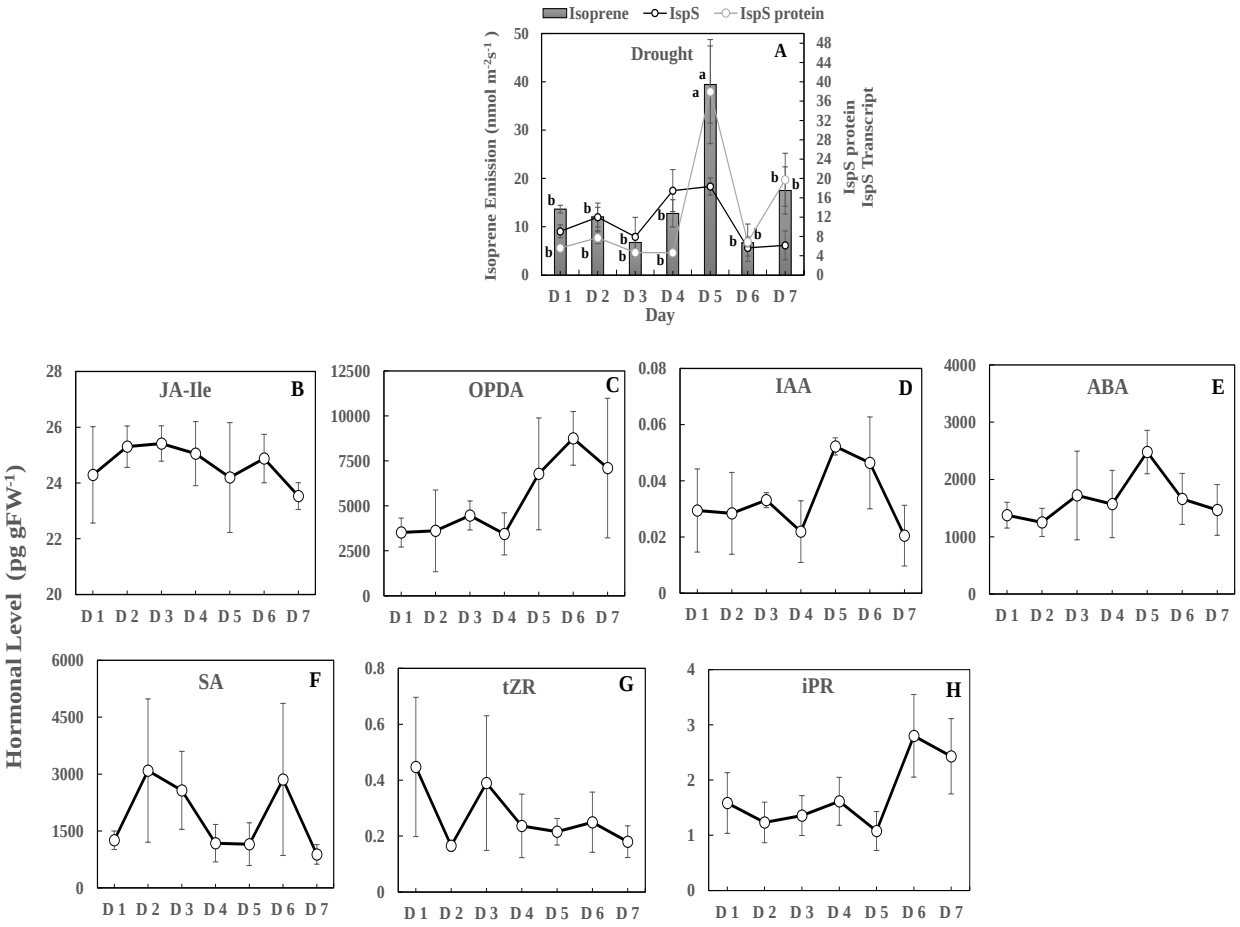

Fig. 2.3-6. Plant hormone profiles in short-term drought stressed *F. septica* leaves. No water was supplied from day 2 to 5, followed by rewatering from day 6 to 7 for recovery under controlled room temperature and light intensity. A, isoprene emission, IspS expression and IspS protein level; B, jasmonoyl isoleucine (JA-Ile); C, 12-oxo-phytodienoic acid (OPDA); D, indole-3-acetic acid (IAA); E, abscisic acid (ABA); F, salicylic acid (SA); G, trans-zeatin riboside (tZR); H, isopentenyl adenosine (iPR). Data are mean±SE of three leaves. Different letters on the data show statistically significant difference ($p < 0.05$ by Turkey test).

Water supply to plants was held for 5 days, and re-watered from day 6 to 7. Figure 2.3-6 shows the hormone concentration of *F. septica* leaves during the short-term drought stress (from

day 2 to 5) and recovery period (from day 6 to 7). There was a sharp rise of isoprene emission on day 5, and this increased emission returned to initial level by rewatering (Fig. 2.3-6 A). IspS protein level also increased on day 5 and decreased by re-watering on day 6 whereas no significant changes were seen in IspS gene expression (Fig.2.3-6 A). No statistically significant difference was noted in the concentration of JA-Ile, OPDA, SA, iPR and tZR concentration throughout the experiment. IAA concentration showed higher tendency on day 5 and day 6 compared to initial level (Fig. 2.3-6 D). Similarly, ABA concentration increased on day 5 and decreased by re-watering from day 6 to 7 (Fig. 2.3-6 E).

Table 2.3-2 summarizes the correlation coefficients between hormone concentration and other parameters related with isoprene biosynthesis and emissions including the gene expression of MEP pathway, hormone signaling, and the concentration of MEP pathway metabolites. No significant correlation was observed between hormone concentration and isoprene emission, IspS gene expression and IspS protein level except for iPR that negatively correlated with IspS gene expression. Among the hormone signaling genes, auxin signaling genes, *IAA, SAUR21, ARF1, TIR1 and GH3* positively correlated with IspS gene expression. Isoprene emission positively correlated with the concentration of MEP pathway enzyme *MCT, MCS, HDS* and *HDR*.

Figure 2.3-7 is the two-dimensional plots of correlation between isoprene biosynthesis related parameters in the *F. septica* leaves under short-term drought stress. Hormone concentration, solely or the ratio against JA-Ile showed scattered distribution away from isoprene emission and IspS gene expression. In contrast, enzymes and metabolite of MEP pathway locates close to isoprene emission and IspS protein level.

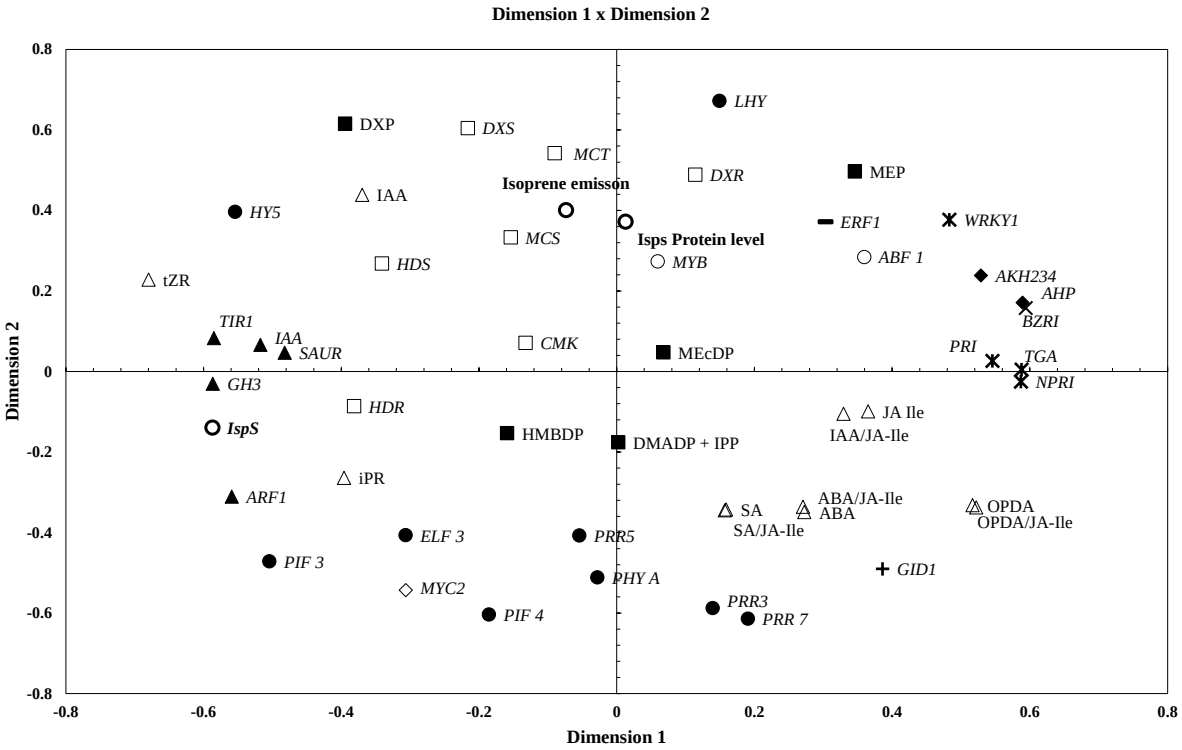

Fig. 2.3-7. Nonmetric multi-dimensional scaling (NMDS) is illustrated using a bidimensional plot in which the points show different variables of short-term drought stressed *F. septica* leaves. Isoprene emission, IspS protein level and *IspS* transcript (⬯); different hormone concentration and their ratio (Δ); ABA signaling genes (○) ; Auxin signaling genes (▲); JA Signaling genes (◊); Giberrellic acid signaling genes (+); Ethylene signaling gene (–); Brassinosteroid signaling genes (×); Cytokinin Signaling genes (♦), SA signaling genes (*); Circadian Rhythm signaling genes (●); MEP pathway metabolites (■); and MEP pathway enzymes (□) are presented. Gene transcription levels are denoted by italic letter. The Kruskal's stress value was 0.269 and 1- | r | was input distance data for NMDS analysis.

2.4 Discussion

The present study analyzed the plant hormone concentration and gene expression of our two experimental models to explore the hormonal control of isoprene biosynthesis: leaves in which transcriptional regulation of *IspS* took place after treatment by JA and leaves experienced short-term drought stress in which post-transcriptional regulation of *IspS* occurred.

JA treatment gave a rise to the concentration of JA and JA-Ile with alterations in the other plant hormone concentrations as shown in Fig. 2.3-2. Among the plant hormones, JA-Ile highly correlated with both isoprene emission and IspS gene expression in support of our postulate that JA signaling plays a pivotal role in the transcriptional control of IspS gene. It was found that JA cannot work independently instead of this; it acts as a complex signalling network with other hormone signalling pathways (Ahmad et al. 2016; Kazan 2015). Biosynthesis of JA-Ile also activates many genes involved in the plants' response and adaptation to the environment by activation of *MYC2* (Ruan et al. 2019). Some studies shows , JA and auxin signal transduction mechanisms have striking similarities involving feedback mechanisms, F-box proteins and repressor protein (Pérez and Goossens 2013). The authors have hypothesized that interaction of JA and auxin modulate IspS gene expression via the transcription factor *MYC2* (Parveen et al. 2019). However, in our case, the IAA/JA-Ile ratio only explained 17.8% of the total variation in *MYC2* gene expression, suggesting the occurrence of an additional transcription factor or intermediate signal transducer to control IspS gene. Of the auxin responsive genes, SAUR21 is proposed as the potential signal-transducing genes in the regulation of IspS gene expression as shown in Fig.2.4-11, because of three reasons below. 1) Similar SAUR molecule of cassava plant (MeSAUR1) has been proven to act as transcriptional factor by binding with auxin responsive

element in the promoter of cassava ADP-glucose pyrophosphatase (Ma et al. 2017). We found 4 auxin responsive elements (AuxRE) in the promoter of IspS gene located close to the transcription start site as shown in Fig. 2.4-8.

