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Comparative Characterization of Isoprene Synthases from Monocot Species for Improved Isoprene Production in Microalgae

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Riassunto

L'isoprene è un emiterpene idrocarburico C5 volatile emesso da molte piante terrestri, che ne costituiscono una delle principali fonti in natura. Viene anche prodotto per via petrolchimica e utilizzato come materia prima per vari prodotti quali adesivi, vernici e principalmente gomma sintetica nell'industria. Al momento, la più alta bioproduzione di isoprene viene ottenuta da *Escherichia coli*, ma c'è ancora molta strada da fare per soddisfare le esigenze su scala industriale a causa della bassa resa e degli alti costi di coltivazione.

I principali colli di bottiglia della resa di isoprene sono l'identificazione di un numero limitato di geni per l'*isoprene sintasi* (*IspS*) e le basse attività catalitiche di IspS. La produzione sostenibile di isoprene utilizzando l'energia solare e la CO_2 da parte delle microalghe eucariotiche è un modo ideale per ridurre i costi di coltivazione. Tuttavia, finora non è stata raggiunta una sintesi eterologa di isoprene nelle microalghe eucariotiche. Pertanto, il nostro progetto mira all'identificazione di nuovi geni *IspS* dalla famiglia delle Arecaceae, alla caratterizzazione funzionale di *IspS* e alla produzione eterologa di isoprene nelle microalghe.

In questo studio, sono stati identificati tre nuovi geni *IspS* da specie di palma, tra cui *Chamaerops humilis*, *Sabal minor* e *Copernicia prunifera*. Le espressioni delle proteine ricombinanti IspS in *E. coli* non hanno avuto successo in quanto non sono state espresse affatto o per lo più precipitate come corpi di inclusione.

Per confrontare le capacità relative della sintesi dell'isoprene ed esplorare le funzioni fisiologiche dell'emissione di isoprene, i geni *IspS* sono stati sovraespressi nella pianta modello *Arabidopsis thaliana*. Lo screening delle emissioni di isoprene ha mostrato che linee di *Arabidopsis* transgeniche sovraesprimenti *ChumIspS* e *CpruIspS* avevano emissioni di isoprene relativamente più elevate rispetto a quelle sovraesprimenti *SminIspS*. Poiché sono disponibili informazioni limitate sulle risposte delle piante emettitrici di isoprene agli stress abiotici ad eccezione degli stress termici e ossidativi,

le linee transgeniche *ChumIspS* o *CpruIspS* sono state utilizzate per studiare le risposte fisiologiche a trattamenti quali la salinità, l'applicazione di ABA esogeno e la siccità.

Sotto stress salino, le linee transgeniche *ChumIspS* o *CpruIspS* hanno avuto una crescita delle piantine simile al tipo selvatico. A seguito del trattamento con ABA, *ChumIspS* ha avuto una germinazione dei semi e una crescita delle piantine simili al tipo selvatico. Questi risultati hanno indicato che l'isoprene non era coinvolto nella mediazione delle risposte alla salinità o all'ABA durante la germinazione dei semi e le fasi post-germinazione.

Sotto stress da siccità, le linee transgeniche *CpruIspS* hanno mostrato risposte morfologiche e fisiologiche positive durante l'intero ciclo di vita della pianta in termini di tasso di germinazione, tasso di formazione ed inverdimento dei cotiledoni, peso fresco, contenuto di clorofilla, tasso di perdita d'acqua e tasso di sopravvivenza. Il livello di espressione relativa di diversi geni reattivi allo stress ha indicato che l'isoprene media le risposte alla siccità in modo tessuto-specifico e probabilmente attraverso percorsi ABA-dipendenti e ABA-indipendenti.

L'espressione di *IspS* non ha comportato un'evidente emissione di isoprene nelle microalghe modello *Nannochloropsis gaditana*, un risultato che potrebbe essere correlato all'espressione di livello relativamente basso o limitazioni metaboliche al momento sconosciute. Per chiarire meglio il potenziale di *Nannochloropsis* per la produzione di isoprene, è necessario esplorare ulteriormente sistemi di espressione e metodi di trasformazione più efficienti, nonché un'ulteriore comprensione dei processi metabolici e delle reti regolatorie in *Nannochloropsis*.

Abstract

Isoprene is a volatile C5 hydrocarbon hemiterpene emitted from many terrestrial plants as a major source in nature. It is produced from petrochemical routes and used as feedstock for various products such as adhesives, paints and primarily synthetic rubber in the industry. At the moment, the highest bioproduction of isoprene is achieved in *Escherichia coli*, while there is still a long way to go to satisfy the industry-scale needs due to the low yield and high cultivation cost.

The major bottlenecks of isoprene yield are limited identification of isoprene synthase (*IspS*) genes available and low catalytic activities of IspS. Sustainable isoprene production using solar energy and CO_2 by eukaryotic microalgae is an ideal way to reduce the cultivation cost. However, there has been no heterologous isoprene synthesis achieved in eukaryotic microalgae so far. Thus, our project aims to identification of novel *IspS* genes from Arecaceae family, functional characterization of *IspS* and heterologous isoprene production in microalgae.

In this study, three novel *IspS* genes were identified from palm species including *Chamaerops humilis*, *Sabal minor* and *Copernicia prunifera*. The expressions of IspS recombinant proteins in *E. coli* were not successful as they were not expressed at all or mostly precipitated as inclusion bodies.

To compare the relative abilities of isoprene synthesis and explore the physiological functions of isoprene emission, the *IspS* genes were over-expressed in the model plant of *Arabidopsis thaliana*. The isoprene emission screening showed that *ChumIspS* and *CpruIspS* transgenic Arabidopsis had relatively higher isoprene emissions than those of *SminIspS* transgenic Arabidopsis. Since limited information is available on the responses of isoprene emitter to abiotic stresses except for heat and oxidative stresses, *ChumIspS* or *CpruIspS* transgenic lines were used to study the physiological responses under salinity, exogenous ABA and drought treatment.

Under salinity stress, *ChumIspS* or *CpruIspS* transgenic lines had similar seedlings growth as wild type. Under ABA treatment, *ChumIspS* had similar seeds germination and seedlings growth as wild type. These results indicated that isoprene was not involved in mediating salinity or ABA responses during seeds germination and post-germination stages.

Under drought stress, *CpruIspS* transgenic lines showed positive morphological and physiological responses throughout the whole plant life cycle in terms of germination rate, green cotyledon formation rate, fresh weight, chlorophyll content, water loss rate and survival rate. The relative expression level of several stress-responsive genes indicated that isoprene mediated drought responses in a tissue-specific manner and probably through ABA-dependent and ABA-independent pathways.

The expression of *IspS* did not result in obvious isoprene emission in the model microalgae of *Nannochloropsis gaditana*, which might be related to the relatively low-level expression or the unknown metabolic limitation. To further explore the potential of *Nannochloropsis* for isoprene production, efficient expression system and transformation methods need to be further explored, as well as a further understanding of metabolic processes and regulatory networks within *Nannochloropsis*.

1 Introduction

1.1 Isoprene

1.1.1 Properties and natural emission of isoprene

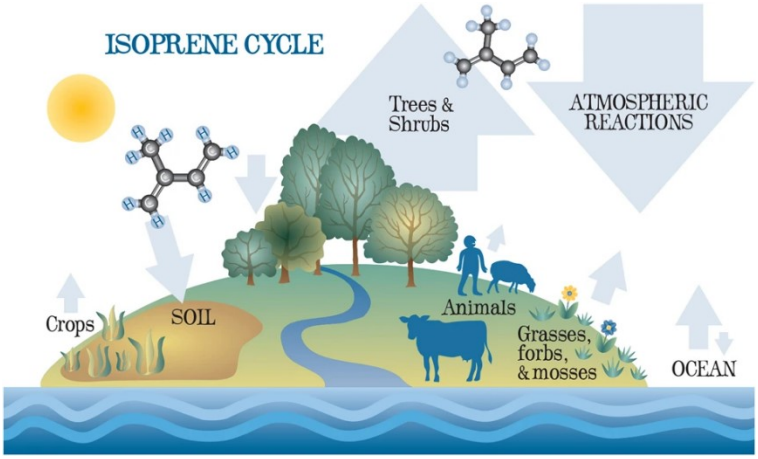


Fig. 1.1 isoprene cycle in the Earth's atmosphere. Arrow thicknesses is related to the estimated amount of isoprene. (Mcgenity et al., 2018)

Isoprene (2-methyl-1,3-butadiene) is a five-carbon compound with a conjugated double bond (Mcgenity et al., 2018). Pure isoprene is colorless and odorless liquid at room temperature with a boiling point of 34°C, thus isoprene is highly volatile (Ye et al., 2017). As the smallest terpene unit, the molecular structure of isoprene was completely determined in 1897 (Hillier et al., 2019), and the identification of isoprene emission in plants was first reported in 1957 (Sanadze 1957). Since then, dramatic progress has been made on the functional characterization of isoprene in plants.

As the dominant biogenic volatile organic compounds (BVOCs), the global isoprene emission was estimated about 600 Tg per year (Guenther et al., 2006), approaching that of methane (Lim et al., 2005). The isoprene emission was identified in some bacteria, fungi, algae and animals (including humans) (Guenther et al., 2006; Mcgenity, et al., 2018), while the majority (about 90%) of isoprene is emitted by the terrestrial systems especially the fast-growing trees such as poplars, oaks and reeds (Gibson et al., 2020; Exton et al., 2010; Dani et al., 2014; Loreto et al.,, 2015) (Fig. 1.1).

Among land plants, almost all mosses that have been examined could emit

isoprene(Hanson et al., 1999). In gymnosperms, all species in the sampled *Ephedraceae* and many species in the genera *Picea abies* produce isoprene (Sharkey et al., 2013). Among angiosperms, isoprene emission has been found mainly in eudicots including many rosids and some asterids. While in monocots, only a small number of species from Cyclanthaceae, Arecaceae and Poaceae are reported to emit isoprene (Ahrar et al., 2015).

1.1.2 Atmospheric effects of isoprene

Isoprene is vulnerable to nitrate (NO_3), hydroxyl radical (OH), ozone (O_3) and halogen radical attack, triggering thousands of subsequent reactions and hundreds of intermediates (Fan et al., 2004; Sharkey et al., 2001; Pacifico et al., 2009). Therefore, the high reactivity of isoprene and the resultant long-lived oxidation products make isoprene important for atmospheric composition, greenhouse effect, acid production and human health in regional and global wide (Fan et al., 2004; Bates et al., 2019; Vasquez et al., 2020).

In polluted environments with high concentration of nitrogen oxides (NO_x), the hydroperoxy radical (HO_2) generated from the oxidation of isoprene by OH could react with NO, resulting in a net production of O_3 and OH recycling (Crombie et al., 2015). The situation is more complicated in unpolluted environments with low concentration of NO_x. In that case, isoprene could react directly with O_3, resulting in the depletion of O_3. While isoprene could also react with OH, resulting in a reduced conversion rate of NO_2 to nitric acid (HNO_3), prompting the production of O_3 (Pacifico et al., 2009).