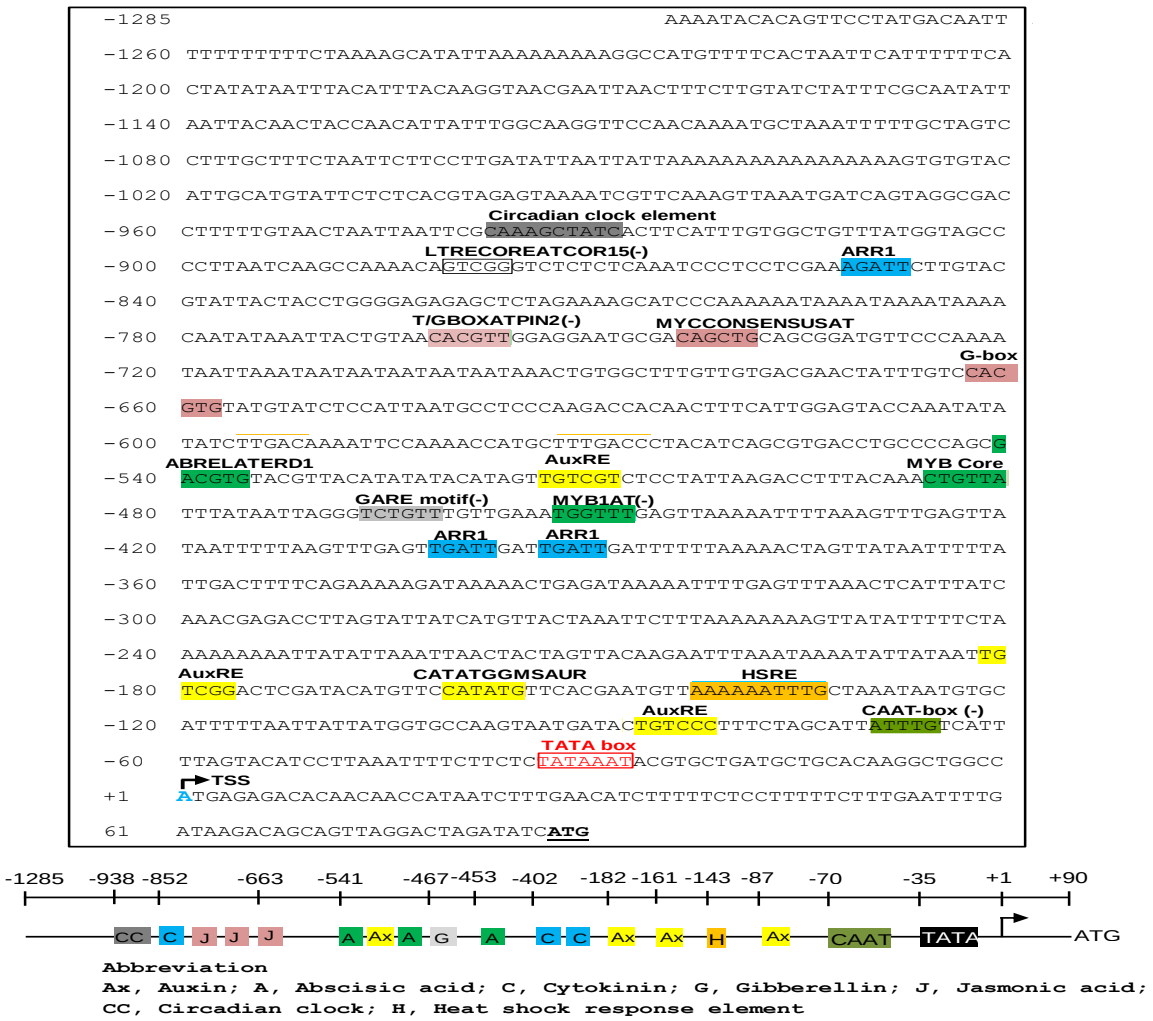

Fig. 2.4-8. DNA sequence and cis-acting elements of *F. septica IspS* promoter. The promoter sequence was analyzed by NEW PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace) and PlantCARE (https://bio.tools/plantcare) for cis-acting DNA-element. For auxin responsive elements,

sequence was manually scanned based on the motif in the literature. Cis-acting elements involved in hormone signaling and abiotic stress are mentioned.

It has been demonstrated that AuxREs are enriched in proximal promoter of the auxin up-regulated genes (Keilwagen et al. 2011). These observations point to involvement of SAUR21 in transcription reaction. 2) SAUR21 comprised of 89 amino acids residues has the conserved SAUR-specific domain (SSD) as shown in (Fig 2.4-9). Furthermore, the total consistency value between amino acid sequence of these two SAURs was found to be 97% by T-COFFEE program (http://tcoffee.crg.cat/) suggesting the high similarity in the protein structure. 3) The gene expression ratio of *SAUR21/MYC2* better correlated with both isoprene emission and IspS gene expression rather than *MYC2* alone, explaining 80% and 61% of total variation in isoprene emission and *IspS* gene expression, respectively. This may support our hypothesis that *SAUR21* in addition to *MYC2* is responsible for the transcriptional regulation of IspS gene. However, it is yet to come to demonstrate the interaction between *SAUR21* and promoter DNA. Studies to test binding of SAUR to IspS promoter are needed to authorize our proposal.

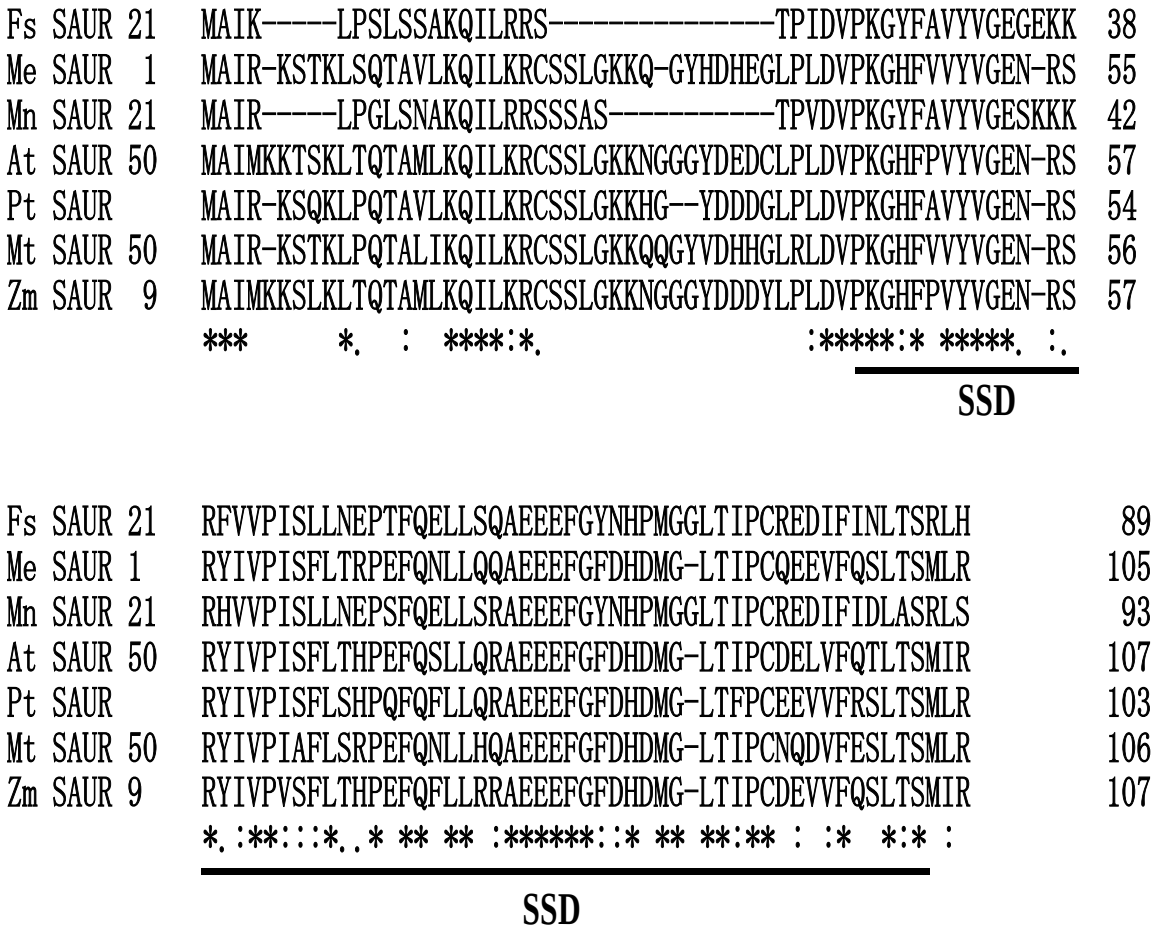

Fig 2.4-9. Amino acid sequence alignment of F. septica SAUR21and related SAURs. The asterisks and dots below alignment denote identical and similar residues, respectively. SSD shows the conserved SAUR specific domain. Fs: Ficus septica Me: Manihot esculenta (MANES_05G149400) Mn: Morus notabilis (XM_010097358.1) At: Arabidopsis thaliana (At4g34760) Pt: Populus tomentosa ((AFT92005) Mt: Medicago truncatula (XP_013462204) Zm: Zea mays (ACG25834).

In contrast to JA treatment experiment, short term drought stress led to no significant changes in plant hormone concentration in the leaves of *F. septica* (Fig. 2.3-6). Moderate

dehydration stress significantly increased ABA concentration, and slightly increased JA-Ile and IAA whereas the dehydration stress decreased the concentration of trans-zeatin (tZ) and salicylic acid (SA) in *Arabidopsis thaliana* (Urano et al. 2017). The drought stress in our experiment could be much more moderate than the conditions applied in the work of Urano et al. 2017 or alternatively due to the difference in the plant species. There was a sharp rise of isoprene emission on day 5 mainly due to increased IspS protein level. On day 5 of our experiment, ABA concentration displayed increasing tendency, as ABA plays an rapid roles and increase during abiotic stress like drought (Rabbani et al. 2003; Seki et al. 2002), but with statistically not significant level (Fig. 2.3-6). In the case of JA-Ile seems not so much variation rather slightly decrease on Day 5 which explain the why there is no correlation with *MYC2* with Isoprene emission. Because, JA-Ile activates plant response and adaptation by activation of *MYC2* (Ruan et al. 2019). Increase of OPDA at day 5 also support deactivation of *MYC2* due to JA-Ile as during drought JA-Ile biosynthesis from OPDA stopped (Savchenko et al. 2014). In the case of Auxin, the concentration of IAA also appeared to be elevated by drought stress on day 5 and this increase may be related with the increase in the auxin responsive gene expression including *SAUR21* . The increase in SAUR21 gene expression however induced no changes in the level of IspS transcript suggesting the importance of the ratio between SAUR21 and MYC2 gene expression, *SAUR21/MYC2*. Thus, the plant hormone signaling appears to play a minor role in the transcriptional modulation of *IspS* under our drought conditions. This view is reflected in the NMDS plots as the hormone related parameters plotted comparatively far from isoprene emission and IspS protein level (Fig. 2.3-7). This pattern is contrasting with the plots showing distribution of hormone related parameters close to isoprene emission and IspS transcripts in JA treated leaves (Fig. 2.3-5). More close relationship of the parameters of MEP pathway with isoprene emission

rather than IspS transcript may indicate that the transcriptional or post-transcriptional regulation of MEP pathway plays a prominent role apart from hormonal control under our drought conditions.

The *SAUR*s are auxin responsive genes with 60-140 members in most higher plant species (Stortenbeker and Bemer 2019). The importance of SAUR genes in the regulation of growth, development and adaptation has been emerged in recent years (Ren and Gray 2015). Although the function of large members has been remained elusive, recent study unveiled the function of SAURs. In Arabidopsis, SAUR genes were found to induce cell elongation via the interaction with PP2C.D phosphatase leading to activation of membrane H^+-ATPass (Spartz et al. 2014). SAUR genes similarly up regulated this phosphatase later identified as important negative regulator of senescence leading to delayed leaf (Ren et al. 2018; Xiao et al. 2015) . Furthermore, Ma et al. found that the cassava SAUR in nucleus can act as a transcription factor of ADP glucose pyrophosphorylase subunit (Ma et al. 2017).

We found 8 (eight) SAUR transcripts in our transcriptome library (Fig 2.4-10). Of these SAURs, *SAUR21* (FsSAUR21) showed the highest correlation with the isoprene emission and IspS gene expression in our previous study (Mutanda, Inafuku, et al. 2016) and confirmed by this study. Four of these SAURs including SAUR21 formed one clade located in the edge of phylogeny tree with Arabidopsis SAUR, AtSAUR77 and AtSAUR45 (Fig 2.4-10). This data suggests the lower similarities of these SAURs with those of Arabidopsis and supports the diversity of these genes between plant species. Although auxin is considered to be the primary regulator of SAUR gene family, their response to auxin exhibited variation, up- or down, depending on the SAUR gene sets in mulberry (HUANG et al. 2016). SAUR gene expression also showed variation in response to drought and heat stress (HUANG et al. 2016). Thus, SAUR

genes appear to be induced by a wide range of internal and environmental stimuli and provide the plant with an extensive toolbox for growth adaptation. In this line, the expression of SAUR genes can be up or down-regulated by many different hormones including auxin, GA, cytokinin JA, as well as by high salt and different light conditions (Stortenbeker and Bemer 2019). It is also worth mentioning that IspS promoter consists of several hormone sensitive cis-acting elements as illustrated in Fig. 2.4-8. There has been a notion that the coupling of hormone sensitive motif provides the basis for integrating the signaling of two pathway and enable fine tuning of transcriptional regulation (Berendzen et al. 2012; Kel et al. 1995; Mironova et al. 2014). *MYC2* and *SAUR21* can bind to cis-acting elements of antagonistic type and produce opposing effects. In this line, *MYC2* and *SAUR21* are antagonistic composite elements and may locate at the crosstalk point to integrate numerous stimuli and hormonal signaling to control *IspS* gene expression.

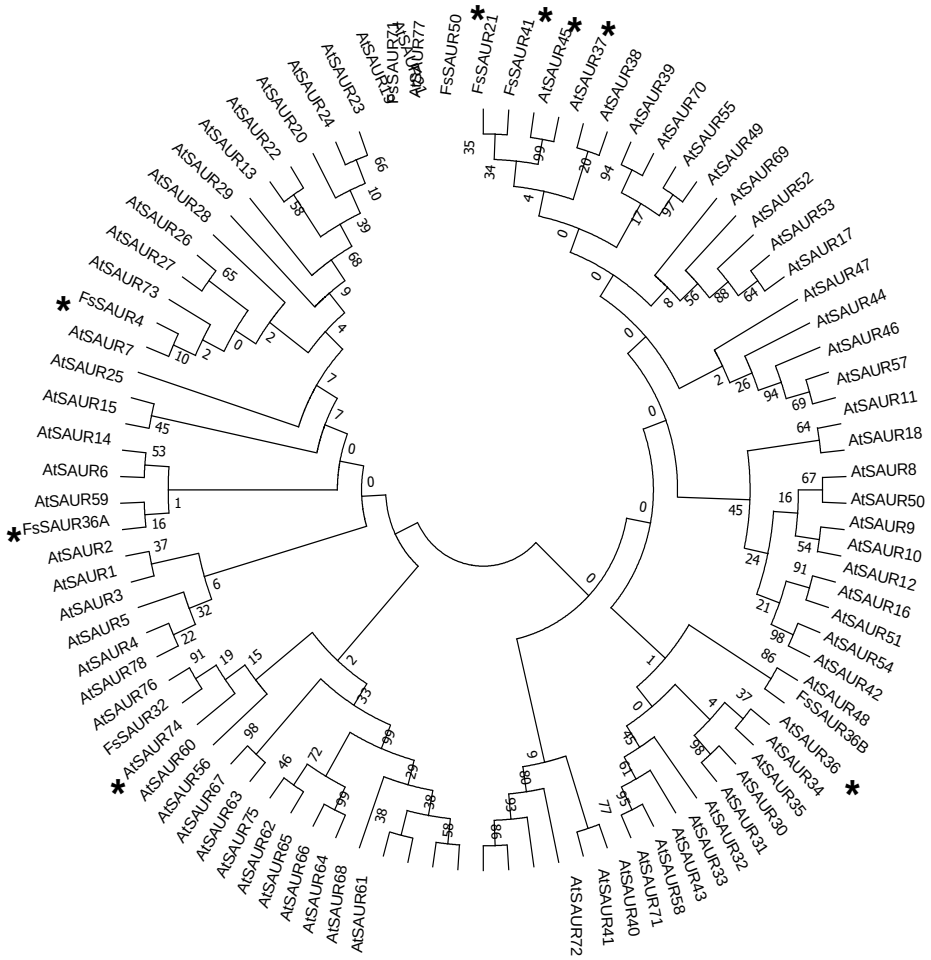

Fig. 2.4-10. Phylogenetic analysis of 78 *Arabidopsis thaliana* (At) and 8 Ficus septica (Fs) SAURs (shown with asterisk *). The topology tree was constructed by neighbor joining method using MEGA 7.0 with 1000 boot strap. DNA sequence of AtSAURs were retrieved from Genebank.

Our previous study suggested the transcriptional regulation of IspS gene under synchronization with the circadian clock gene *LHY* (Mutanda, Inafuku, et al. 2016). We hypothesized that this protein binds to the promoter and regulates IspS gene expression. However, the ratio of IAA/JA-Ile only accounted for small proportion of total variation in LHY, the positive

or negative regulator of IspS, suggesting its minor role in the IspS gene expression under this condition. In this relevance, SAUR gene was found to be regulated by the clock genes PSEUDO-RESPONSE REGULATOR 5 (PRR5) and *PRR7* (Nakamichi et al. 2010). Both factors can bind to many Arabidopsis SAUR genes and negatively regulate their expression (van Mourik et al. 2017). *PRR5* and 7 positively correlated with both *IspS* (Table 2.3-1) and *SAUR21* gene expression . It therefore appears in our case that *PRR5* and 7 positively regulate the expression of *SAUR21* reflecting the different environmental and light conditions. Thus, SAUR genes in accordance with *MYC2* and probably *LHY* appear to consist the downstream core regulation component in the IspS gene expression as illustrated in Fig. 2.4-11. Studies on molecular interaction between these components are needed to test this postulate and merit further investigation.

Among plant hormones, the function of cytokinin in the regulation of IspS gene expression is somewhat puzzling. Zuo et al. 2019 reported that the transcriptome associated with CK signaling does affect the CK production during isoprene mediated changes. tZR concentration exhibited positive correlation with isoprene emission and IspS gene expression whilst cZR negatively correlated with these parameters in JA treated leaves (Table 2.3-1). Moreover, the cytokinin signaling gene *AHP* and *ARR-A* are positively correlated with the isoprene emission and IspS gene expression in this study (Table 2.3-1). These results may suggest some potential role of cytokinin in the regulation of isoprene biosynthesis and emission. tZ type cytokinins, tZR, biosythesized from adenosine phosphate (ATP, ADP or AMP) and DMADP in root is transported to shoot via the xylem sap, whereas iP and cZ type cytokinins are biosynthesized probably in the leaves and reverse transported from shoot to root through the phloem (Kieber and Schaller 2018; Sakakibara 2006). Recently, it has been shown that tZR is reactivated to tZ, the active form, in

situ at the site of action by the cytokinin ribose 5'-monophosphate phosphoribohydrolase (LOG) mediated pathway. The concentration of active form cytokinins tZ and cZ is lower than the detection limit in our analysis and this low concentration may explain the puzzling opposite spectrum of its precursor in the regulation of IspS gene expression. Analysis of the active form of cytokinin tZ or cZ with higher sensitivity may answer the questions whether tZR or cZR is responsible for the changes in the hormone signaling and hence their involvement in the regulation of isoprene biosynthesis.

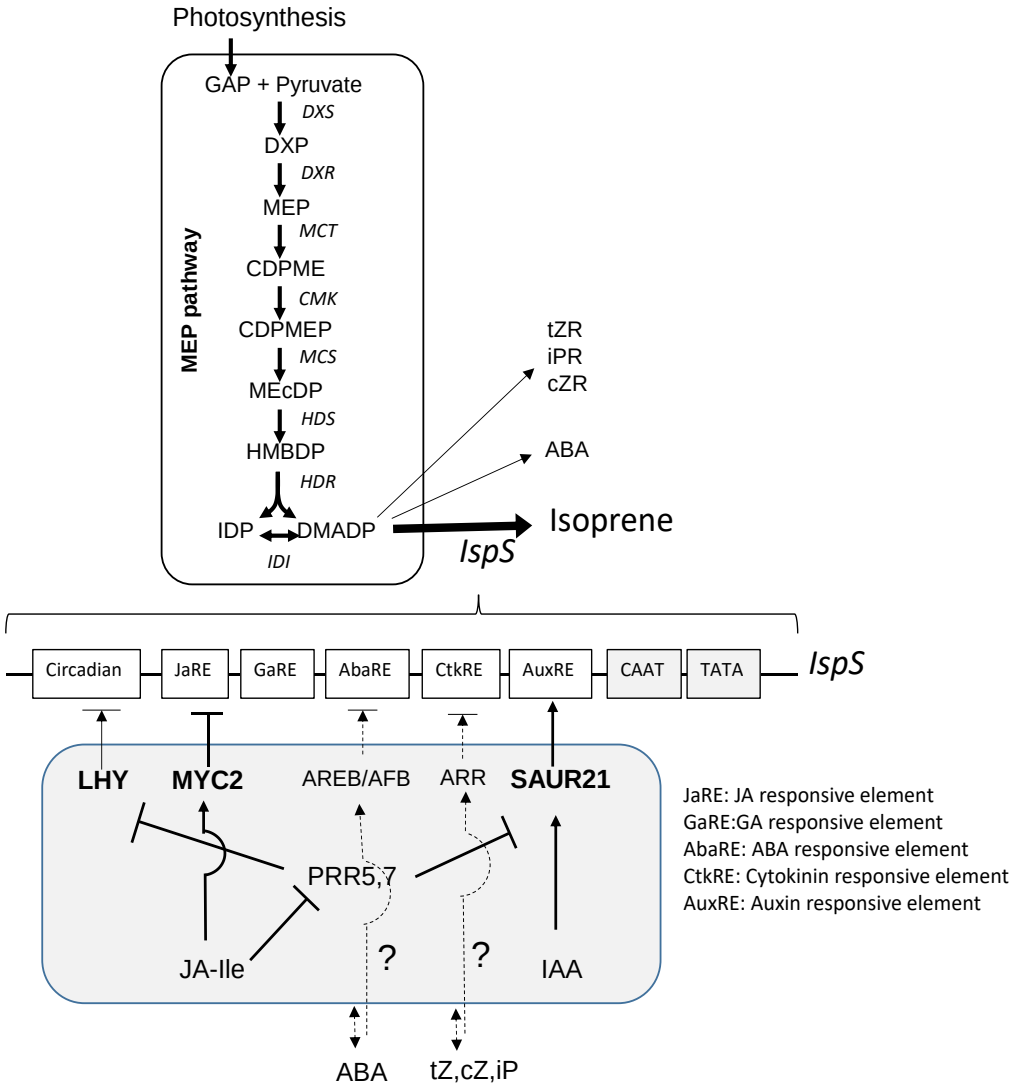

Fig. 2.4-11. Schematic representation of molecular regulatory mechanism of IspS gene expression by plant hormone. The core components of transcription factors consist of LHY, MYC2 and SAUR21 and interact with the composite motif of IspS promoter. JA-Ile up-regulate MYC2 and down regulate LHY and SAUR21 while IAA upregulate SAUR 21. MYC2, SAUR21 and LHY can bind to cis-acting element of antagonistic type (JaRE, AuxRE and Circadian) and produce opposing effect integrating the signaling of two pathways. ABA and cytokinins could

regulate IspS gene expression directly or via the interaction with the core components of JA or auxin signaling.

2.5 Conclusion

In conclusion, analysis of plant hormone and gene expression of signaling pathway and interpretation of the relationship with IspS gene expression allowed the authors to propose a core regulation component in the isoprene biosynthesis (Fig. 2.4-11). We hope that this proposal provides a view guiding future study on molecular regulatory mechanism of isoprene biosynthesis.

CHAPTER III

Seasonality of isoprene emission and its relevance with plant hormone profile in tropical plant *Ficus septica.*

3.1 Introduction

Isoprene is the most prominent volatile compound emitted by the terrestrial plant and considerably impacts on tropospheric chemistry (Arneth et al., 2008; Guenther et al., 1995, 2006). Isoprene in the atmosphere easily reacted with hydroxy radicals and nitrogen oxide to produce tropospheric ozone and influences tropospheric oxidation chemistry (Poisson et al., 2000; Sharkey et al., 2008). Moreover, isoprene plays a potential role in the formation of secondary aerosol that acts as cloud condensation and influences the global radiation budget (McFiggans et al., 2019). Isoprene emission also represents a loss of carbon fixed by photosynthesis (Harley et al., 1996; Sharkey et al., 1991). Isoprene emission on the other hand benefit plants by counteracting heat and oxidative stress to confer thylakoid membrane stabilization under high light and temperature condition (Loreto and Velikova, 2001; Singsaas et al., 1997; Velikova et al., 2008).