In addition, isoprene is also a precursor for secondary organic aerosol (SOA), which has an important impact on air quality and climate change (Hantson et al., 2017; Achakulwisut et al., 2015; Henze et al., 2006). Therefore, it is important to understand the factors that affect isoprene emission and its emission pattern in the future.

1.1.3 Isoprene applications

Isoprene is highly reactive and easy to polymerize due to the conjugated double bonds (Pollastri et al., 2021). Polyisoprene is the first elastic polymer to be commercially produced and has been widely used in industry (Kind et al., 2012). The yearly industrial production of isoprene is about 1 million tons, over 95% of which is used to produce

cis-1,4-polyisoprene, the main components of natural rubber (Kim et al., 2016). The natural rubber primarily extracted from the latex of the *Hevea brasiliensis* tree is widely used in tires and surgical gloves due to its high elasticity (Sansatsadeekul et al., 2011; Borges et al., 2017). In 2020, the total global production of natural and synthetic rubber was approximately 27 million metric tons, of which synthetic rubber was accounted for 52% (Gerez et al., 2022). The synthetic polyisoprene alleviates the problems of natural rubber supply, limited land resources and sustainable agricultural development (Chiarelli et al., 2020).

Different polymerization modes of isoprene molecules and additives lead polymers to have specific physical and mechanical properties (Worch et al., 2019). Trans-1,4-polyisoprene (TPI) obtained from natural plants such as *Eucommia ulmoides* could be used as biomedical material due to its high deformation recovery degree and accuracy (Zhao et al., 2022a; Zhang et al., 2021b). Besides, the high content of 3,4-polyisoprene could improve the water resistance and hermeticity of the synthetic rubber (Chen et al., 2013; Basalova et al., 2021). In addition to its major use for synthetic rubber, isoprene is also widely used in the production of adhesives, elastomers, medicines, pesticides, as well as a fuel additive (Zhao et al., 2011; Ye et al., 2016).

1.1.4 Industrial isoprene production

Although the yearly isoprene emission from the plants is around 500 million tons of carbon, which would be more than enough to satisfy the industrial needs if they are used for tires production, it is not technically feasible to collect isoprene due to its high volatility (Whited et al., 2010).

At present, the industrial production of isoprene is mainly separated as a by-product from the C5 cracking fractions during the pyrolysis of hydrocarbons into ethylene (Ye et al., 2016). It can be achieved by two specific methods and the first one is through extractive distillation. The isoprene concentration in the C5 stream is usually 15-20% (Fedotov et al., 2019), and the yearly productivity of isoprene through this method is about 30 thousand tons (Morais et al., 2015). The second one is by dehydrogenation of isopentane and isoamylenes (methyl butene), which is energy-intensive and the conversion of methyl butene is considerably reduced with the presence of CO_2 (Safin et al., 2012). As a by-product of ethylene, isoprene yield is pretty low, equivalent to 2%–5% of ethylene yield (Valenzuela et al., 2008).

Four primary convergent synthetic routes for commercial isoprene production include: 1) liquid-phase condensation of isobutylene with formaldehyde followed by second-vapor-phase decomposition of 4,4-dimethyldioxane-1,3 (DMD) into isoprene (Sushkevich et al., 2012), 2) Dimerization of propylene and subsequent demethanation (designed by Goodyear company) (Lopez-Arenas et al., 2019), 3) condensation of acetylene and acetone, 4) dismutation of isobutene and 2-butene followed by dehydrogenation (Morais et al., 2015).

1.2 Isoprene biosynthesis

1.2.1 Isoprene biosynthetic pathways

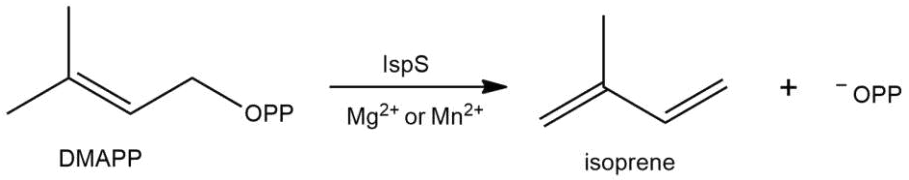


Fig. 1.2 Biocatalytic reaction of DMAPP to isoprene catalyzed by IspS.

Isoprene is synthesized by isoprene synthase (IspS) which catalyzes the elimination of the diphosphate group from dimethylallyl diphosphate (DMAPP) using Mg^{2+} or Mn^{2+} as the cofactor (Zhao et al., 2011; Li et al., 2019b) (Fig. 1.2). The cleavage of the diphosphate group generates a resonance-stabilized allylic carbocation, leading to the formation of a carbon-carbon bond and finally resulting in the isoprene unit (Gao et al., 2012).

Based on the isoprene units, terpenoids of different lengths and structures are obtained through carbon chain extension and structural modifications such as cyclization, hydroxylation, methylation and isomerization (Jin et al., 2020), which makes DMAPP and its isopentenyl pyrophosphates (IPP) the universal building blocks for more than 60000 terpenoids (Guan et al., 2015; Zhou et al., 2013).

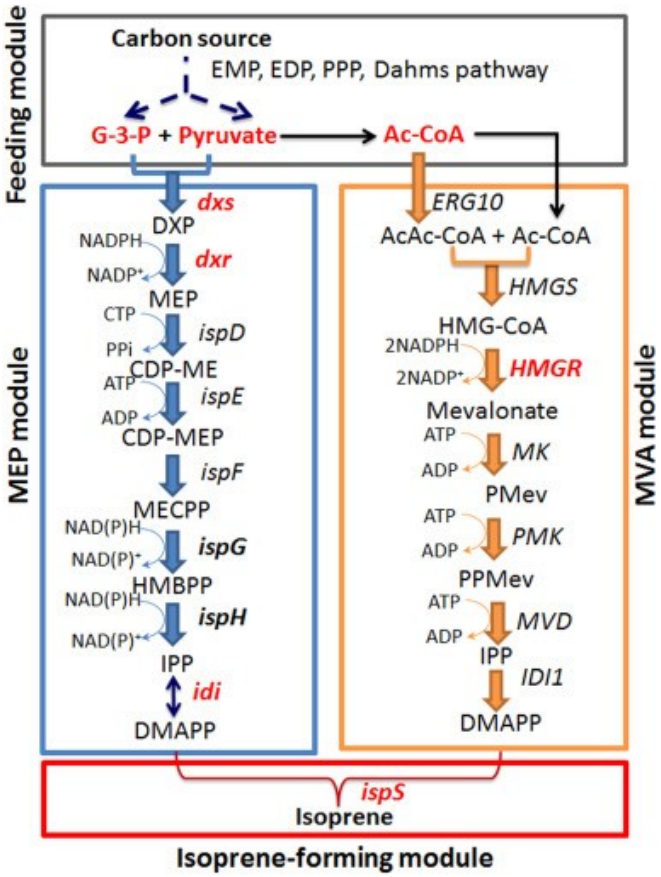


Fig. 1.3 Isoprene biosynthesis pathways. (Ye et al., 2016)

DMAPP is synthesized via mevalonate (MVA) pathway or methylerythritol phosphate (MEP) pathway in vivo (Li et al., 2018). The MVA pathway has been studied for more than 40 years and it includes six anabolic steps converting acetyl-CoA to IPP (Bergman et al., 2019; Boucher et al., 2000). MVA functions in most eukaryotes and archaebacteria and some gram-positive bacteria (e.g., *Streptomyces* and *Streptococcus*) (Vranová et al 2013; Niinemets et al., 2013b; Kaneda et al., 2001; Yoon et al., 2009).

In the mid-1990s, the MEP pathway was found in *Bacillus subtilis* (Guan et al., 2015). MEP pathway starts with the pyruvate and D-glyceraldehyde 3-phosphate (G3P) and involves eight enzymatic steps to generate DMAPP and IPP (Isar et al., 2022). In the final step, the isomerization reaction between DMAPP and IPP is catalyzed by isopentenyl diphosphate isomerase (IDI) (Wang et al., 2017a). Unlike MVA pathway, the IPP and DMAPP are generated with a ratio of 5:1/3:1 during the reductive dehydration of 4-hydroxy-3-methyl-butenyl 1-diphosphate (HMBPP) in the MEP pathway (Singh et al., 2007). So the IDI is not necessary for the MEP pathway in some organisms (Zhao et al., 2016). MEP pathway exists in apicomplexan protozoa (e.g.,

malaria parasites) and the majority of eubacteria (e.g., *Escherichia coli*) (Phillips et al., 2008) (Fig. 1.3).

Especially, the MVA and MEP pathways co-exist in plants and most microalgae, while functioning in different cell compartments (Rodríguez-Concepción et al., 2002; Mao et al., 2020; Huang et al., 2021a). The MVA pathway functions in the cytosol, offering precursors for sesquiterpenes, triterpenes, sterols and ubiquinone (Rodríguez-Concepción et al., 2015). On the contrary, the MEP operates in the plastid, providing building blocks for hemiterpene, monoterpenes, diterpenes, carotenoids, and the side chain of plastoquinone (Dudareva et al., 2005; Skorupinska-Tudek et al., 2008). Some unicellular algae such as the green alga *Chlamydomonas*, and the stramenoplie *Nannochloropsis* sp. have lost MVA pathway in the evolutionary process and now only use MEP pathway to provide isoprenoids (Davies et al., 2015).

Besides, the MVA and MEP pathways also differ significantly in terms of stoichiometry, energy and cofactor consumption (Steinbüchel 2003). To produce one molecule of IPP/DMAPP, 1.5 molecule of glucose are consumed and four molecules of NAD(P)H are produced in the MVA pathway; while one molecule of glucose, three molecules of ATP and two molecules of NAD(P)H are consumed in the MEP pathway (Li et al., 2020a). Thus, the MVA pathway is more energy-efficient, while MEP pathway has a higher theoretical mass yield on glucose (30.2% vs. 25.2%) (Lv et al., 2016).

In general, the MVA and MEP pathways operate independently, while cross-talk is found in the production of isoprenoid secondary metabolites (especially monoterpenes and sesquiterpenes) (Opitz et al., 2014; Hemmerlin et al., 2012).

It is still under debate why plants utilize separated MVA and MEP pathways to provide precursors for different types of isoprenoids (Bergman et al., 2019). The reasons might be that the two separated pathways are beneficial for optimizing the isoprenoid synthesis based on precursors and ATP availability or rapid production of specific end products in response to different environmental selective pressures (Vranová et al., 2013).

1.2.2 Biochemical properties of isoprene synthase

Based on the reported sequences of three fragments of isoprene synthase purified from aspen (*Populus tremuloides*) leaves (Silver et al., 1995), the first complete

functional isoprene synthase gene was isolated from poplar (*Populus alba* × *Populus tremula*) in 2001 (Miller et al., 2001). Since then, more and more Isoprene synthases have been reported from plants such as several poplar species (Sasaki et al., 2005; Schnitzler et al., 2005; Wiberley et al., 2007), kudzu (*Pueraria montana*) (Sharkey 2005) and oak (*Quercus petraea*) (Lehning et al., 1999). Among them, the exon regions of poplar genes share pretty high sequence identity (97-100%) (Lv et al., 2016). Although various microorganisms have been found to emit isoprene, and some of them, especially the *Bacillus subtilis*, can release a large amount of isoprene, similar to that of plants (based on cell weight) (Fall et al., 2000), there has been no isoprene synthase gene identified from the microorganisms so far (Li et al., 2018).