Isoprene is biosynthesized from DMADP via plastid-localized methylerythritol phosphate (MEP) pathway using reducing power supplied from photosynthesis (Lichtenthaler et al., 1997; Zeidler Johannes et al., 1997). Concurrently, the MEP pathway provides DMADP as the precursor of plant hormones such as cytokinin (CK), abscisic acid (ABA) and secondary metabolite including b-carotene and xanthophylls (Dani et al., 2016). A trade-off between isoprene and these products has been proposed (Dani et al., 2016), and these endo products of MEP pathway comprise a regulatory network to control various biological process of plants: growth, senescence, defense and stress responses (Pollastri et al., 2021). Increased isoprene emission elevated cytokinin concentration and hastened the plant development in both *Arabidopsis* and poplar (Dani et al., 2021). Moreover, the multitasking role of isoprene as a signaling molecule has been proposed (Zuo et al., 2019). By analogy, isoprene exposure induced resistance to bacterial

infection through salicylic acid mediated mechanism in *Arabidopsis* suggesting a defense priming role of isoprene (Frank et al., 2021). Interaction between isoprene emission and stress hormones mediates plant stress responses (Behnke et al., 2010; Mutanda et al., 2016; Parveen et al., 2019).

Isoprene emission from tropical trees as well as evergreen temperate vegetation undergoes temperature and light-dependent seasonal variation (Langford et al., 2022; Monson et al., 1999; Mayrhofer et al., 2005; Petron et al., 2001). Given the metabolic proximity of isoprene biosynthesis and its potential interactions with vital biological process in plant cells, the seasonality of isoprene emission would impact on the metabolic homeostasis and plant hormone profile of the plants. In this context, our previous study explored the transcriptional regulation of *IspS* gene by cold temperatures and revealed that the cold temperature perceived by the cellular sensor likely activated the pathway of auxin (IAA) and jasmonic acid (JA) signaling and down-regulated the *IspS* gene expression in *Ficus septica* (Mutanda et al., Parveen et al.). The hormonal balance between IAA and JA-Ile was further demonstrated to be important for the transcriptional regulation of *IspS* integrated via the coupling of the hormone-sensitive cis-element for the transcriptional factor SAUR21 and MYC2 (Iqbal et al. 2022). It is likely that IAA and JA-Ile balance plays a central role in the short-term transcriptional control of *IspS* to cope with rapid daily temperature changes adopted in our experimental regimen. However, to data, few information has been available on the seasonality of plant hormone profile linked with isoprene emission.

The chapter III extended the study of chaper II and analyzed the full spectrum of plant hormone concentration in the leaf of *F. septica* every month throughout a year to explore the reciprocal relationship between isoprene emission and plant hormone profile. Interpretation of the correlation between parameters of hormone concentration, signaling gene expression, MEP

pathway metabolite and related enzyme gene expression provides a view for long-term temperature and light-dependent control of isoprene emission and plant hormone profile in a tropical tree.

3.2 Materials and Methods

3.2.1 Plant material

Three clones of *F. septica* (2 years old) saplings were used in this experiment. Plants were grown in plastic pots (30 L) and placed in the open field of the University of the Ryukyus (26°15'N, 127°46'E), Okinawa, Japan. During the experiment, plants were regularly supplied with water and plant nutrition (Hyponex, www.hyponex.co.jp), so that saplings do not encounter any drought or water logging stress during the experiment.

A branch of *F. septica* consists of 6 or 7 node leaves indicating that abscission takes place at 7^{th} or 8^{th} node from the apex. Life span of *F. septica* leaf from emergence to abscission is almost 3 months and the cotyledon leaf grows for 3 months to maturation and senescence with apical extension. Photosynthesis rate decreased with aging due to the degradation of chlorophyll (Sastak et al., 1977). Thus, the chlorophyll content of leaves can be used as a hallmark of aging. In support of this view, the chlorophyll content of *F. septica* leaves expressed by SPAD value (Minolta SPAD-502-meter, Spectrum Technologies Inc.) increased from the juvenile to mature stage, almost constant from day 20 to 60 and gradually decreased with further aging (Fig. 3.2-1 A). The chlorophyll content from 3^{rd} to 6^{th} node was almost comparable suggesting that the final abscission process takes place at node 7^{th} or 8^{th} (Fig. 3.2-1 B). Based on these observations, a mature appearance leaf was collected from 3rd or 4^{th} node after measurement of isoprene emission. Isoprene emission measurement and leaf sampling were done in every month from April 2019 to

March 2020. Thus, SPAD values (chlorophyll content) of leaves sampled every month were largely comparable supporting that the growth stages of leaves were comparable (Fig. 3.2-1 C).

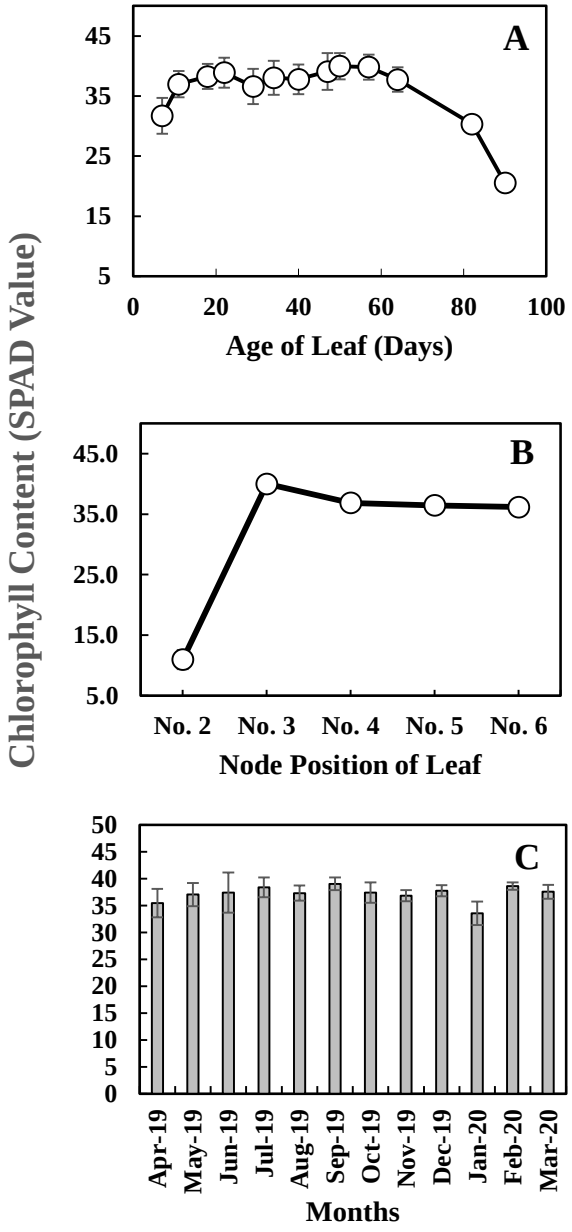

Fig 3.2-1. Chlorophyll content (SPAD value) in the leaves of *F. septica* as a function of age (A), leaf position (B) and time of the year (C).

3.2.2 Growth condition of filed Temperature and Light intensity.

Growth conditions of field temperature and light intensity were monitored throughout a year by use of HOBO^{R} Temperature/Light Data logger (Onset Computer Corporation, MA, USA). Temperature and illumination intensity (Lux) were recorded every 30 min, and average of a day for temperature and average of daytime (7 to 18 hr) for light intensity was taken as the measure of the day and processed further as described below. Illumination (Lux) records on the logger were converted into photosynthetically active radiation (PAR) by the relationship between Lux and PAR under comparable conditions as described previously (Oku et al., 2021). Our previous study suggested that past weather condition influence the isoprene emission rate: past 3 days before measurement for temperature and past 10 days for light intensity. Thus, temperature of 3 days before measurement and light intensity of past 10 days including measurement day were averaged and used for correlation analysis.

3.2.3 Measurement of Isoprene Emission and SPAD value

Three healthy and mature leaves of each sapling were randomly selected for isoprene measurements. Isoprene emission was measured between 11.00 to 2.00 pm using leaf cuvette (PLC-4C, ADC BioScientific, Hertfordshire, UK) and a real-time isoprene analyzer (KFCL-500, Anatec Yanaco, Kyoto, Japan) as described previously (Oku et al.,2014). Leaves were irradiated with LED light (1,000μmolm^{-2} s^{-1} PPFD) for 15 min at ambient temperature and isoprene emission of the last 5 min was taken as isoprene emission rate in this study throughout. No control of leaf temperature was made to minimize the temperature gap between field and laboratory condition. Chlorophyll content (SPAD) value was measured by a Soil Plant Analysis

Development meter (SPAD–502, Minolta Camera Co., Osaka, Japan) after isoprene emission measurement. As soon as these measurements finished, the leaf was frozen in liquid nitrogen and stored at -80 ℃ for further analysis of plant hormones, MEP pathway metabolites, gene expression and IspS protein.

3.2.4 Metabolite analysis of MEP pathway

The procedure described in chapter II was applied.

3.2.5 Plant Hormone analyses

The procedure described in chapter II was applied.

3.2.6 Gene expression analysis

The procedure described in chapter II was applied.

3.2.7 Western blot for Isoprene synthase (IspS) protein analysis

The procedure described in chapter II was applied.

3.2.8 Statistical analysis

Statistical significance of the differences in gene expression levels and hormones were performed using the Tukey test by XLSTAT (Addinsoft) software. Nonmetric multi-dimensional scaling (NMDS) was performed by BellCurve for Excel based on the distance of 1- | r |.

3.3 Results

This study monitored the isoprene emission from *F. septica* leaves, plant hormone concentration and signaling gene expression, MEP pathway metabolite concentration and related enzyme gene expression throughout a year starting from April 2019 to March 2020 under field condition of subtropic Okinawa Japan. Average daily temperature for 3 days before the measurement day was the lowest of 13.8 ℃ in Feb. 2020 and the highest of 29.5 ℃ in Aug. 2019 (Fig. 3.3-1 A). The average light intensity of past 10 days of the measurement was the highest of 504 μmol/s/m^2 in Sep. 2019 and the lowest of 221 μmol/s/m^2 in Jan. 2020. Temperature and light intensity almost paralleled throughout a year. However, the fluctuation in light intensity was much larger than that of temperature. Moreover, temperature change appeared to show more distinctive difference between summer (Jul. to Oct.) and winter (Dec. to Mar.) season.

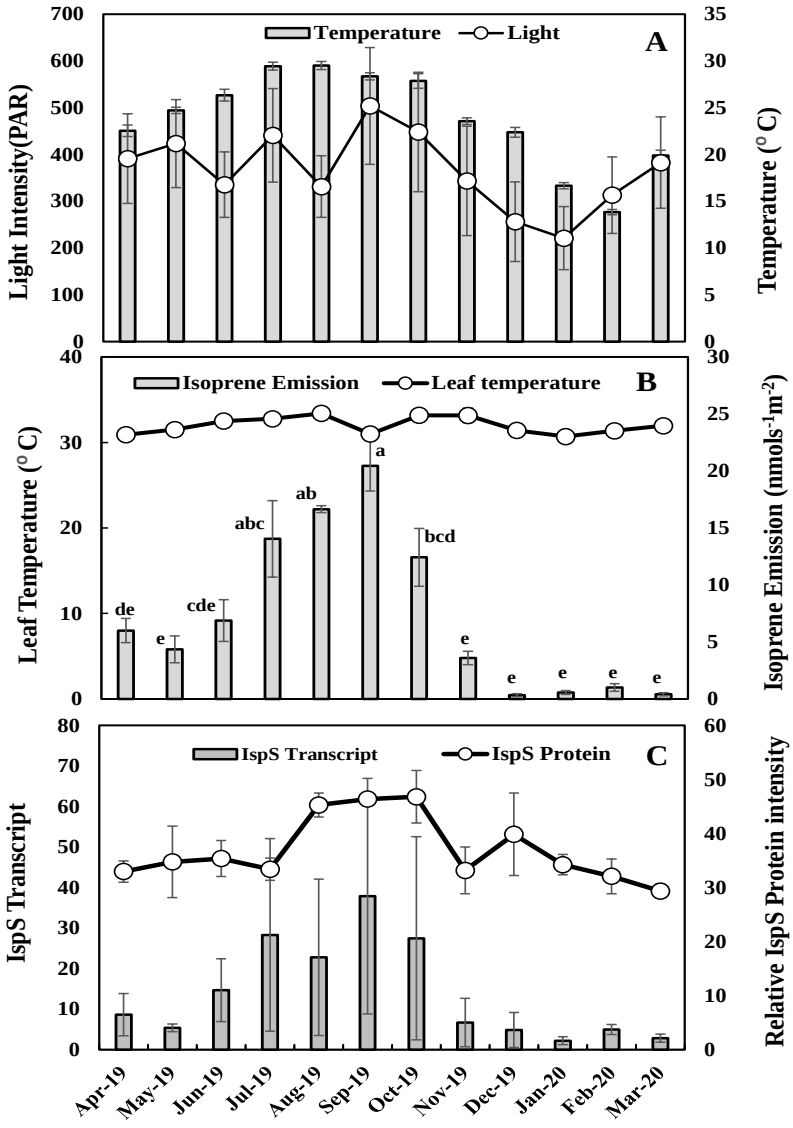

Fig 3.3-2. Seasonal variation of growth condition (A), isoprene emission (B), IspS transcript and IspS protein level (C) for the leaves of tropical plant *Ficus septica.* Measurements were done every month from April 2019 to March 2020. Each value represents mean ± SE (n = 3) and different lowercase letters on bars indicate statistical significance ($P < 0.05$, Tukey's test).

Seasonality of isoprene emission showed largely similar pattern as demonstrated for growth temperature or light intensity of the field (Fig. 3.3-2 A). Leaf temperature irradiated with 1000 μmol/s/m^2 of LED light fluctuated from 31 (in Jan.) to 33 ℃ (in Aug.) as shown in Fig. 3.3-2 B probably due to variation in ambient temperature of the measurement day. Isoprene emission rate was the highest in Sep. 2019 and decreased with air temperature or light intensity of field towered winter season.

Isoprene synthase plays a pivotal role in the leaf level control of isoprene emission (Mayrhofer et al., 2005; Sharkey et. Al., 2008; Wiberley et al., 2005). Seasonal variation in *IspS* gene expression and IspS protein level was similar to the pattern demonstrated for seasonality of isoprene emission (Fig. 3.3-2 C).

Plant hormone profile related to the seasonality of isoprene emission is shown in Fig. 3.3-3. Statistically significant changes were noted with salicylic acid (SA), trans-zeatin O-glucoside (tZOG), trans-zeatin riboside (tZR), cis-zeatin O-glucoside, cis-zeatin O-glucoside riboside (cZROG) and cis-zeatin riboside (cZR). No statistically significant changes were observed with other plant hormones. However, it worth noting that JA-Ile showed lower tendency in the summer season while opposite trends for OPDA (Fig. 3.3-3 E and F). Despite statistically significant changes, no trends of seasonality were seen with the profile of tZOG and tZR. In contrast, seasonal variation manifested clearly in the profiles of cZOG, cZROG and cZR showing lower concentration in summer season (Jul. to Oct.) compared to those in winter season (Dec. to Mar).

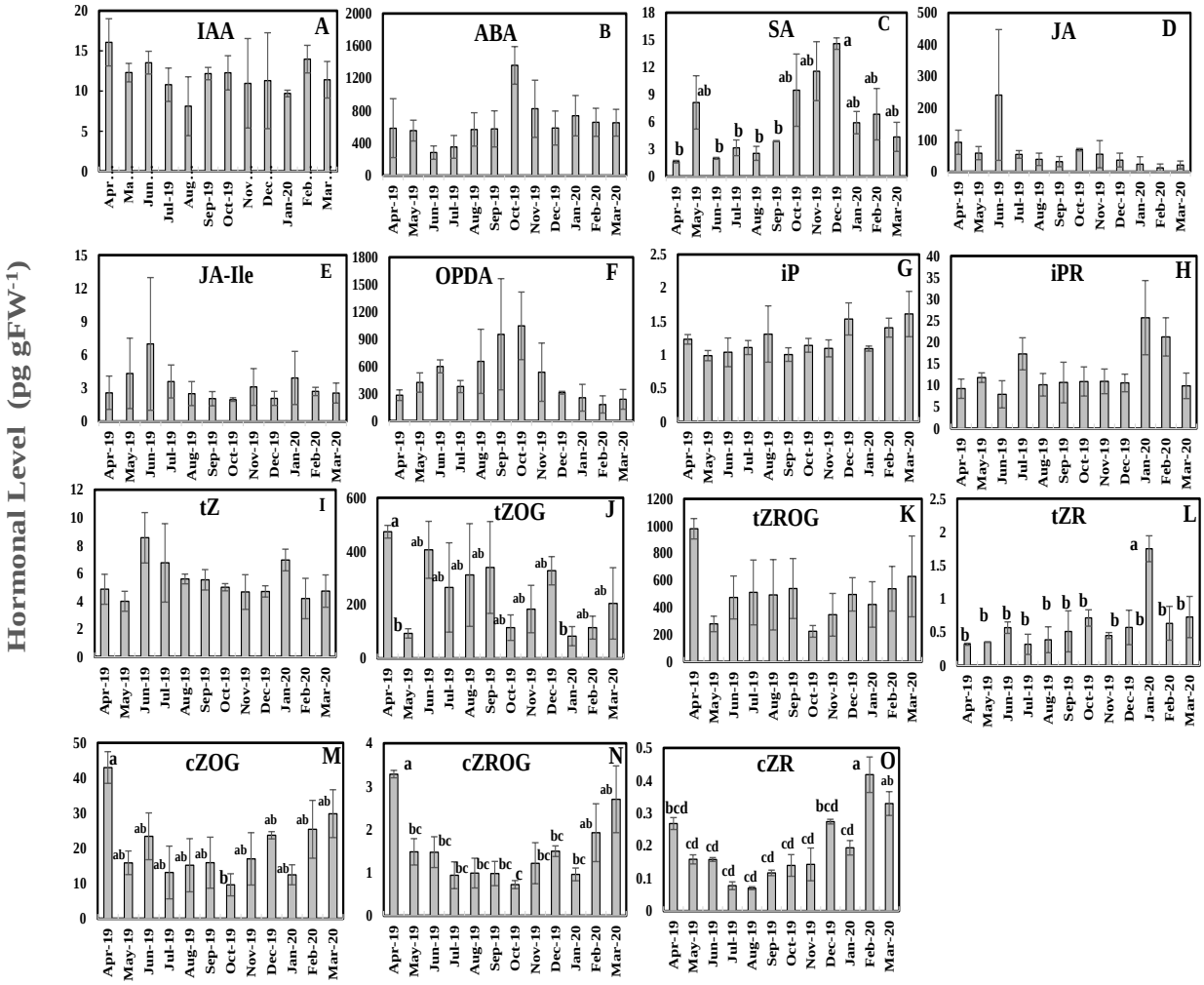

Fig. 3.3-3. Seasonal variation of plant hormone level in the leaves of tropical plant *Ficus septica*. Hormone levels were measured every month from April 2019 to March 2020. A, indole-3-acetic acid (IAA); B, abscisic acid (ABA); C, salicylic acid (SA); D, jasmonic acid (JA); E, jasmonoyl isoleucine (JA-Ile); F, 12-oxo-phytodienoic acid (OPDA); G, isopentyl adenine (iP); H, isopentyl adenosine (iPR); I, trans-zeain (tZ); J, trans-zeatin-O-glucoside (tZOG); K, trans-zeatin riboside-O-glucoside (tZROG); L, trans-zeatin riboside (tZR); M, cis-zeatin-O-glucoside (cZOG); N, cis-zeatin riboside-O-glucoside (cZROG); O, cis-zeatin riboside (cZR). Data are mean±SE of three

leaves. Different letters on the data show a statistically significant difference ($p < 0.05$ by Turkey test).

Metabolic flux through MEP pathway is an important factor to control isoprene emission (Andrea et al., 2014). Figure 3.3-4 shows the seasonal variation in concentration of MEP pathway metabolite of *F.septica* leaves through a year. Concentrations of DXP, MEP and MEcDP in summer season were significantly higher than those in winter season with statistical significance. No statistically significant changes were seen in the concentration of HMBDP and DMADP. Nonetheless, concentration of DMADP showed higher tendency in Sep. to Dec. compared to other months of the year (Fig. 3.3-4 E). Close examination of seasonal variation of MEP metabolites found that the profile of HMBDP depicted U shape and differs from other metabolites DXP, MEP, MEcDP and DMADP those show a bell shape pattern with higher concentration in the summer season (Fig. 3.3-4). Furthermore, this trend was more clearly demonstrated by their correlation with the concentration of cZR. The concentration of cZR showed statistically significant ($p < 0.05$) negative correlations with that of DXP, MEP and MEcDP while a positive correlation tendency ($p = 0.09$) for HMBDP.

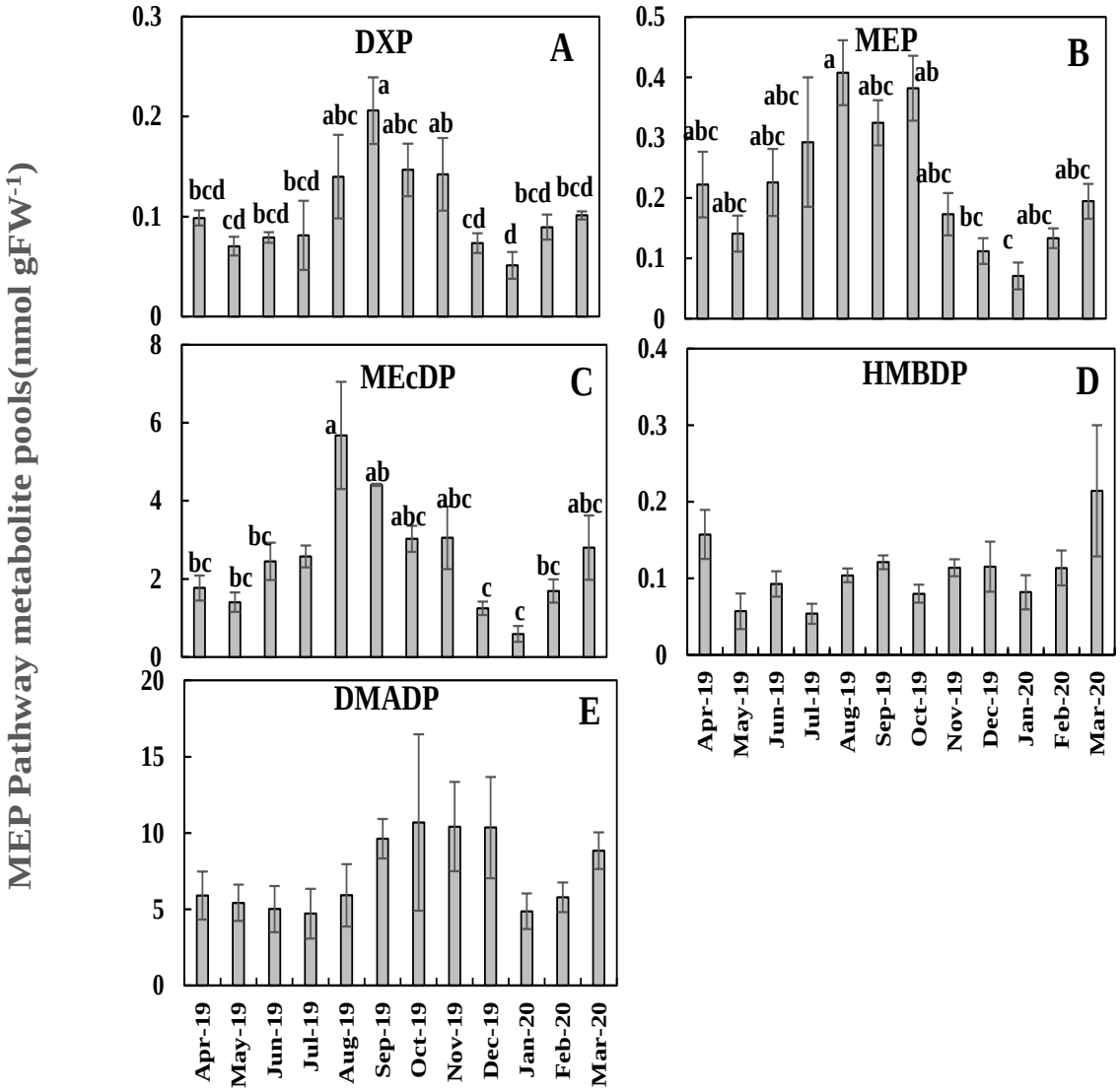

Fig 3.3-4. Seasonal variation of MEP pathway metabolites in the leaves of tropical plant *Ficus septica.* Metabolite concentrations were measured every month from April 2019 to March 2020. A, DXP, 1 - deoxy - D - xylulose - 5 - phosphate; B, MEP, 2 - C - methyl - D - erythritol 4 - phosphate; C, MEcDP, 2 - C - methyl - D - erythritol 2, 4 - cyclodiphosphate; D, HMBDP, 4 - hydroxy - 3 - methylbut - 2 - butenyl 4 - diphosphate; and E, DMADP, dimethylallyl pyrophosphate. Each value represents mean ± SE (n = 3) and different lowercase letters on bars indicate statistical significance ($P < 0.05$, Tukey's test).