In plants, the isoprene synthase enzyme is localized in the chloroplast, which is consistent with its inclusion of a chloroplast transit peptide (Lv et al., 2013). The soluble isoforms of isoprene synthases have been found in all species examined, while the thylakoid-bound isoforms are found in most of these species, meaning that not all species have both two isoforms of the isoprene synthases (Vickers et al., 2010). The catalytic properties of soluble and thylakoid-bound isoforms of isoprene synthase from Willow (*Salix discolor* L.) were similar (Wildermuth et al.,1998). But it is still unclear whether the conversion between the two isoforms is possible and the potential benefits and effects (Kulshrestha et al., 2016).

Although around 20% of perennial vegetation has been found to emit isoprene, only a few isoprene synthase genes have been identified and characterized (Loreto et al., 2015; Li et al., 2017). The enzymatic characterization has been investigated in several isoprene synthases from plants such as *Populus alba* (Sasaki et al., 2005), *Ficus septica* (Oku et al., 2015) and *Ipomoea batatas* (Li et al., 2019b). At the moment, most characterized isoprene synthases seem to be monomers with the molecular mass of around 60-100 kDa (Sharkey et al., 2001; Lantz et al., 2015). But the crystal structure of PcISPS indicates the dimeric quaternary structure of isoprene synthase (Köksal et al., 2010).

In general, the enzymatic properties of the isoprene synthases from different plant species are similar. The optimal pH is between 7-10, indicating that the isoprene synthases prefer alkaline conditions (Wildermuth et al.,1998; Schnitzler et al., 1996). The limited data shows that the optimal temperature for isoprene synthase ranges from 35 to 40°C (Lantz et al., 2015), with an exception of isoprene synthase from *Quercus robur* L., whose enzymatic activity reached its highest at 50°C (Lehning et al., 1999).

The relatively high K_m values (millimolar range) indicate that the isoprene synthases don't have high affinity for the DMAPP, as K_m equals the concentration of the substrate at which the reaction rate reaches half the maximum (Eisenthal et al., 2007; Wilson et al., 2018). The catalytic rate constant k_{cat} is relatively low, ranging from 0.011 to 5 s^{-1} (Li et al., 2018; Zurbriggen et al., 2012). Thus, from the bioproduction of isoprene, the high K_m means high concentration of DMAPP required and low k_{cat} means low isoprene production per unit time, which is not an advantage for large-scale production(Chaves et al., 2017).

1.2.3 Genetic and crystal structure of isoprene synthase

The multiple sequence alignment indicates that the isoprene synthase genes contain two conserved metal ion-binding motifs ("aspartate-rich" motif DDXXD and the "NSE/DTE" motif NDXXSXXXE) (Vickers et al., 2010; Yeom et al., 2018). The DDXXD and NDXXSXXXE motifs are also characteristic sequences of all monoterpene synthases and other plant terpene synthases (Huang et al., 2021b; Chen et al., 2011). These two motifs flank the entrance of the active site cavity and are involved in binding divalent metal ions to initiate the reaction (Aaron et al., 2010; Christianson 2006). For example, DDXXD motif in the active center of class I terpene cyclase could bind to Mg^{2+} and attack the pyrophosphate group of the substrate through the ionization of metal cations, thereby promoting the release of the pyrophosphate group (Huang et al., 2021b).

Besides, two conserved Phe residues F338 and F485 (number based on the IspS from *Populus alba*) in the active site are thought to be unique to isoprene synthases in angiosperms, which can be used to distinguish isoprene synthases from other terpene synthases (Sharkey et al., 2013). It is demonstrated that both F338 and F485 make van der Waals contacts with the substrate and are responsible for reducing the size of substrate binding pocket to ensure the substrate specificity for DMAPP (Sharkey et al., 2013). Especially, compared with F485 residue, the F338 residue has a greater impact on isoprene emission (Li et al., 2020b). Because F338 is strictly conserved in all known isoprene synthases and the neofunctionalization to monoterpene synthase was only observed in F310 mutant (F338 in *P. alba*) rather than F457 mutant (F485 in *P. alba*) by site-directed mutagenesis of IspS from *Arundo donax* (Li et al., 2017). The three-dimensional model analysis of AdoIspS WT and F310A suggests that the effect of the F310 residue on substrate specificity might be related to the significant reduction in

cavity size caused by the protruding aromatic ring of F310 (Li et al., 2017).

F338, F485, together with another two residues S446 (serine) and N505 (asparagine) (number based on the IspS from *Populus alba*) were evaluated as the diagnostic tetrad (FSFN) of isoprene synthase previously (Ilmén et al., 2015). The S446 residue is in the middle of the triple serine at a bend of helix G and could provide some surface for the active site (Ilmén et al., 2015; Sharkey et al., 2013). But some variations at residues S446 and N505 (Oku et al., 2015; Ilmén et al., 2015) were indicated from the recently identified isoprene synthase genes.

Table 1.1 IspS diagnostic tetrads from different families in angiosperms (Li et al., 2020b)

Diagnostic Tetrad	Family	Type
FVFK	Casuarinaceae	Dicotyledons
FVFN	Convolvulaceae, Elaeocarpaceae, Fagaceae	Dicotyledons
FSFN	Fabaceae, Moraceae, Myrtaceae, Salicaceae	Dicotyledons
FSFS	Anacardiaceae, Poaceae	Dicotyledons, monocotyledons
FVFT	Arecaceae	Monocotyledons

Briefly, in the angiosperms, the corresponding diagnostic tetrad present in monocots include FSFS and FVFT, while the ones in dicots includes FVFK, FVFN, FSFN and FSFS (Li et al., 2020b). Among them, FSFS is present in both monocots and dicots (Table 1.1). By comparison, it can be seen that the second residue of the tetrads both in monocots and dicots could be either V (valine) or S (serine), while the fourth residue could be T (threonine) or S (serine) in monocots and K (lysine), N (asparagine), S (serine) in dicots. Therefore, the fourth residue of the tetrad seems to be more flexible. The structure modeling indicates that the first three residues of IspS tetrads are close to each other in the active pocket site, while the fourth residue located as the last amino acid of the H-α1 loop is away from the other three residues (Li et al., 2020b). Besides, the site-directed mutagenesis results reveal that the fourth residue might have a significant effect on enzyme activity (Li et al., 2020b).

Till now, the only known crystal structure of isoprene synthase was reported in 2010 from gray poplar hybrid *Populus* × *canescens* (Köksal et al., 2010). This PcISPS structure is determined as a complex with Mg^{2+} and the unreactive substrate analogue dimethylallyl-S-thiolodiphosphate (DMASPP) and the active-site pocket of PcISPS is hydrophobic, but shallower than that of monoterpene cyclase, consistent with its

specificity for the smaller substrate DMAPP (Gao et al., 2012).

The PcISPS folds into two domains: the non-catalytic N-terminal domain adopts an α-barrel class II terpenoid synthase fold, and the catalytic C-terminal domain adopts the α-helical class I terpenoid synthase fold (Köksal et al., 2010). Especially, structural studies showed that this class I terpenoid synthase fold was also found in the enzymes that catalyze four coupling steps (chain elongation, branching, cyclopropanation and cyclobutanation) during irregular isoprenoid biosynthesis (Thulasiram et al., 2007), indicating that this class I terpenoid synthase fold belongs to the evolutionary template for the enzymes involved in isoprenoid biosynthesis (Köksal et al., 2010). Besides, the PcIspS structure analysis suggests that the synthesis of isoprene is via a *syn*-periplanar elimination mechanism in which the released diphosphate anion could serve as the catalytic base (Köksal et al., 2010).

1.2.4 Evolution of isoprene emission in angiosperms

Many plant species emit isoprene in the plant kingdom (Alves et al., 2014). In a survey of mosses and ferns, isoprene emission was pretty common as it was found in 94% of mosses and 50% of ferns sampled (Hanson et al., 1999). In gymnosperm, isoprene emission was detected in all species of the Ephedraceae examined and many species in the genera *Picea abies* (Sharkey et al., 2013).

In angiosperms, isoprene-emitting species are dispersed across the phylogenetic tree (Monson et al., 2013). Briefly, isoprene emission is mainly found in the eudicots especially the rosids and also detected in some grasses and palms (monocots) (Sharkey et al., 2013). It is considered that isoprene emission ability has arose independently in angiosperms and gymnosperms (Sharkey et al., 2013), as the identified isoprene synthases from angiosperms belonged to the Tps-b clade of terpene synthases, which contain 6 introns and 7 exons, whereas isoprene synthases from gymnosperms contain 9 introns and 10 exons (Li et al., 2017).

However, the possible explanations for the sparse phylogeny of isoprene emission in angiosperms are still under debate. One opinion is that the isoprene synthase occurred once in the early stage of irradiation and then underwent multiple losses (Sharkey et al., 2013). This observation was based on the tradeoffs between metabolic cost and environmental challenges (such as paleoclimatic fluctuations of atmospheric CO_2) since isoprene emission may exceed 15% of photosynthesis when photosynthesis is

inhibited (Sharkey et al., 2013). The other opinion proposes that isoprene synthase genes may experience multiple gains and losses at the family or even genus level since the independent gains of isoprene emission trait are much higher. It is based on the assumption that a small number of amino acid mutations can lead to the conversion between isoprene synthase and other terpene synthases (Li et al., 2017). Understanding the overall evolutionary trajectory of the isoprene synthase genes in angiosperms requires information of the actual number of mutations for conversion of other terpene synthases to isoprene synthase and the true ancestral enzymes (Li et al., 2017).

1.2.5 Palms are important isoprene emitters

In angiosperms, isoprene emission from monocots is much less studied than the one in dicots (Ahrar et al., 2015), and so far, isoprene emission has been found mainly in Arecaceae and Poaceae family among the monocots (Ahrar et al., 2015; Li et al., 2017).

The Arecaceae, also known as the palms, is a family with 181 genera and 2600 species, mainly distributed in tropical and subtropical regions (Fehr et al., 2020; Baker et al., 2016). Some of these species are widely cultivated because of their economic value. For example, as an important oil crop, the oil palms (*Elaeis guineensis*) are planted on 28.7 million hectares worldwide, becoming a major contributor to the economy in many countries such as Indonesia and Malaysia (Nambiappan et al., 2018; Chang et al., 2022); coconut palms (*Cocos nucifera*) are important food and economic crops, which can provide raw materials for food, cosmetics, construction and pharmaceutical fields, with a global planting area of more than 12 million hectares (Xiao et al., 2017).

The isoprene emission was estimated to be present in 20%-80% of the species in the palm family, with abundant isoprene emitters such as *Elaeis guineensis*, *Calamus gracilis* and *Salacca secanda*, probably in response to the tropical environments with high irradiance and temperature (Geron et al., 2006; Wiedinmyer et al., 2004; Jones et al., 2011).