The enzyme activity of MEP pathway is a critical factor to control the metabolic flux and was shown in Fig. 3.3-5. Activities of MEP pathway enzymes except for HDS peaked in Sep. and depicted a largely similar pattern as shown for seasonality of isoprene emission (Fig. 3.3-2 C). The metabolic flux through MEP pathway is mainly controlled by transcriptional and post-transcriptional regulation of DXS and HDR in response to several environment stimulus including changes in temperature and light intensity (Rodoriguez-Concepcion, 2006; Cordoba et al., 2009). Although seasonality in the activity of DXS was less evident, DXR exhibited clear seasonality with the peak in Sep. Thus, MEP pathway metabolite concentration (Fig. 3.3-4) and related enzyme activity (Fig. 3.3-5) indicated that entire metabolic flux rate largely compares with the seasonality profile of isoprene emission.

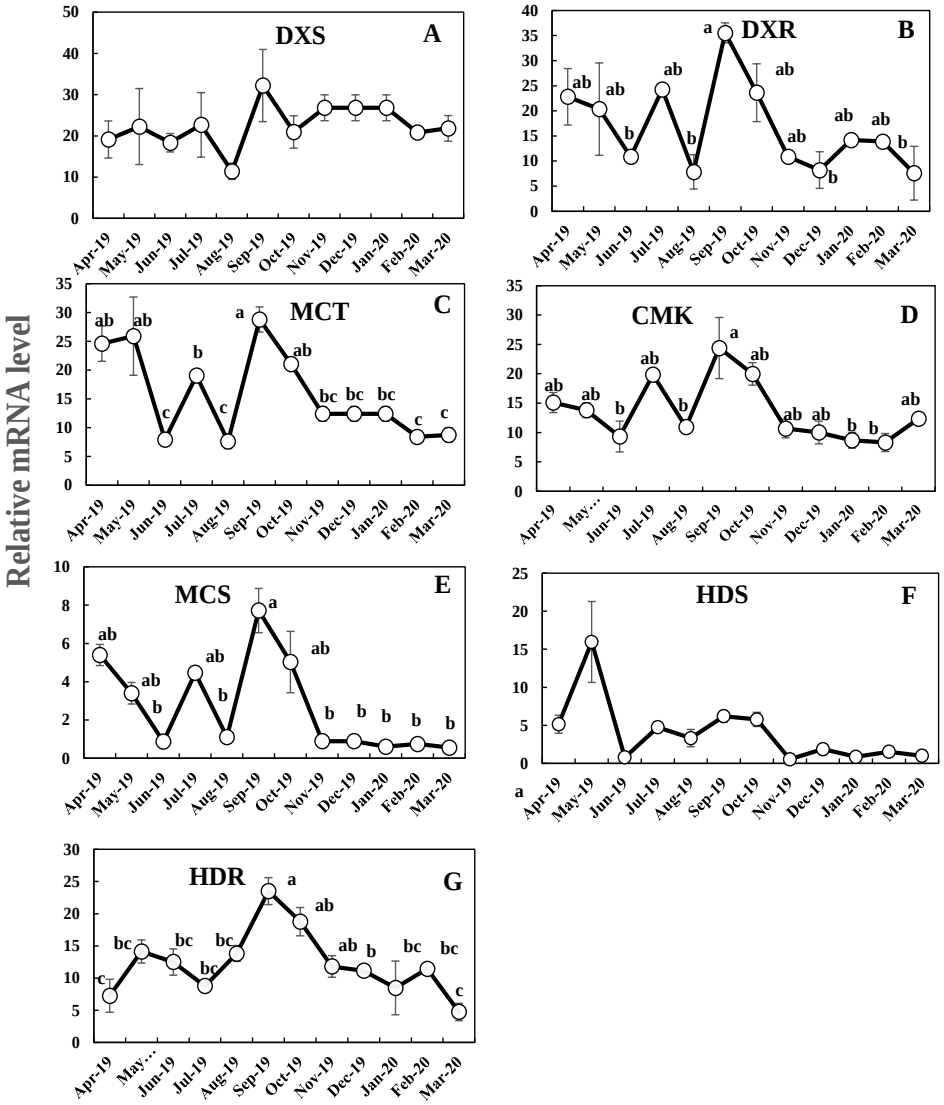

Fig 3.3-5. Seasonal variation of MEP pathway gene expression in the leaves of tropical plant *Ficus septica.* Measurements were done every month from April 2019 to March 2020. A,1-deoxy-D-xylulose-5-phosphate synthase (DXS); B, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR,); C, MEP cytidyltransferase (MCT); D, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-ME) kinase (CMK); E, 2-Cmethyl-D-erythritol 2,4-cyclodiphosphate (MEcDP) synthase (MCS); F, 4-hydroxy-3-methyl-2-butenyl4-diphosphate (HMBDP) synthase (HDS); G, HMBDP reductase (HDR). Values are means ± SE (n = 3) and different lowercase letters on bars indicate statistical significance ($P < 0.05$, Tukey's test).

To get more insight into the hormonal control of isoprene emission, the authors studied correlations between growth condition, *IspS* gene expression, IspS protein level, plant hormone concentration, hormone signaling related transcriptional factor, MEP metabolite concentration and related enzyme gene expression throughout a year (Table 3.3-1).

Table 3.3-1. Correlation coefficient analysis between plant hormone concentration, plant hormone signaling genes, isoprene emission, *IspS* transcripts, IspS protein, temperature, light and other parameters.

	Temperature	Light	Isoprene emission	*IspS* Transcript	IspS Protein
Temperature	1.00	**0.61*** **	**0.79*** **	**0.39***	**0.57****
Light	**0.61*** **	1.00	**0.64*** **	**0.34***	**0.43****
Isoprene emission	**0.79*** **	**0.64*** **	1.00	**0.45****	**0.70*** **
IspS Transcript	**0.39***	**0.34***	**0.45****	1.00	0.29
IspS Protein	**0.57****	**0.43****	**0.70*** **	0.29	1.00
Plant Hormones					
IAAH	-0.11	0.11	-0.11	-0.09	-0.23
ABA	-0.06	0.06	0.06	0.15	0.07
JA	0.18	0.02	0.10	0.14	0.02
JA-Ile	0.02	-0.07	-0.01	0.08	-0.20
OPDA	**0.42***	**0.34***	**0.47****	**0.65****	0.26
SA	-0.18	-0.24	-0.28	-0.16	-0.10
tZ	0.16	-0.09	0.03	-0.01	0.19
tZOG	0.25	0.08	0.14	-0.17	0.17
tZROG	-0.12	0.00	-0.08	-0.25	-0.07
tZR	-0.21	**-0.35***	-0.09	0.24	-0.06
cZOG	-0.28	-0.08	-0.32	**-0.33***	-0.25
cZROG	**-0.36***	-0.04	**-0.39***	-0.26	**-0.34***
cZR	**-0.80*** **	**-0.35***	**-0.63*** **	**-0.37***	**-0.42****
iP	-0.25	-0.22	-0.28	-0.02	-0.16
iPR	**-0.40***	-0.29	-0.18	0.21	-0.29
Plant Hormone Signaling Gene					
SAUR	-0.11	0.11	-0.15	-0.05	-0.11
MYC2	0.17	0.07	0.12	0.27	0.19
ABF1	0.08	-0.11	-0.05	0.11	0.10
PR1	**0.34***	0.20	0.25	**0.36***	0.19
ARRB	0.02	0.13	-0.07	-0.17	-0.12
LHY	0.25	0.25	0.27	**0.33***	0.05
PRR7	0.17	0.05	0.10	0.16	-0.03
MEP Pathway Metabolites					
DXP	**0.42***	**0.52****	**0.62*** **	**0.44****	**0.51****
MEP	**0.66*** **	**0.52****	**0.69*** **	**0.46****	**0.52****
MEcDP	**0.57*** **	**0.39****	**0.66*** **	0.25	**0.56*** **
HMBDP	-0.24	-0.02	-0.19	-0.15	-0.18
DMADP + IPP	0.07	0.11	0.10	-0.13	0.15
MEP Pathway genes					
DXS	-0.10	0.06	-0.19	-0.14	-0.20
DXR	0.30	**0.60*** **	**0.36***	0.22	0.19
MCT	**0.34***	**0.65*** **	**0.34***	0.20	0.19
CMK	**0.52****	**0.73*** **	**0.51****	0.14	**0.46****
MCS	**0.49****	**0.77*** **	0.57	0.31	**0.37***
HDS	0.29	**0.48****	0.19	0.02	0.20
HDR	**0.44****	**0.44****	**0.57****	**0.43****	**0.57****

Letters in boldface indicate statistical significance by Student's t-test ($P < 0.05$): ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

Table 3.3-2. Correlation coefficient analysis between Cytokine signaling genes, isoprene emission, *IspS* transcripts, IspS protein, Temperature, and light in all the year-round isoprene emission of JA sprayed *F. septica* leaves. Letters in boldface indicate statistical significance by Student's t-test (P < 0.05): ***P < 0.001; **P < 0.01; *P < 0.05

	Temperature	Light	Isoprene emission	IspS Transcript	IspS Protein	*AHP*	*AKH234*	*ARR-A*	*ARRB*
Temperature	1								
Light	**0.51****	1.00							
Isoprene emission	**0.88*****	**0.60*****	1.00						
IspS Transcript	**0.42***	0.29	**0.45****	1.00					
IspS Protein	**0.59****	**0.58****	**0.70*****	0.29	1.00				
AHP	-0.07	0.01	-0.05	0.12	-0.18	1.00			
AKH234	0.19	0.06	0.12	0.04	0.01	**0.62*****	**1.00**		
ARR-A	0.31	0.27	0.16	**0.38***	0.24	**0.42***	**0.48****	1.00	
ARRB	-0.01	0.21	-0.07	-0.17	-0.12	-0.06	0.15	-0.27	1.00

Of the plant hormones, OPDA, and cytokinins (cZOG, cZROG and CZR) showed statistically significant positive and negative correlation with isoprene emission, *IspS* transcript and IspS protein level, respectively. Most strong correlation was noted between cZR concentration and isoprene emission. However, no significant correlation was noted with the effector transcription factor ARR-B in the cytokinin signaling. In contrast, PR1 involved in SA signaling exhibited positive correlation with *IspS* transcript. *LHY*, a circadian clock gene similarly showed positive correlation with *IspS* transcript.

MEP pathway metabolites, DXP, MEP and MEcDP positively correlated with air temperature, light intensity, isoprene emission, IspS transcript and IspS protein level. DMADP concentration was not this case and showed no statistically significant correlation with any other parameters mentioned in Table 3.3-1. In accordance with the correlation profile of metabolite,

gene expression of MEP pathway showed similar trend of positive correlation with growth condition and isoprene emission related parameters listed in Table 3.3-1.

Cytokinin level, especially tZOG, cZROG and cZR appeared to be related with the seasonal variation of isoprene emission as shown in Fig. 3.3-2 and Table 3.3-1. Nevertheless, no correlation was noted between isoprene emission and *ARR-B* the effecter transcription factor of this signaling pathway. This prompted author to examine more detailed gene expression in cytokinin signaling. Table 3.3-2 list the correlation of intermediate genes of the pathway with growth condition, isoprene emission, *IspS* transcript and IspS protein level. *ARR-A* is the negative regulator of *ARR-B* and showed positive correlation with *IspS* transcript, *AHP* and *AHK234*. This coordinated correlation of signal transduction pathway may suggest that signaling of cytokinin was activated even no correlation was observed between cytokinin concentration and *ARR-B* gene expression.

Our previous study postulated that hormonal balance between IAA and JA-Ile plays an important role in the short-term regulation of isoprene emission from *F. septica* challenged by rapid temperature change in the laboratory conditions (Iqbal et al., 2022). The relative proportion of the transcription factor *SAUR21* and *MYC2* were suggested to be involved in this signal transduction. However, hormone balance of *IAA/JA-Ile* nor *SAUR21/MYC2* showed no correlation with IspS transcript and isoprene emission under long-term natural conditions in this study (Fig. 3.3-7).

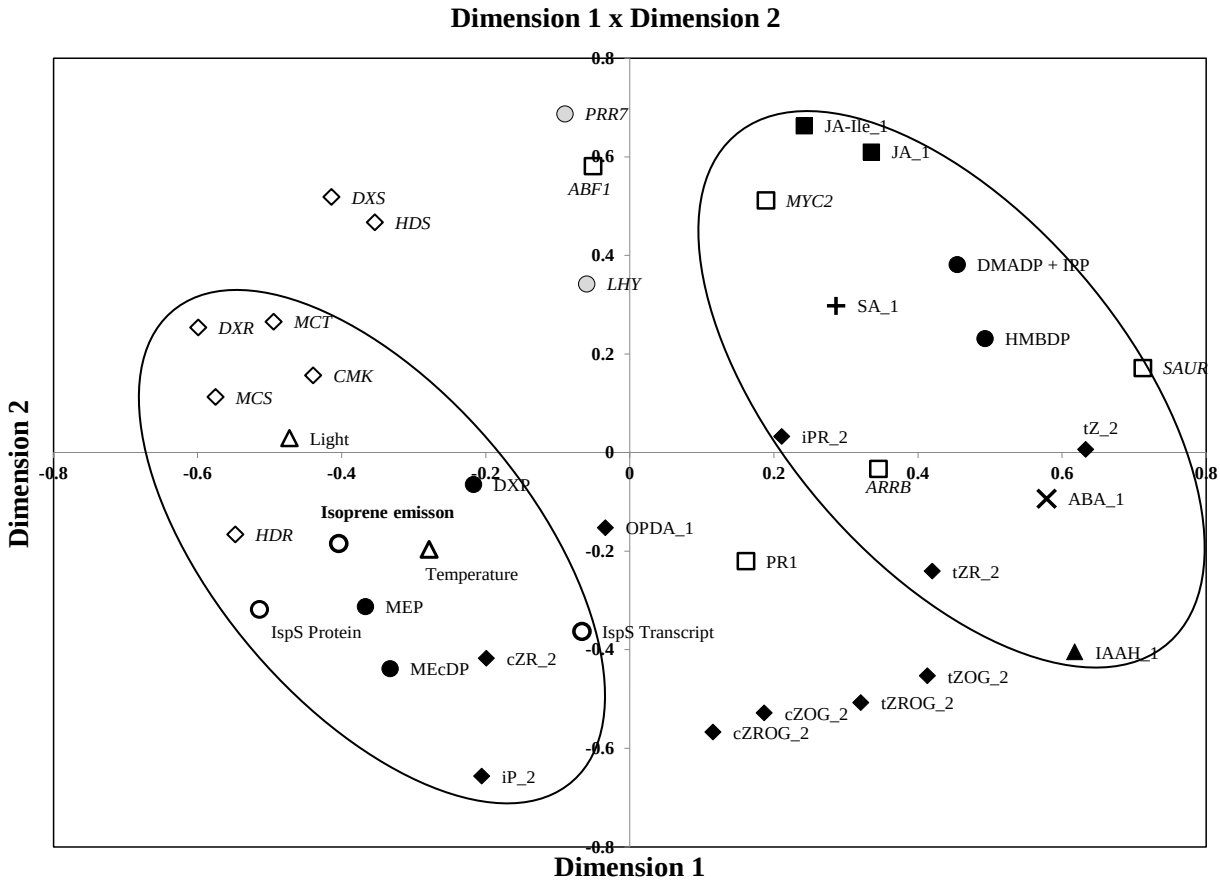

Fig. 3.3-6. Nonmetric multi-dimensional scaling (NMDS) for different variables in this study. Isoprene emission, IspS protein level and IspS transcript (◯); temperature and light intensity (Δ); MEP pathway metabolites (●); MEP pathway genes (◊); cytokinins (♦); jasmonic acid (■); auxin (▲); absisic Acid (×); salisylic acid (+); hormone signaling genes (□); and circadian clock genes ( ). Gene names are italicized. 1- | r | was input distance data for NMDS analysis.

Finally, correlations between parameters analyzed in this study were summarized in two dimensions by non-metric multidimensional scaling as shown in Fig.3.3-6 (close location in the plot indicate close relationship). There seems to be two major groups: group A, that showed close relationship with isoprene emission including *IspS* transcript and IspS protein. Isoprene emissions

and its main regulatory factors are surrounded with growth conditions, MEP pathway metabolites and related enzyme genes indicating more importance of these factors in controlling the isoprene emission. Of the plant hormones, cZR and iP were classified into this group. On the other hand, the group B was comprised of factors of lower correlation with isoprene emission including several plant hormones and signal transduction genes. DMADP against our expectations belongs to this group suggesting a rather minor role in the regulation of isoprene emission. It should be also mentioned that there were several factors located between these two groups including circadian clock genes and some O-glucosylated cytokinins such as cZROG, cZOG, tZROG and tZOG.

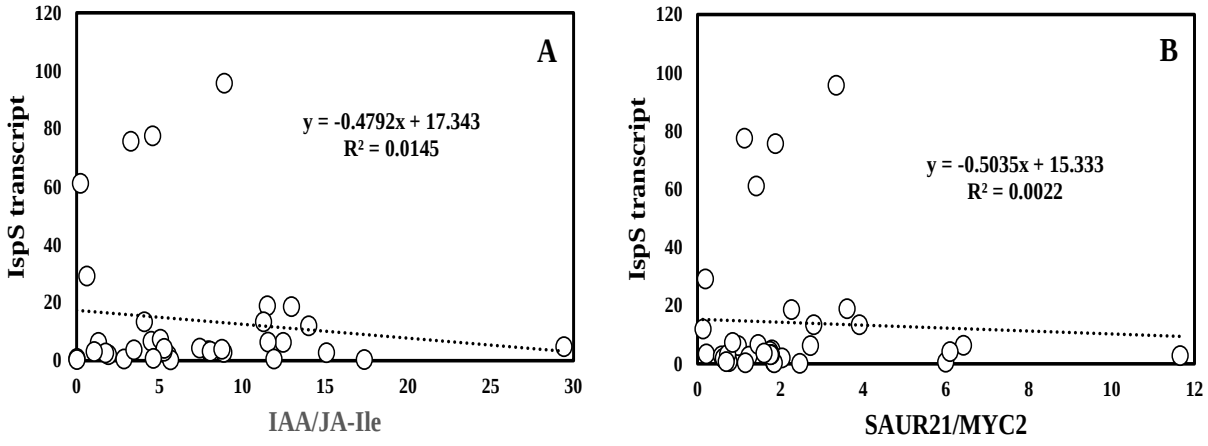

Fig 3.3-7. Relationship between IspS transcript, ratio of IAA/JA-Ile or ratio of *SAUR21*/*MYC2* gene expression. A, *IspS* transcript vs ratio of IAA/JA-Ile; B, *IspS* vs ratio of *SAUR/MYC2*. Statistical significance was evaluated by Student's t -test

3.4 Discussion

Isoprene emission from *F.septica* leaves displayed typical seasonal variation as illustrated in Fig. 1 with high emission rate in summer season (Jul. to Oct.) and low emission rate in winter season (Dec. to Mar.) in subtropic Okinawa. It is likely that the seasonality of isoprene emission is driven by temperature and light dependent changes in substrate availability via the MEP pathway and transcriptional and post-transcriptional regulation of *IspS*.

Substrate supply through flux of MEP pathway is mainly controlled at the steps of DXS that catalyze formation of DXP from GAP and pyruvate, and HDR that catalyze conversion of HMBDP to DMADP or IDP (Rodriguez-Conception, 2006; Cordoba et al., 2009) as illustrated in Fig.3.3-6. Profiles of seasonal variation in metabolite concentration (Fig. 3.3-4) and involved enzyme gene expression (Fig. 3.3-5) are largely similar to that of seasonality of isoprene emission suggesting that the substrate availability is a limiting factor for isoprene emission throughout the year.

Among the MEP metabolite, DMADP showed lower correlation with isoprene emission as was observed in previous our (Parveen et al., 2019a; Parveen et al., 2019b) and another study (Wiberley et al., 2008). DMADP is biosynthesized in chloroplast via the MEP pathway and in cytosol by mevalonic acid (MVA) pathway (de Souza et al., 2018). In this context, it has been reported that chloroplastic pool of DMADP constitutes about one-third of the total pool (Loreto et al., 2004). Our present data is total amount of DMADP representing both chloroplastic and cytosolic pool and therefore may explain the lower correlation of DMADP with isoprene emission rate in this study. Furthermore, there is metabolite cross talk between MEP and MVA pathway and it could be possible that DMADP synthesized by cytosolic MVA pathway contributes to the

chloroplastic pool and influence the substrate availability of IspS. However, it is an accepted view that there is limited cross talk between the MEP pathway and MVA pathway metabolite and isoprene is synthesized exclusively from newly fixed carbon in the chloroplast. Seasonality of MEP pathway metabolites confined in the chloroplast (Fig. 3.3-4) thus well compare the isoprene emission profile and may demonstrate the basal driving force of MEP pathway for control of isoprene emission under non-stressed natural conditions.

Plant hormones constitute the fundamental signal transduction system of living plant cell and control development, stress response and senescence. It has been reported that isoprene emission modulates the plant hormone profile and influences the growth and stress response of plant cell. Increased isoprene emission elevated cytokinin concentration and accelerated the plant development in both *Arabidopsis* and poplar (Dani et al., 2021). Similarly, the multitasking role of isoprene as a signaling molecule has been proposed (Zuo et al., 2019). Furthermore, isoprene exposure induced resistance to bacterial infection through salicylic acid mediated mechanism in *Arabidopsis* suggesting a defense priming role of isoprene (Frank et al., 2021). Thus, isoprene emission indirectly or isoprene molecule itself modulates the plant hormone profile and control the biological event of plant cells.

Seasonal variation of isoprene emission modulated the plant hormone profile of *F. septica* leaves (Fig. 3.3-3). This was rather clearly demonstrated with cZ-type cytokinins such as cZOG, cZROG and cZR than tZ- or iP type cytokines in this study. The tZ type cytokinins are biosynthesized in the root and transported to shoots via the xylem sap whereas iP and cZ type are biosynthesized in the leaves and reverse-transported from the shoot to the root through the phloem (Sakakibara 2006; Kieber and Schaller 2018). Thus, it appears true that seasonality of cZ type cytokinin is a reflection of input variation via the in-situ biosynthesis in *F. septica* leaves.

The first step of cytokinin is N-prenylation of AMP, ADP or ATP with isoprenoid moieties donated by DMADP or HMBDP (Krall et al., 2002). DMADP is an intermediate metabolite of MVA pathway in the cytosol whilst HMBDP is an intermediate of the MEP pathway. Among the MEP metabolites, only HMBDP showed tendency to correlate with cZR, and this prompted the authors to postulate the relationship between isoprene emission and plant hormone profile in *F. septica*, with the lack of any experimental evidence (Fig.3.4-8). Isotopic labeling studies with *Arabidopsis thaliana* seedling demonstrated that the prenyl side chains of tZ- and iP-ype cytokinins are mainly provided by MEP pathway whilst significant proportion of cZ-type is synthesized through MVA pathway (Kasahara et al., 2004). Thus, it could be possible that tZ type cytokinin is firstly biosynthesized in plastid by direct prenylation of AMP, ADP or ATP with HMBDP derived from MEP pathway and then converted to cZ by cis-trans isomerase of zeatin as demonstrated in *Phaseoulus vulgaris seed* (Bassil et al., 1993). However, this cis-trans conversion appeared to be refuted by examination of cytokinin distribution after application of exogenous cytokinins in *Arabidopsis* (Hosek et al., 2020). Another experiment similarly casted doubt on the existence of zeatin cis-trans isomerase by examination of corresponding enzyme properties in maize (Hluska et al., 2017). Nevertheless, it cannot be excluded that isomerization might take place under specific conditions or in particular plants (Schafer et al., 2015). The authors hope that our postulate triggers studies of this line.