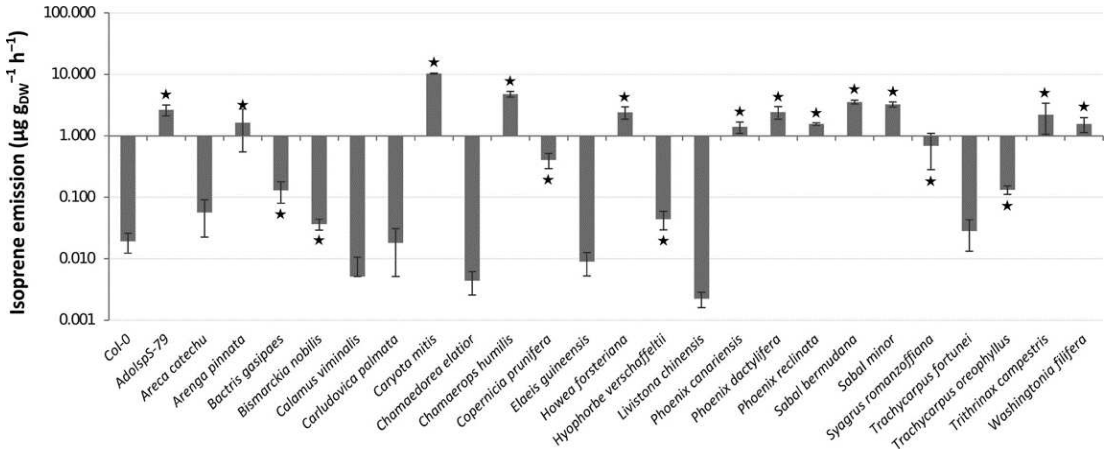


Fig.1.4 Isoprene emission level expressed in logarithmic form among different species of Arecaceae family. (Li et al., 2020b)

It was suggested from a recent survey that isoprene emission was a common trait in the palm family by investigating 23 species in 18 genera (Li et al., 2020b) (Fig. 1.4). In the same study, six isoprene synthase genes were isolated and identified from six different genera. They have basic features of isoprene synthases: two phenylalanine marker residues and heavy-metal binding motifs (DXXD and DTE/NSE) (Sharkey et al., 2013; Li et al., 2017).

However, the diagnostic tetrad, FVFT, of these six isoprene synthases is different from those identified in dicots species and another monocot species, *A. donax* (Li et al., 2020b). Besides, the FVFT tetrad only exists in the isoprene synthases from Arecaceae by analyzing 257 reviewed TPS proteins and 8,000 unreviewed TPS proteins in UniProt (Li et al., 2020b). The site-directed mutagenesis and structure modeling results showed that the fourth threonine residue of the tetrad seemed to be critical for enzyme activity (Li et al., 2020b). Thus, this threonine residue could be used as a mutation site to enhance enzyme activity and therefore isoprene yield since the low catalytic activities of identified isoprene synthases is one of the bottleneck of isoprene synthesis (Gao et al., 2016; Li et al., 2018). Furthermore, the widespread capacity of isoprene emission in palm family implies the possibility of identifying novel and highly catalytic isoprene synthases.

1.2.6 Biochemical factors controlling isoprene emission

It is generally considered that DMAPP pool size and the activity of isoprene synthase are two important factors controlling foliar isoprene emission in plants (Copolovici, et al., 2009). DMAPP and its isomer IPP are synthesized through MVA and MEP

pathways (Zhao et al., 2022b). The isoprene synthases are chloroplast-located, so the DMAPP generated from MEP pathway is important for isoprene synthesis (Kaidala et al., 2016). Since there is crosstalk between the MVA and MEP pathways, some carbons used for isoprene synthesis may come from the cytosol (Liao et al., 2016). However, lacking early MEP pathway genes was reported to prevent Arabidopsis from developing normally, suggesting that the crosstalk between MVA and MEP could be a pretty limited (Estevez et al., 2000).

Although the DMAPP concentration varies widely among plant species regardless of natural isoprene emitters and non-emitters, in general, isoprene emitters contain higher concentration of DMAPP (Rosenstiel et al., 2002). The DMAPP pool was estimated to be as high as 8,000 to 13,000 nmol m^{-2} in aspen leaves by *in vitro* analysis. In contrast, only 10% to 15% of the total leaf DMAPP pool is related to isoprene formation, which is consistent with the fosmidomycin inhibition results (Rasulov et al., 2009b). Because DMAPP is also used in synthesizing of various isoprenoids, primarily for the higher isoprenoids such as carotenoids (including photosynthetic pigments) and gibberellic acid, which are essential for the growth and survival of plants (Owen et al., 2005).

DMAPP productions in isoprene emitters are light-dependent. Based on acid-catalyzed hydrolysis to determine DMAPP, it was found that DMAPP productions in oak leaves were regulated by circadian clock, and leaf DMAPP content was positively correlated with net assimilation and isoprene emission rates, indicating that the cellular DMAPP concentration might directly regulate the isoprene emission under light (Nicolas et al., 2002). A similar phenomenon was reported by Magel et al., where the light–dark transition of poplar leaves caused isoprene emission rates to drop to zero within a few minutes, accompanied by a rapid drop in foliar DMAPP content (Magel et al., 2006).

To understand the dependence of isoprene emission on DMAPP availability more in depth, Rasulov et al. determined the in vivo DMAPP pool and fitted the kinetic curve of isoprene synthase relative to in vivo DMAPP pool (Rasulov et al., 2009b). The V_{max} and K_m were derived as 110 nmol m^{-2} s^{-1} and 0.28 mM, respectively. The K_m was quite different from the previously reported one (8 mM) derived from the in vitro characterization of poplar-derived isoprene synthase, possibly due to the loss of certain cofactors or enzyme inactivation during extraction and purification (Rasulov et al., 2009b; Sanadze 2004). Considering that the V_{max} of isoprene synthase was four to five times higher than the actual rate of isoprene production, it was hypothesized that

isoprene emission was dependent on the DMAPP availability rather than enzyme activity under short-term light, CO_2 or O_2 changes (Rasulov et al., 2009b).

The in vivo temperature dependence of isoprene emission from hybrid aspen (*Populus tremula* × *Populus tremuloides*) suggested the combined effect of DMAPP limitations and isoprene synthase activity (Rasulov et al., 2010). It was reported that at a temperature below the threshold for thermal damage, the DMAPP pool size was the potential cause to determine isoprene emission rate (Rasulov et al., 2010). While the optimum temperature for isoprene emission (39.3°C) was higher than the temperature (35.3°C) at which the steady-state DMAPP pool was reached the maximum, indicating that the exponential temperature response of isoprene synthase kinetics dominates isoprene emission relative to DMAPP pool reduction over a narrow temperature range above 35°C (Rasulov et al., 2010).

Another study suggested that the early season isoprene emission depending on growth conditions such as temperature fluctuation was closely related to the transcriptional control of isoprene synthase in kudzu (Wiberley et al., 2005). It was reported that the isoprene emission and isoprene synthase expression was much faster in the kudzu leaves grown at high temperature (30°C) than those grown at low temperature (20°C) (Wiberley et al., 2005). The importance of isoprene synthase for isoprene expression was also reflected in the phenomenon that the down-regulation of isoprene synthase by RNAi resulted in stable non-isoprene emitting poplars throughout the growing season despite high DMAPP concentration (Behnke et al., 2010a).

Promoter sequence analysis of isoprene synthase from *Populus* × *canescens* identified a number of putative heat shock, circadian elements and light-responsive boxes, demonstrating the potential correlation of isoprene emission with temperature, photo period and light intensity (Loivamäki et al., 2007). The circadian regulation of isoprene emission and isoprene synthase transcript level has been demonstrated in some poplar trees including *Populus* × *canescens* and *Populus trichocarpa* (Loivamäki et al., 2007; Wiberley et al., 2009). By fusing the enhanced green fluorescent protein (E-GFP) and β-glucuronidase (GUS) reporter genes to the *PcISPS* gene promoter, the direct light induction of the *PcISPS* gene in leaves was observed (Cinege et al., 2009).

As for the isoprene emission on plant hormones responses, it has been found that isoprene emission was closely correlated with isoprene synthase gene transcription

and expression levels during jasmonic acid (JA) treatment (Parveen et al., 2019). It was further supported by exploring the relationships among plant hormone concentrations, isoprene synthase gene expression and hormone signaling transcription factors (Iqbal et al., 2022). These observations suggest that plant hormones are also involved in the regulation of isoprene emission.

1.3 The impact of environmental conditions on plant-derived isoprene emission

1.3.1 Environmental factors affecting isoprene emission

Isoprene emission rates from plants are affected by various environmental factors including light, temperature, ambient CO_2 concentration and mechanical damage (Lantz et al., 2019b).

1.3.1.1 Light

The isoprene emission is light-dependent, and the isoprene release rate in light is nearly two orders of magnitude higher than that in darkness (Sanadze 2004). In general, the light dependence of isoprene emission is similar to photosynthesis, but isoprene emission saturates at higher light intensity than photosynthesis (Arena et al., 2016). Regarding the light quality effect, isoprene emission is consistent with photosynthesis, being the highest in the red light spectral range, and significantly suppressed by blue light, especially at high light intensities (Pallozzi et al., 2013; Sanadze 2004). The light-dependent phenomenon was speculated to be due to the availability of photochemical products generated from photosynthesis (NADPH and ATP), as the isoprene emission ceased when leaves were fed the DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) to inhibit the photosynthetic electron transport (Garcia et al., 2019).

Under darkness treatment, the synthesis of DMAPP ceased almost immediately, while the release of isoprene in poplars and oaks exhibited a "post-illumination burst" due to the stored DMAPP used for the isoprene synthesis (Rasulov et al., 2011). Whereas the synthesis rate dropped to almost zero within a few minutes due to limited reducing power (Lantz et al., 2019b). Then it was followed by a second burst of isoprene

emission with the source of the MEP pathway metabolites, especially the methylerythritol cyclodiphosphate (MEcDP) (Li et al., 2013). While it was suggested that the "post-illumination burst" were species-specific since the phenomenon was not observed in the Arabidopsis transformed with isoprene synthase from *Eucalyptus globulu*s (Zuo et al., 2019).

1.3.1.2 Temperature

In vivo, isoprene emission is temperature-dependent, and within a certain temperature range, isoprene emission increases with rising leaves temperature (Lantz, et al., 2019b). The isoprene emission rate at a temperature below 20°C was very low, 5 to 20 times less than under optimal conditions (Wiberley et al., 2005; Rasulov et al., 2010); at steady state, isoprene emission rate increased exponentially with temperature up to 40°C-42°C, followed by a decrease in isoprene emission rate with increasing temperature (Singsaas et al., 2000; Sharkey et al., 1996). When the temperature was raised to 45°C-50°C, the isoprene emission rate dropped to 0 due to irreversible thermal damage (Zimmer et al., 2000; Rasulov et al., 2010). However, the inhibition of isoprene emission rate by high temperature was reversible between 40°C and 45°C, i.e., isoprene emission rate increased when the leaves were returned to lower temperature (Rasulov et al., 2010).

It has been reported that at steady state, the temperature response of isoprene emission is affected by changes in the size of the DMAPP pool and the rate constant of isoprene synthase (Lantz et al., 2019b). In hybrid aspen, DMAPP pool size was reported to reach a maximum at 35°C, and the increased isoprene emission rate was associated with increased DMAPP pool size and enhanced isoprene synthase activity up to 35°C (Rasulov et al., 2010). At temperatures above 35°C, the DMAPP pool size decreased, but the effect of higher enzyme activity by increasing temperature outweighed the decrease in substrate availability, causing the isoprene emission rate continued to increase with increasing temperature, albeit at a slower rate (Rasulov et al., 2010). However, between 40°C and 45°C, the isoprene emission rate was mainly affected by the DMAPP pool size, which was deduced from the unchanged rate constant of isoprene synthase under transient conditions (Rasulov et al., 2010).

Unlike steady state, under transient conditions, isoprene emission rate may increase to a maximum even at a temperature as high as 45°C (Singsaas et al., 1999). Such difference in the temperature response of isoprene emission rate under transient and

steady states may be affected by different temperature responses of other MEP pathway reactions (Rasulov et al., 2010).

The optimal temperatures of isoprene emission rate under both steady and transient states were 10°C to 20°C higher than that of photosynthesis, so the intermediates provided by photosynthesis were insufficient to maintain isoprene synthesis, and the photorespiration was indicated as an alternative carbon source for isoprene emission (Rasulov et al., 2010; Jardine et al., 2014). In this case, the reason why plants still maintain high isoprene emission rates may be related to protection from oxidative stress (Jardine et al., 2013) and activation of stress-related signaling processes (Cappellin et al., 2019).

1.3.1.3 CO_2

In general, isoprene emission from plants decreases as CO_2 concentration increases (Rosenstiel et al., 2003; Feng et al., 2019; Wilkinson et al., 2009; Way et al., 2013), although some studies reported that the increased CO_2 content didn't reduce the isoprene emission (Rapparini et al., 2004; Calfapietra et al., 2007). The effects of CO_2 on isoprene emission include shoer-term response and long-term effects, and affecting canopy development, thus modifying isoprene emission (Rasulov et al., 2016; Sun et al., 2013a).

Based on the assumption that pyruvate production in the chloroplast was dependent on the import of phosphoenolpyruvate (PEP) from the cytosol, there were two widely discussed hypotheses to explain the inhibition of isoprene emission by the short-term high concentration of CO_2 (Sun et al., 2013b). The first one was the PEP carboxylase competition theory. The enhanced activity of PEP carboxylase at high CO_2 concentration resulted in reduced PEP transport from the cytosol to the chloroplast and limited pyruvate production, thereby reducing isoprene synthesis (Wilkinson et al., 2009). However, a recent study using malate feeding and PEP carboxylase inhibitors rejected this hypothesis (Rasulov et al., 2018).

Another hypothesis was related to the energy status of the chloroplast (Morfopoulos et al., 2014). Under high CO_2 conditions, triose phosphate utilization (TPU) limitation affected photosynthesis, resulting in reduced ATP and NADPH availability, in turn, reduced production of the DMAPP (Rasulov et al., 2009a; McClain et al., 2019). Although CO_2 sensitivity of isoprene emission has been found to be TPU-related in

some species, it has also been shown that TPU limitation was not necessary for CO_2 inhibition (Lantz et al., 2019a). For example, strong inhibition of isoprene by CO_2 was also observed under conditions with constant photochemical electron transport and without TPU limitation in *Populus alba* (Lantz et al., 2019a).

Lantz et al. proposed that the CO_2 sensitivity of isoprene emission might be related to changes in the MEP pathway resulting from direct CO_2 sensing in the stomata or indirect sensing through calcium release from the apoplast (Lantz et al., 2019a). But the theory is not yet clarified. The calcium effects related to the stomatal responses have been observed in the isoprene responses to mechanical damage, probably because mesophyll cells have similar mechanisms as guard cells to sense CO_2 concentration and regulate the MEP pathway (Lantz et al., 2019b).

However, the long-term effects of high concentrations of CO_2 on isoprene emission are widely divergent. Some studies showed that elevated CO_2 caused a decreased isoprene emission in plants such as *Phragmites australis* (Scholefield et al., 2004), *Platanus orientalis* L. (Velikova et al., 2009), *Liquidambar styraciflua* L. and aspen (Monson et al., 2007), *Populus deltoides* and *Populus tremuloides* (Wilkinson et al., 2009).

While isoprene emission didn't decrease with elevated CO_2 in other studies. For example, in hybrid poplars, the analysis of isoprene emission integrated over the whole plant profile showed no difference in plants grown in ambient and elevated CO_2, while the maximum isoprene emission rate and isoprene emission per unit of leaf area decreased in elevated CO_2, accompanied by more leaf formation (Centritto et al., 2004). In hybrid aspen (*Populus tremuloides* Michx. x *P. tremula* L.), the elevated CO_2 had no significant effects on isoprene emission rate under moderately high light and 30°C (Sun et al., 2012). However, plants grown under elevated CO_2 concentration had a higher maximum isoprene emission rate than plants grown under ambient CO_2 concentration when the light and CO_2 concentration satisfied the greatest isoprene emission rate (Sun et al., 2012).

The reduced isoprene synthase activity and DMAPP pool size were reported to be related to decreased isoprene emission (Possell et al., 2011). Whereas, the biochemical and physiological causes of these complex phenomena in different species remain to be discovered.

1.3.1.4 Mechanical damage

In nature, plants are faced with mechanical damage caused by adverse weather such as rain, snow and wind, herbivores and pathogen invasion (Benikhlef et al., 2013). One of the responses of plants is to release volatile organic compounds (VOCs) (Loreto et al., 2014), which help to reduce the performance of herbivores (Ament et al., 2004), protect tissues from pathogens infection (Saunier et al., 2020), and induce defensive responses in non-injured tissues (Niinemets et al., 2013a).

The reduction of isoprene emission immediately after leaf damage was found in some studies (Portillo-Estrada et al., 2015; Copolovici et al., 2017). Besides, simultaneous reductions of net assimilation rate and constitutive isoprene emission were observed in herbivore-fed (Copolovici et al., 2017; Loreto et al., 1993) and fungal-infected leaves (Copolovici et al., 2014; Toome et al., 2010; Jiang et al., 2016). This may be related to limited DMAPP availability and untimely activation of alternative carbon sources (Copolovici et al., 2017; Rasulov et al., 2011).

The long-term response of isoprene emission after damage could differ from the immediate response (Visakorpi et al., 2018; Maja et al., 2014). Loreto et al. found a 10-minute burst of isoprene emission in damaged leaves of *Phragmites australis* about 5 minutes after the damage (Loreto et al., 2006). Isoprene release lasted for at least 30 minutes in the lateral leaflet-damaged velvet bean, although less than before damage (Loreto et al., 1993). Portillo-Estrada et al. found that isoprene emission rate and CO_2 assimilation rate decreased slightly after leaf wounding in *Populus tremula*, but both of them did not reach pre-stress levels even after several hours (Portillo-Estrada et al., 2015).

The reduction of constitutive isoprene release and photosynthetic activity may be related to electrical signals upon leaf damage (Sharkey et al., 2001; Gallé et al., 2013). It has been speculated that wounding can generate a variation potential (VP) which triggers membrane depolarization leading to inhibition of photosynthesis. The resulting reduced supply of the DMAPP pool coupled with the delayed activation of alternative carbon sources finally led to a reduction of isoprene emission (Portillo-Estrada et al., 2015).

On the contrary, Visakorpi et al. observed significantly higher isoprene emission rate of wounded leaves in oak trees over a period of time (Visakorpi et al., 2018). It was

speculated that increased isoprene emission might be stimulated by water stress induced by mechanical damage (Aldea et al., 2005; Sharkey et al., 1993). Brilli et al. found an enhanced isoprene emission from the cut leaves of the strong isoprene-emitter *Populus alba*, which lasted for one hour and was independent of photosynthesis (Brilli et al., 2011). The isoprene emission of cut leaves was recovered after a long darkening, indicating the alternative carbon sources used for isoprene emission (Brilli et al., 2011; Brilli et al., 2007). Additionally, the enhanced isoprene emission was speculated to be related to higher isoprene synthase activity and stimulated by higher leaf temperature resulting from progressive stomatal closure (Brilli et al., 2011). But it is still hard to explain the phenomenon from an ecological view.

1.3.2 Physiological functions of isoprene emission against abiotic stresses

In isoprene-emitting plant species such as poplars, the isoprene emission normally consumes 0.5-2% of the carbon fixed by photosynthesis, and when the plants are faced with adverse growing conditions such as high temperature, high light intensity and water stress, the proportion could be even higher than 15% (Sharkey et al., 2013; Guidolotti et al., 2011). Thus, isoprene could be important for plant growth in harsh conditions.

1.3.2.1 Sun fleck

Sun fleck is a short and intermittent period of high photon flux density (PFD) that can cause dramatical fluctuations in leaf temperature (Way et al., 2012; Vickers et al., 2009a). Many plants that thrived in sun fleck-prone environments could emit isoprene, while plants grown in long-term high temperature and light intensity produce little isoprene (Way et al., 2012; Porcar-Castell et al., 2012).

Due to the co-stimulation of high light intensity and leaf temperature, isoprene emission rapidly increased during the sun fleck and decreased at the end of sun fleck (Behnke et al., 2010b; Way et al., 2011). Isoprene emission could help to maintain photosynthesis under sun fleck. By the end of the combined treatment with sun fleck and salt, the photosynthetic performance of the RNAi-mediated non-isoprene emitting poplars was negatively affected as indicated by the slightly higher increase in non-photochemical quenching (NPQ) and a decrease in electron transport rate (ETR)

(Behnke et al., 2013). Besides, significant accumulation of H_2O_2 in vivo and associated down-regulation of phenolic biosynthesis under high temperature and high light intensities were observed in PcISPS-RNAi lines of *Populus* × *canescens*, which could interfere with the environmental stress resistance (Behnke et al., 2010a).

In addition, the concentration of CO_2 affected the protective effect of isoprene on photosynthesis under combined heat and light stresses to mimic sun fleck (Way et al., 2011). At high concentration of CO_2, the photosynthetic responses to sun fleck were similar between isoprene emitters and non-isoprene emitters, while at low concentration of CO_2, isoprene emitters had less photosynthetic capacity lost during the treatment and more photosynthetic capacity recovered after the treatment than non-isoprene emitters (Way et al., 2011). This positive response of isoprene emitters was consistent with its greater ability to recover NPQ and ETR from two sun fleck treatments (Way et al., 2011).

There may be multiple mechanisms by which the isoprene emission enhances sun fleck tolerance in plants (Way et al., 2012). Velikova et al. found the direct evidence demonstrating that isoprene emission helps to stabilize thylakoid membranes under heat stress(Velikova et al., 2011). Besides, isoprene may also help to protect leaves from oxidative damage caused by reactive oxygen species and ozone (Gove et al., 2018; Velikova et al., 2004).

1.3.2.2 Heat stress

Numerous studies have shown that isoprene emission could protect plants against heat stress and accelerate recovery (Vanzo et al., 2016; Velikova et al., 2005; Singsaas et al., 2000). Behnke et al. observed reduced net assimilation rate and photosynthetic electron transfer rate under thermal stress in transgenic non-isoprene-emitting poplars, as well as increased NPQ indicating more heat dissipation required (Behnke et al., 2007). Under heat treatment at 60°C for 2.5 h, transgenic Arabidopsis with overexpression of the *Populus alba* isoprene synthase showed apparent tolerance, while leaves of wild-type were severely damaged. Besides, the high isoprene emission was accompanied by a decrease in leaf temperature (Sasaki et al., 2007), suggesting that isoprene emission plays an important role in protecting plants against heat stress (Sasaki et al., 2007).