A trade-off between isoprene emission and plant hormone is postulated (Dani et al. 2016). Thus, MEP pathway hypothesized to provide DMADP or HMBDP to synthesize iP or tZ type cytokinins in plastid (Dani et al., 2016). In this context, it is interesting to note that gene expression of HDS responsible for conversion of MEcDP to HMBDP displayed different seasonal variation profile compared to other MEP pathway enzyme: no elevated level was observed in

summer season in contrast to the pattern as seen for other enzymes (Fig. 3.3-5). Given that the cZ type cytokinins are biosynthesized in plastid of *F. septica* leaves, HDS could play a role in the channeling of HMBDP to cZ type cytokinin biosynthesis despite the entire MEP pathway flux is controlled at the steps of DXS or HDR controlling the entire substrate supply for isoprene emission (Fig. 3.3-5). This view also could be tested by examining the gene expression and enzyme activity along with the profiling of cytokinin concentration under more controlled laboratory conditions in a future study.

Our previous study proposed that hormonal balance between IAA and JA-Ile plays an important role in the short-term transcriptional regulation of isoprene emission from *F. septica* subjected to rapid temperature change in the laboratory conditions (Iqbal et al., 2022). In our previous study, cZR concentration negatively correlated with isoprene emission in jasmonic acid treated *F. septica* leaves as was seen in current study. The relative proportion of the transcription factor *SAUR21* and *MYC2* were suggested to be involved in this signal transduction predominating over the cytokinin signaling. Our following experiment examined the interaction of *SAUR21* and *MYC2* protein with promoter region of *F. septica* IspS gene and confirmed the binding of these proteins with IspS promoter region (unpublished observation). This observation prompted the authors to expect the involvement of this signaling system even in the long-term control of *IspS* gene expression and isoprene emission. However, hormone balance of IAA/JA-Ile nor respective transcriptional factor *SAUR21/MYC2* showed no correlation with IspS transcript and isoprene emission under long-term natural conditions in this study (Fig. 3.3-7). This observation clearly demonstrated that IAA/JA-Ile ratio plays a major role in the short-term transcriptional regulation but not in the long-term seasonal variation in growth conditions.

Seasonal variation in isoprene emission modulated the hormone profile in particular the homeostasis of cZ type cytokinins (Fig. 3.3-3). Cytokinins play an essential role in plant development and growth (Marquez-Lopez et al., 2019) stress responses (Shi et al., 2012), pathogen resistance (Choi et al., 2010; Grosskinsky et al., 2011) and plant-herbivore interaction (Straka et al., 2010). Biological activity of cZ-type has been considered to be much lower compared to iP or tZ-type (Romanov et al., 2006; Stolz et al., 2011). The leaves of *F. septica* are in abundance of tZ type cytokinins and active form of cZ base was lower than detection limit of our analysis system, and no information on the seasonal variation of cZ was presented in this study. However, gene expression of *ARR-A* the negative regulator of *ARR-B* positively correlated with the *IspS* transcript in accordance with the increased expression of *AHP* and *AHK234* (Table 3.3-2) suggesting that signaling pathway was activated in response to the increased level of cZ type cytokinins. This could be in part due to the biological activity of cZR as signaling molecule conferring metal resistance in halophyte plant species (Zhou et al., 2019; Daudu et al., 2019). It is therefore plausible that cytokinin signaling constitutes a feed-forward loop to control isoprene emission probably via HMBDP level of MEP pathway as illustrated in Fig. 3.4-8. With acknowledging the lack of evidence, as well as many uncertainties in this hypothesis, the authors hope that this view merit further investigation in future study.

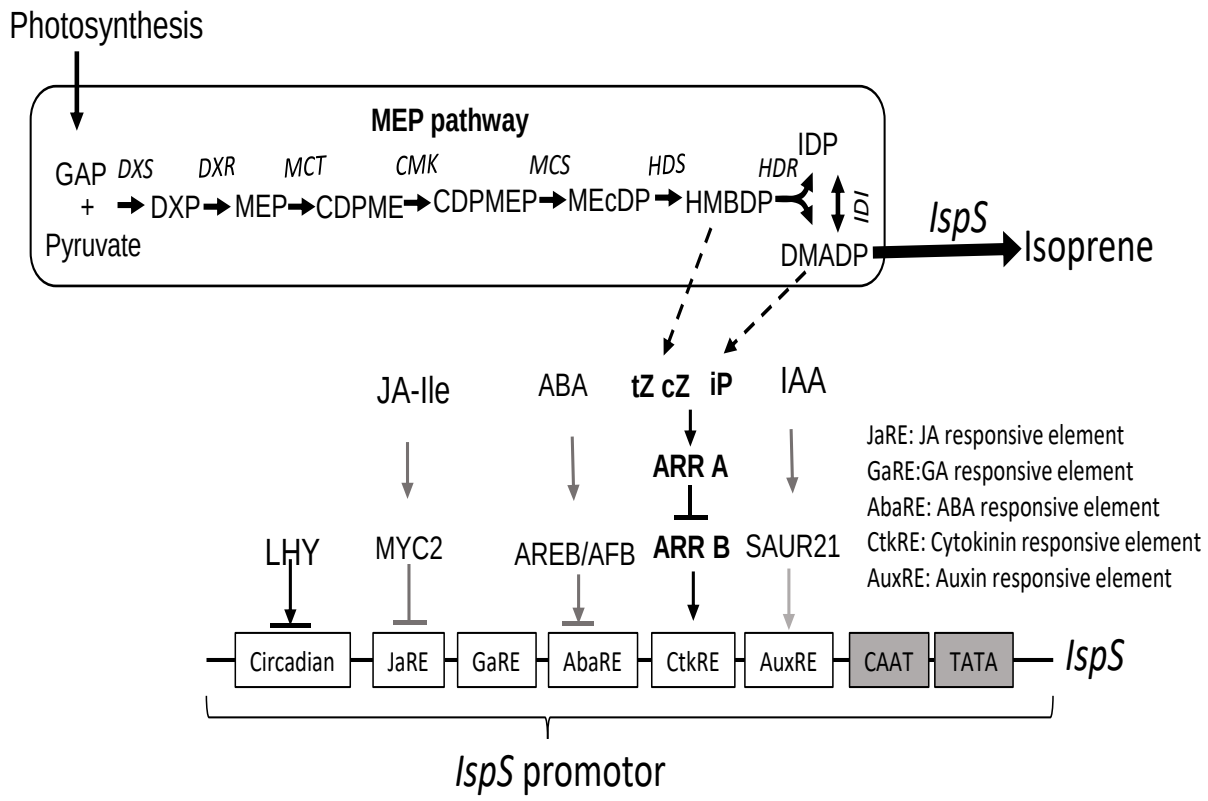

Fig. 3.4-8. Schematic representation of long-term molecular regulatory mechanism of *IspS* gene expression by plant hormones. The core components of transcription factors consist of *LHY, MYC2,* AEWB/FAB *ARR B* and *SAUR21*. The cytokinin signaling predominated over the short-term regulation by the ratio of *SAUR21/MYC2* genes and could form a feed-forward loop to inhibit *IspS* gene expression.

During this experiment, plant *F. septica* was grown under natural condition without any experimental stress of drought and temperature or light. Observed changes therefore represent the natural consequences in the field. Although cytokinins have activities to control many biological processes in plant cells, it should be mentioned that link between isoprene emission and cytokinins were most evident with the less-active glucoside derivatives of cytokinins rather than active base. There seems to be some redundancy in the biological activity of cZ and this pointed

a direction that cZ might modulate the activity of tZ by competing the receptor binding thereby preserving the specific function (Schafer et al., 2015). Accumulation of cZ type cytokinins in winter season thus might function as modulators to fine-tune the cytokinin pathway signaling and maintain the homeostasis. Alternatively, the accumulation of cZ type could be a deposit for coming leaf development or other responses against potential abiotic or biotic stresses.

In addition to cytokinins, OPDA (12-oxo-phytodienoic acid) showed statistically significant positive correlation with temperature, light intensity, isoprene emission and IspS transcript (Table 3.2-1). OPDA is a member of oxylipin and has been considered to a precursor for the biosynthesis of JA-Ile. Recent study unveiled that OPDA possess a signaling role distinctive from JA-Ile (Dave and Graham, 2012; Monte et sl., 2020). However, OPDA in our previous study negatively correlated with isoprene emission, IspS protein and *IspS* transcript. This completely opposite regulatory spectrum of OPDA signaling casted doubt on a relevance of this correlation with the constitutive role of this molecule in isoprene emission.

3.5 Conclusion

In conclusion, seasonality of isoprene emission appeared to be mainly driven by MEP pathway flux and transcriptional and post-transcriptional regulation of *IspS* gene. The seasonality of isoprene emission rate modulated the plant hormone profile. This was most clearly rendered with the profile of cytokinins with decrease in summer and increase in winter season. It is likely that isoprene emission and cytokinin homeostasis constitute a feed-forward loop to control isoprene biosynthesis and could be representing long-term fine-tuning regulation of isoprene emission via the cytokinin metabolism. However, modulation of cytokinin homeostasis could influence many cross talks between hormone signaling pathways including ABA pathway (Marquez-Lopez et al., 2019). Furthermore, recent study unveiled the promoter structures of MEP pathway genes and found numerous target sites for typical transcriptional families and light responsive cis-element (Zhu et al., 2022). Although, whole pictures of signal transduction encompassing substrate supply and biosynthesis of isoprene is till elusive, this study extended our previous study and revealed the difference between short- and long-term hormonal regulation of isoprene emission from tropical tree *F. septica*.

CHAPTER IV

GENERAL CONCLUSION AND FUTURE DIRECTIONS

The functional reasons for isoprene emission are still a matter of hot debate. A theory of the purpose of isoprene is that it is used as a mechanism by the plant to combat abiotic stresses, such as heat stress (Peñuelas et al. 2009). Siwko et al. (2007) provided evidence that isoprene stabilized lipid membranes and blocking "heat-induced phase transitions," helping the plant regulate temperature. Isoprene may additionally protect thylakoid membranes by scavenging reactive oxygen species. Despite the fact isoprene benefit the plant, an abundance of isoprene can cause indirect adverse effects on current and future air quality in an already polluted atmosphere.

It is essential to improve our understanding of the regulatory mechanism of isoprene emission, because present and predicted climate change causes a concurrent increase in the formation of these gases. Isolation of IspS upstream promoters from poplars, in silico analysis and reporter assays, gave signs on temperature and circadian regulation of IspS gene expression. It demonstrated that heat shock elements (HSEs), circadian motifs and different heat and light sensitive components prevailed in those promoters (Cinege et al. 2009; Schnitzler et al. 2010; Wiberley et al. 2009). Putative binding motifs of transcription factors of circadian rhythm and hormone signaling pathway was situated in the IspS upstream promoter sequences of *F. septica*. Likewise, the other terpene genes, there is a probability that *IspS* can be modulated by phytohormones by means of signal transduction, such as JA, CK auxin, ethylene, and salicylic acid (SA) signaling that were shown to regulate genes of MEP, monoterpene, and sesquiterpene pathways (Ginis et al. 2012; Martin et al. 2002; Martin et al. 2003; Pateraki and Kanellis, 2010).

With the progression in plant science, it has been built up that phytohormones have the potential in reducing the detrimental impacts postured by abiotic stresses (Khan et al. 2013). Crosstalk between hormones is an incredibly dynamic sector of research that has profited from

the current illustration of hormone signaling pathways. Our insight into the hormone signaling molecules to regulate isoprene biosynthesis contributed to better understanding of leaf level control of isoprene emission by plant hormones. JAs have been involved especially to come up with plant stress responses due to its contribution as a signal of developmentally or naturally controlled articulation of different genes related to stress resilience (Kazan, 2015; Wasternack and Hause, 2013). Our study unveiled the possibility of auxin, IAA, as signal transduction molecules in transcriptional control of *IspS* gene antagonizing the signaling by JA-Ile. This interaction appeared to be important for the short-term responses to cope with the rapid temperature changes such as hour to hour or day to day, and predominated over other long-term signaling effected by accumulation of cytokinins via the feed-forward loop between isoprene and cytokinin biosynthesis. Furthermore, there are numerous interactions between plant hormones controlling not only isoprene biosynthesis but also stress responses, growth and development of plant. These biological events are controlled in harmony with optimization of physiological state of plant cell. Hormonal control of isoprene biosynthesis could be a small tip of the huge and complicated biological network puzzle, however, could revise the entire signal networking maps. Clarification of the control mechanism of isoprene biosynthesis at leaf level is prerequisite for estimation of regional isoprene emission and continuous effort in this line will contribute to better understanding the global dynamics of isoprene emission.

CHAPTER V

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