The thermal protection mechanisms of isoprene have been widely discussed. One

hypothesis that has been directly demonstrated by Velikova et al. was about the involvement of isoprene in protecting thylakoid membranes thus protecting photosynthesis from thermal stress (Velikova et al., 2011). In addition, Pollastri et al. found that isoprene could also help maintain membrane cohesiveness and lipo-protein structure at physiologically high temperatures (28-37°C), thereby increasing PSII photochemical efficiency and reducing the need for heat dissipation (Pollastri et al., 2014). It indicated the rationale for substantial temperature-dependent carbon investment in isoprene in unstressed leaves (Pollastri et al., 2014).

Another hypothesis was that isoprene was involved in reducing the accumulation of reactive oxygen species (ROS) (Velikova et al., 2012). On the one hand, the stable chloroplast structure under thermal pressure could avoid the formation of ROS caused by inefficient photosynthetic electron transport (Velikova et al., 2005). On the other hand, the highly reactive isoprene could react directly with hydroxyl radicals in vivo (Logan et al., 2000). However, this effect may not be significant due to the pretty low concentration of isoprene within the leaves (Harvey et al., 2015; Lantz et al., 2020).

In addition, isoprene was reported to affect the S-nitrosylation level of ROS-metabolizing enzymes, thus indirectly reducing the ROS formation and affecting the response rate and resistance degree under different environmental stresses (Vanzo et al., 2016). Velikova et al. observed that inhibition of isoprene biosynthesis in *Phragmites australis* resulted in increased H_2O_2 and MDA concentrations, as well as enhanced catalase and peroxidase activities, although the mechanism related to this phenomenon remains to be elucidated (Velikova et al., 2005). Overall, the enhanced thermal tolerance of isoprene-emitting leaves was associated with a stable membrane structure and scavenging of ROS.

1.3.2.3 Drought stress

Compared with sun fleck and heat stress, the drought response of isoprene emission is less studied and varies by species and environmental conditions (Dani et al., 2015; Ryan et al., 2014). Tattini et al. found that the isoprene emission of *PaISPS* transgenic tobacco was induced under mild drought which was defined as a fraction of transpirable soil water (FTSW) of 68% (Tattini et al., 2014). It was consistent with recent results that an increased ratio of photosynthetic ETR to net carbon assimilation rate (NAR) supported increased isoprene emission rate under drought following a species-specific drought tolerance threshold (Dani et al., 2015).

When the drought stress became severe (FTSW = 34%), the isoprene emission was suppressed (Tattini et al., 2014). In some studies, isoprene biosynthesis decreased under drought stress, but to a lesser extent than photosynthesis (Pegoraro et al., 2004; Fortunati et al., 2008; Brüggemann et al., 2002). For example, during severe drought when the fraction of transpirable soil water (FTSW) reached 5%, the photosynthesis of aspen was severely inhibited, while the isoprene emission was 51% of the pre-treatment level, indicating an alternative carbon source available for isoprene synthesis under severe drought stress (Brilli et al., 2007). Besides, after rewatering for one week, the isoprene emission was even 60% higher than the pre-treatment level (Brilli et al., 2007).

The opinions about effect of isoprene on the drought response of plants are pretty diverse. The *PaIspS* transgenic Arabidopsis which showed apparent heat tolerance didn't appear significantly different from the wild type in the drought treatment (Sasaki et al., 2007). While in the *PaIspS* transgenic tobacco, the photosynthetic apparatus was well protected and water use efficiency was higher in terms of leaf and whole-plant, but the biomass was less relative to the wild type (Ryan et al., 2014).

Additionally, the increase in ROS content was much lower in the transgenic lines than wild type line, but the efficiency factor of PSII was similar between transgenic and wild type lines, suggesting that isoprene might be used to protect membrane structure under drought stress rather than offering additional sink for excess energy (Ryan et al., 2014). The photosynthesis was less inhibited and the recovery rate was higher in the *PaIspS* transgenic tobacco than wild type line under drought stress (Tattini et al., 2014).

The isoprene emission was also found to be associated with enhanced stomatal closure, thereby reducing transpiration and increasing leaf and whole-plant water use efficiency (WUE) (Ryan et al., 2014). Guidolotti et al. also found the positive correlation between isoprene emission and WUE (Guidolotti et al., 2011).

Isoprene may play different roles in response to drought severity (Tattini et al., 2014). Isoprene acted as a direct protectant under mild drought, while under severe drought, isoprene production might trigger the synthesis of non-volatile isoprenoids, especially de-epoxidated xanthophylls and abscisic acid (ABA), regulating the metabolic pathways to enhance drought tolerance (Tattini et al., 2014).

Biochemically, the gene transcription and enzymatic activity of isoprene synthase vary

among species. Brüggemann reported that the in vivo isoprene synthase activities of oaks including *Quercus pubescens* and *Quercus robur* were not affected under severe drought stress (Brüggemann et al., 2002). However, the isoprene synthase activity of *Populus alba* was sensitive to drought stress, and the gene expression and protein concentration of isoprene synthase reduced with further increase in water stress (Brilli et al., 2007). After rewatering, the activity and concentration of isoprene synthase quickly recovered to pre-drought levels, while gene expression was still low, indicating that the isoprene emission regulation might occur at the post-transcriptional level (Brilli et al., 2007). In *Populus nigra*, the gene transcription, concentration and activity of isoprene synthase changed in concert with isoprene emission during drought stress (Fortunati et al., 2008).

1.3.3 Possible impact of climate change on isoprene emission

Highly reactive isoprene is involved in the formation of secondary oxidants such as organic peroxy radicals (RO_2), ozone and secondary organic aerosols (SOA) in the atmosphere (Hallquist et al., 2009; Schwantes et al., 2020; Rollins et al., 2009), which could affect air quality, regional climate and human health (Henninger 2012). Especially in the air-polluted cities with high levels of NO_X, isoprene could cause a substantial increase in secondary pollutants (Wang et al., 2013b).

Approximately 90% of atmospheric isoprene is produced by terrestrial plants worldwide (Carrión et al., 2020). Environmental factors such as temperature, radiation and CO_2, as well as human activities such as afforestation and land-use change can affect isoprene production (Lathière et al., 2006; Hantson et al., 2017). Regarding human activities, the growing demands for bioenergy crops such as large reeds and short-rotation forests such as poplars result in enhanced isoprene emission, with regional specificity (Morrison et al., 2016).

However, due to the complex impact of climate on isoprene release, it is difficult to predict future isoprene emission (Sharkey et al., 2014). It was predicted that global temperature will increase by around 2.0-4.5°C and atmospheric CO_2 concentration will be over 800 ppm by 2100 (Munday et al., 2009; Albright et al., 2013). On one hand, increasing global temperature could contribute to a higher isoprene level as isoprene biosynthesis is positively correlated with temperature (Heald et al., 2009). On the other hand, elevated concentration of CO_2 could inhibit isoprene emission (Scholefield et al., 2004).

In addition, the aggravated drought stress caused by global warming has complex effects on isoprene emission (Jiang et al., 2018). Studies based on different models have different views on the forecast trend of isoprene emission (Lantz et al., 2019b; Monson et al., 2007; Young et al., 2009; Sanderson et al., 2003). For example, by modeling the combined effects of increasing atmospheric CO_2 concentration, climate warming and anthropogenic land use, Wu et al. estimated that the global isoprene emission could increase between 2000 and 2100 (Wu et al., 2012). By considering the enhanced photosynthesis by CO_2, increasing BVOC emissions together with the direct inhibition of CO_2 on leaf BVOC emissions, Hantson et al. predicted a strong decrease in isoprene emission in the next 100 years (Hantson et al., 2017; Lantz et al., 2019b). However, it has also been suggested that the increased leaf area index with elevated CO_2 concentration could offset the CO_2 inhibition on leaf isoprene emission (Sharkey et al., 2014).

In summary, the interaction of the above factors increases the difficulty of predicting the future isoprene emission (Potosnak et al., 2014). Besides, changes in time and region cannot be ignored. For example, there could be more isoprene emission in the early growing season (Pétron et al., 2001). In the area with severe warming, high-temperature stimulation may override the CO_2 inhibition (Monson et al., 2016; Lahr et al., 2015), and in some regions with relatively mild temperature increase, the CO_2 suppression may predominate (Rosenstiel et al., 2003). Therefore, predictions for future isoprene emission are also region-specific. In addition, a deeper understanding of the regulation mechanisms of isoprene emission such as the effects of CO_2 and drought will be more helpful (Lantz et al., 2019b; Monson et al., 2007).

1.4 Bio-isoprene production in different hosts

Biosynthesis of isoprene is gaining increasing attention, with increasing energy demand and continuous consumption of non-renewable fossil resources and the resulting environmental impacts (Ezinkwo et al., 2013; Wang et al., 2017c). Due to the prevalence of MVA and/or MEP pathways in all organisms, it is feasible to make non-isoprene emitter produce isoprene by introducing heterologous isoprene synthase (Xu et al., 2020) (Table 1.2).

Table 1.2 Isoprene biosynthesis optimization in different hosts

Source organism	Host	Optimization strategy	Titer ($g\ L^{-1}$)	Productivity ($mg\ L^{-1}\ h^{-1}$)	Cultivation type	Reference
P. montana	*Bacillus subtilis*	Heterologous expression of IspS	$1.434 * 10^{-3}$	N.A.	Sealed culture	(Gomaa et al., 2017)
P. alba	*E. coli*	Introduction of hybrid MVA pathway; increasing carbon flux	60	2000	Fed-batch fermentation	(Whited et al., 2010)
P. nigra	*E. coli*	Overexpression of heterologous MEP genes	0.314	N.A.	Fed-batch fermentation	(Zhao et al., 2011)
P. montana	*E. coli*	Overexpression of hybrid MVA pathway	0.32	N.A.	Sealed culture	(Zurbriggen et al., 2012)
P. alba	*E. coli*	Introduction of MVA pathway	0.532	N.A.	Fed-batch fermentation	(Yang et al., 2012a)
P. alba	*E. coli*	Introduction of hybrid MVA pathway; mutated mvaS gene	6.3	N.A.	Fed-batch fermentation	(Yang et al., 2012b)
P. alba	*E. coli*	Optimization of MEP feeding modules	0.221	N.A.	Sealed culture	(Liu et al., 2013)
P. alba	*E. coli*	Expression regulation of MEP genes	0.020	N.A.	Sealed culture	(Li et al., 2014)
P. alba	*E. coli*	Combination of De Ley–Doudoroff and MEP pathway for isoprene production from galactose	0.264	N.A.	Sealed culture	(Ramos et al., 2014)
P. alba	*E. coli*	Overexpression of both MEP and MVA pathway	24.0	N.A.	Fed-batch fermentation	(Yang et al., 2016a)
P. trichocarpa	*E. coli*	Overexpression of MVA pathway; genome manipulation to reduce by-products	1.832	N.A.	Sealed culture	(Kim et al., 2016)
NA	*E. coli*	Introduction a novel MVA-mediated pathway without IspS	0.62	6.46	Fed-batch fermentation	(Yang et al., 2016b)
P. alba	*E. coli*	Expression regulation of MVA genes; knock out acetate-producing genes	0.092	N.A.	Sealed culture	(Liu et al., 2019a)

I. batatas	*E. coli*	Expression regulation of MVA genes	0.698	N.A.	Sealed culture	(Li et al., 2019b)
P. alba	*E. coli*	Coupling the production of isoprene with 1,3-propanediol to balance redox cofactor	0.665	N.A.	Sealed culture	(Guo et al., 2019)
P. alba	*E. coli*	Introduction of hybrid MVA pathway; expression regulation of MVA genes	0.587	N.A.	Sealed culture	(Liu et al., 2019b)
P. montana	*S. cerevisiae*	Multiple independent integrations of the IspS gene	> 0.5 * 10^{-3}	> 7 * 10^{-3}	Sealed culture	(Hong et al., 2012)
P. alba	*S. cerevisiae*	Up-regulation of precursor supply; down-regulation of the competing pathway	0.037	N.A.	Sealed culture	(Lv et al., 2014)
P. alba	*S. cerevisiae*	Dual regulation of cytoplasmic and mitochondrial acetyl-CoA utilization	2.527	N.A.	Fed-batch fermentation	(Lv et al., 2016)
P. alba	*S. cerevisiae*	Promoter engineering to enhance IspS expression; directed evolution of IspS	3.7	N.A.	Fed-batch fermentation	(Wang et al., 2017b)
P. alba	*S. cerevisiae*	Directed evolution of IspS; diploid strains with reconstructed metabolic balance	11.9	N.A.	Fed-batch fermentation	(Yao et al., 2018)
P. montana	Cyanobacterium	Application of a gaseous/aqueous two-phase photobioreactor	1.5 * 10^{-4}	2 * 10^{-3}	Gaseous−aqueous two-phase cultivation	(Bentley et al., 2012)
P. montana	Cyanobacterium	Heterologous expression of the MVA Pathway	~ 3 * 10^{-4}	N.A.	Sealed culture with aliquots of 100% CO_2 every 24 h	(Bentley et al., 2014)
E. globulus	Cyanobacterium	Overexpression of IDI; direct fusion of IDI and IspS; heterologous expression of IspG	1.26	4.26	continuous culture aerated with air containing 5% CO_2	(Gao et al., 2016)

E. globulus	Cyano bacterium	Combining several genes upstream of the MEP pathway with Dxs and Idi and IspS	1 * 10^{-3}	N.A.	Sealed culture	(Englund et al., 2018)
P. montana	Cyano bacterium	Fusion construct of IspS and highly expressed cpcB with linkers	N.A.	2.89 * 10^{-2}	Gaseous−aqueous two-phase cultivation	(Chaves et al., 2017)
P. montana	Cyano bacterium	Overexpression of heterologous Idi with cpcB*linker*IspS fusions	N.A.	4.6 * 10^{-2}	Gaseous−aqueous two-phase cultivation	(Chaves et al., 2018a)

1.4.1 In *Bacillus subtilis*

The highest natural isoprene production in microorganisms was found in *Bacillus subtilis* with a production rate of 12.78 nmol g^{-1} h^{-1} (Wilson et al., 2018). In this organism, isoprene was found to be formed by the MEP pathway rather than MVA pathway through ^{13}C- and ^{2}H-labeling analysis (Wagner et al., 2000).

Although the enzymatic conversion of DMAPP to isoprene was demonstrated in permeabilized cells and partially purified cell extracts of *B. subtilis*, the activity was very labile and no isoprene synthase gene has been identified in *B. subtilis* so far (Sivy et al. 2002; Julsing et al., 2007). It is unclear whether the isoprene synthesis in *B. subtilis* is catalyzed by an enzyme with unique or multiple catalytic abilities (Xue et al., 2011).

Therefore, only plant-derived isoprene synthases can be applied in the protein engineering and metabolic engineering for isoprene production optimization in *B. subtilis* at the moment (Xue et al., 2011). The introduction of the kudzu isoprene synthase (kIspS) gene in *B. subtilis* DSM 402 resulted in three-fold higher isoprene production (1434.3 µg L^{-1}) than the wild type (Gomaa et al., 2017). A 40% enhancement in isoprene yield was observed in the Dxs overexpression strain compared to the wild-type *B. subtilis*, indicating that the Dxs was the key modification candidate (Xue et al., 2011).

1.4.2 In *Escherichia coli*

In terms of translation level, the optimization of ribosome binding site (RBS) and codon usage could be effective strategies (Zhou et al., 2004; Nieuwkoop et al., 2019). Especially, for the expression of plant-derived isoprene synthases *in E. coli*, the codon usage between prokaryotes and eukaryotes could significantly affect gene expression levels. For example, codon optimization and strong RBS resulted in a significant 2.5-fold increment in isoprene production due to enhanced expression of isoprene synthase gene (Kim et al., 2016).

In terms of the metabolic pathway, regulating the endogenous MEP pathway in *E. coli* is an effective strategy. It could be achieved by the overexpression of endogenous or exogenous rate-limiting enzymes (e.g, 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) and isopentenyl diphosphate isomerase (IDI)) and weakening the endogenous enzymes in competing pathway (e.g., farnesyl diphosphate synthase (IspA)).

For example, by overexpression of exogenous *dxs* and *dxr* which catalyze the first and second step of the MEP pathway, respectively, 2.3-fold production enhancement (94 to 314 mg L^{-1}) of isoprene was achieved (Zhao et al., 2011). The IDI catalyzes the conversion of IPP into DMAPP by reversible isomerization, adjusting the ratio of IPP and DMAPP (Berthelot et al., 2012). Overexpression of *dxs*, *dxr*, and *idi* in the order consistent with the metabolic pathway contributed more for isoprene production, indicating that optimizing the expression of multiple genes according to the biosynthetic pathway was an effective approach (Lv et al., 2013). The endogenous farnesyl diphosphate synthase (IspA) converts DMAPP to GPP in *E. coli* (Wang et al., 2013a), competing with isoprene for substrate DMAPP. By weakening the expression of *ispA* gene, enhancing the expression of exogenous *Idi* gene and endogenous *dxs* gene, the isoprene titer reached 19.9 mg L^{-1} (33-fold enhancement) (Liu et al., 2014a).

Besides the modification of native MEP pathway, the introduction and modification of exogenous MVA pathway into *E. coli* also showed interesting results (Yang et al., 2012b; Liu et al., 2019a; Kim et al., 2016). The heterologous over-expression of the entire MVA pathway in *E. coli* could introduce a bypass in the cellular substrate flux to IPP and DMAPP, thereby alleviating cellular constraints on innate regulation of the MEP pathway, resulting in a significant 800-fold increase in isoprene production to 320 mg L^{-1} (Zurbriggen et al., 2012). The synergy between MEP and MVA pathways also

had advantages of coupling the complementary reducing equivalent and ATP demands (Yang et al., 2016a).

The highest isoprene production in *E. coli* has been reported by whited so far (Whited et al., 2010). By construction of the bacterial/yeast hybrid MVA pathway and the overexpression of phosphogluconolactonase (PGL) which is related to increasing carbon flux in the pentose phosphate pathway thus increasing isoprene titer, the isoprene titers reached more than 60 g L^{-1} with a volumetric productivity of 2 g L^{-1} h^{-1} and a yield of 11% isoprene from glucose in a fed-batch fermentation (Whited et al., 2010).

1.4.3 In *Saccharomyces cerevisiae*

The *S. cerevisiae* is a eukaryotic model organism that synthesizes the isoprenoids through the MVA pathway (Cao et al., 2020). Introduction of codon-optimized isoprene synthase gene and its multi-copy integration into the rDNA region demonstrated for the first time the feasibility of applying the MVA pathway to produce isoprene in *S. cerevisiae* (Hong et al., 2012).

Currently, the isoprene production optimization in *S. cerevisiae* mainly includes enhancing precursors and cofactors supplies of acetyl-CoA pathway and MVA pathway and improving the enzymatic activities of isoprene synthase. By up-regulation of precursor supply of native acetyl-CoA and MVA modules, up-regulation of isoprene branch flux, and down-regulation of the competing metabolic flux, isoprene production was increased to 37 mg L^{-1} (approximately 782-fold increment) with glycerol-sucrose as carbon source (Lv et al., 2014). Through dual metabolic regulation of cytoplasmic and mitochondrial acetyl-CoA utilization, 2.527 g L^{-1} isoprene was achieved with the dual engineered diploid strain under aerobic fed-batch fermentation (Lv et al., 2016).

Based on the previously constructed precursor-supply-enhanced *S. cerevisiae* strain (Lv et al., 2014), Wang et al. further exploited up-regulation of P_{GAL}-driven isoprene synthase expression and enhanced catalytic activity of isoprene synthase by directed evolution to obtain isoprene titer of 3.7 g L^{-1} under aerobic fed-batch fermentation (Wang et al., 2017b).

By dual regulation of MVA pathway in mitochondria and cytoplasm to enhance precursor supply, directed evolution to increase the catalytic activity of the isoprene

synthase, and copy number adjustment of MVD1/IDI1 to reestablish the metabolic balance, the biomass was restored due to the relief of IPP cytotoxicity and the isoprene production was significantly increased to 11.9 g L^{-1} in the diploid *S. cerevisiae*, which is the highest isoprene titer ever reported in engineered eukaryotic organisms (Yao et al., 2018).

1.4.4 In cyanobacteria

The application of autotrophic organisms such as cyanobacteria to synthesize isoprene is of ecological importance and holds great promise when facing energy issues in the future (Machado et al., 2012; Chaves et al., 2018b). Heterologous expression of the codon-optimized *Pueraria montana* (kudzu) isoprene synthase gene in the cyanobacterium *Synechocystis* together with the use of the photosynthetic *psbA2* promoter for light-intensity-dependent isoprene synthase expression achieved the isoprene yield of 50 μg/g per dry cell weight via the endogenous MEP pathway (Lindberg et al., 2010).

From the view of reactor engineering, Bentley et al. designed a fed-batch gaseous/aqueous two-phase photobioreactor to collect isoprene and supplement CO_2 from cyanobacterial culture simultaneously and it was estimated to reach a consistent rate of 2 μg isoprene L^{-1} culture h^{-1} (Bentley et al., 2012).

In terms of metabolic engineering, introduction of the entire exogenous MVA pathway in *Synechocystis* increased isoprene yield by approximately 2.5-fold, as it bypassed the flux limitation of native MEP pathway and increased carbon flux to DMAPP (Bentley et al., 2014). For the MEP pathway, the widely-accepted bottlenecks were the enzymes DXS and IDI (Rodrigues et al., 2021). Gao et al. found IspG to be another bottleneck by kinetic flux profiling and improved isoprene titer to 1.26 g L^{-1} through direct fusion of IDI and isoprene synthase, together with overexpression of DXS and IspG (Gao et al., 2016). Englund et al. found that the enzymes DXS, IDI and IspD had the greatest effect on isoprene production in the cyanobacterium *Synechocystis* PCC 6803 by metabolic network analysis of MEP pathway (Englund et al., 2018). They obtained a stable strain that expressing IspS together with Dxs and Ipi resulting in isoprene yields of 2.8 mg g^{-1} (40-fold increment) (Englund et al., 2018).

In terms of protein engineering, as the low expression of recombinant isoprene synthase was one of the major bottlenecks (Zhang et al., 2021a), Chaves et al. tried

the fusion construct of isoprene synthase and highly expressed cpcB (the β-subunit of phycocyanin) with or without different linkers (Chaves et al., 2017). The best fusion construct led to the isoprene accumulation rate of 28.9 μg L^{-1} h^{-1}, with 275-fold increase in isoprene synthase protein accumulation, although the specific activity of isoprene synthase was reduced to 10.65% of the unfused enzyme (Chaves et al., 2017).

In later studies, by combining the heterologous expression of an isopentenyl isomerase from *Streptococcus pneumoniae* (FNI) with cpcB*linker*ISPS fusions for enhancement of both substrate availability and isoprene synthase concentration, the isoprene yield increased by 62-fold to 12.3 mg g^{-1}, constituting a step forward in the platform optimization for renewable photosynthetic isoprene production (Chaves et al., 2018a).

Although some progress has been made in both prokaryotes and eukaryotes, especially *E. coli* and *S. cerevisiae*, optimization of the bio-isoprene yield to meet industrial production needs is still a challenge, either through direct evolution to increase the activity of rate-limiting enzymes or to find novel highly active enzymes and/or metabolic engineering strategies. Despite that the current isoprene productivity and cell density of autotrophs such as cyanobacterium are much lower than the heterotrophs such as *E. coli* and *S. cerevisiae*, the advantages of CO_2 fixation, high photosynthetic efficiency, and reduced land-use make microalgae promising for sustainable production of various biochemicals and biofuels (Onyeaka et al., 2021).

1.4.5 *Nannochloropsis gaditana* could be a promising organism for isoprene bioproduction

Nannochloropsis gaditana belongs to unicellular microalgae with the cell size of 2-4 μm, and a single chloroplast occupying most of the cell volume (Cecchin et al., 2020; Rocha et al., 2003). *N. gaditana* is tolerant to a wide range of environmental conditions including varying pH, temperature and salinity concentrations and can be cultivated to high densities (>10 g L^{-1}), making it suitable for large-scale industrial cultivation (Radakovits et al., 2012).

Nannochloropsis has a broad potential for biotechnological applications and is mainly used in the aquafeed industry. *Nannochloropsis* can also be used in production of

biofuels and high-value polyunsaturated fatty acids, in addition to wastewater treatment (Al-Hoqani et al., 2017). However, heterologous isoprene production has not been achieved in eukaryotic microalgae, which is mainly limited by the efficient transformation and high expression of heterologous genes.

N. gaditana was the first *Nannochloropsis* species with fully sequenced nuclear, plastid and mitochondrial genomes (Radakovits et al., 2012). The availability of all three genome sequences and molecular tools facilitated genetic engineering in *N. gaditana* (Jinkerson et al., 2013).

The successful methods of nuclear transformation in *Nannochloropsis* include electroporation (Kilian et al., 2011; Osorio et al., 2019), *Agrobacterium*-mediated conjugation (Vavitsas et al., 2021) and biolistics (Kang et al., 2015). On one hand, nuclear transformation has advantages such as post-translational modifications such as glycosylation (Jinkerson et al., 2015). On the other hand, nuclear transformation may lead to position effects affecting heterologous gene expression due to random integration, as well as gene-dosage effects caused by variable copy numbers integrated into the genome (Lerche et al., 2013). Although plastid transformation can achieve precise insertion of heterologous genes through homologous recombination and high expression levels, there is no report about the successful plastid transformation in *Nannochloropsis* (Gan et al., 2018).

Despite the difficulties of efficient transformation and expression of transgenes, considering that *Nannochloropsis* are the major source of isoprenoids such as chlorophyll, zeaxanthin, canthaxanthin and astaxanthin (Cancela et al., 2019), *N. gaditana* may have a higher DMAPP flux than *E. coli* and yeast, and theoretically has great potential for efficient heterologous isoprene production (Lauersen 2019). Besides, it is possible to improve isoprene production in *N. gaditana* by metabolic engineering. On the one hand, it has been reported that over-expression of the bottleneck enzymes (e.g., DXS and IDI) of the native MEP pathway is efficient for isoprene production in *E. coli* and cyanobacteria (Lv et al., 2013; Englund et al., 2018; Gao et al., 2016; Rodrigues et al., 2021). On the other hand, the introduction of an entire exogenous MVA pathway turned out to be effective in *E. coli* and cyanobacteria (Yang et al., 2012b; Liu et al., 2019a; Zurbriggen et al., 2012; Bentley et al., 2014), which might alleviate flux limitation of native MEP pathway and increase carbon flux to DMAPP (Bentley et al., 2014).

2 Materials and methods

2.1 Materials and growth conditions

2.1.1 *Escherichia coli* strains and culture conditions

E. coli DH10B, Rosetta(DE3), Rosetta(DE3)pLysS, BL21(DE3), BL21Star(DE3) and BL21-CodonPlus were used for recombinant plasmid transformation and protein expression.

For liquid culture, *E. coli* strains were grown with Luria-Bertani (LB) medium supplemented with specific antibiotics when necessary (50 mg L^{-1} Kanamycin or 100 mg L^{-1} Ampicillin) at 37°C by shaking at 200 rpm. LB medium is composed of 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone and 10 g L^{-1} NaCl.

For cell maintenance, plates were prepared with LB medium containing 15 g L^{-1} agar with specific antibiotics when necessary. For blue-white screening of *E. coli* colonies, the X-Gal/IPTG LB Agar plates were prepared with LB medium by adding 15 g L^{-1} agar, 32 μM IPTG, 32 mg L^{-1} X-Gal and 100 mg L^{-1} Ampicillin.

Table 2.1 Summary of *E. coli* strains used in this study.

Strain name	Purpose	Antibiotic resistance
DH10B	Plasmid propagation	-
Rosetta(DE3)	High-level expression of eukaryotic proteins	Chloramphenicol
Rosetta(DE3)pLysS	expression of eukaryotic proteins, expression of toxic proteins	Chloramphenicol
BL21(DE3)	Expression of non-toxic proteins	-
BL21Star(DE3)	High-level expression of non-toxic proteins	-

BL21-CodonPlus	High expression of eukaryotic proteins	Chloramphenicol

2.1.2 Plant materials and pot soil mixture

The *Arabidopsis thaliana* Col-0 wild-type was used and the IspS transgenic plants were generated from Col-0. The Cauliflower Mosaic Virus (CaMV) 35S promoter was used for the constitutive over-expression of isoprene synthase from *Copernicia prunifera*.

The pot soil was prepared by mixing Schildkrötensubstrat soil (Floragard products, Germany), Profi Substrat soil (Einheits Erde® Classic, Einheitserde-Werke, Germany) and perlite with a ratio of 3:3:1 (v:v:v). Then soil was aliquoted into each pot (8x8x8 cm) and soaked with water for 30 min followed by watering from the top.

2.1.3 Seeds sterilization and growth conditions

Seeds were sterilized as previously described (Li et al., 2021). In brief, 70% ethanol was added into 1.5 mL Eppendorf tubes containing prealiquoted seeds, mixed by gently shaking for 5 min and aspirated out. Then 5% (v/v) commercial bleach was added into the tubes and gently shaken for 5 min. After washing twice with sterile deionized water, the seeds were sown on 1/2 Murashige-Skoog (MS) agar medium containing 2.2 g L^{-1} Murashige and Skoog Salt Mixture, 10 g L^{-1} sucrose and 8 g L^{-1} phyto-agar (pH 5.7).

The plates were sealed and kept at 4°C for 72 h and moved to the growth chamber. The chambers were set at 23°C with light intensity of 100-120 µmol m^{-2} s^{-1} and relative humidity of 40-50% under a 16 h light/8 h dark photoperiod.

After germinating on plates for seven days, the seedlings were transferred into soil and grown in the chamber.

2.1.4 *Nannochloropsis gaditana* strains and culture conditions

N. gaditana strain 849/5 was used for heterologous isoprene synthase expression. For the liquid culture, cells were grown in F/2 medium at 25°C with continuous lighting of 100 µmol m^{-2} s^{-1}. F/2 medium was prepared with 32 g L^{-1} sea salts, 40 mM TRIS HCl (pH 8), Guillard's (F/2) marine water enrichment solution (Sigma) and specific antibiotics when necessary (Simionato et al., 2011).

For cell maintenance, the colonies were streaked on the plates prepared with F/2 medium and 10 g L^{-1} of plant agar. The plates were kept in the chamber set at 23°C with light intensity of 100-120 µmol m^{-2} s^{-1}, relative humidity of 40-50% and a long-day photoperiod (16 h light/8 h dark).

2.2 Plasmid construction and transformation into *E. coli*

The palm leaves of *Chamaerops humilis*, *Sabal minor* and *Copernicia prunifera* were sampled in the Botanic Garden of the University of Padova. The full-length cDNAs of isoprene synthases from these three palms were identified by Dr. mingai Li. The genomic DNA isolation, total RNA extraction and cDNA synthesis were performed as previous description (Li et al., 2020b). Briefly, the genomic DNA was extracted from fresh leaf with the DNeasy Plant Mini Kit (Qiagen). Total RNA was extracted from frozen leaf with TRIzol reagent (Invitrogen) and then treated with Amplification-Grade DNase I (Sigma-Aldrich®). cDNA was synthesized with the extracted total RNA as template using SuperScript™ III Reverse Transcriptase (Invitrogen™). The full-length coding sequence of isoprene synthase was amplified with the previously synthesized cDNA as a template using Phusion High-Fidelity DNA Polymerase (Thermo Fisher) and primers designed with GenomeWalker™ Universal Kit (Clontech). The sequences of *SminIspS* are recorded in GenBank with accession number MT512621. The sequences of *ChumIspS* and *CpruIspS* are not published yet.

Table 2.2 Summary of used vectors for transformation into *E. coli*

Vector	Purposes	Affinity tag	Antibiotic resistance
pGEM-T	Efficient ligation of PCR products into the plasmid	-	Ampicillin
pET-28b	High gene expression level, simple protein purification	N-terminal and optional C-terminal 6×His tag	Kanamycin
pGEX-4T-1	High gene expression level, simple protein purification	N-terminal GST tag	Ampicillin
pQE-30 Xa	Gene expression and simple protein purification	N-terminal 6×His tag	Ampicillin

According to the vectors used for expression in *E. coli*, the primer pairs were designed and used for the gene cloning of target isoprene synthases without putative chloroplast transit peptide. The coding sequences of IspS were amplified by polymerase chain reaction (PCR) using the plasmid containing IspS cDNA as the template, dNTPs, corresponding primer pairs, Phusion High-Fidelity DNA Polymerase (Thermo Fisher). PCR program was set as: denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 60 s and the final elongation at 72°C for 10 min. The PCR products were loaded into a 1% (w/v) agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM Na_2EDTA). After separation at 120 V for around 30 min, the PCR products were visualized using a UV transilluminator, and the sizes of amplicons were estimated with GeneRuler DNA ladders (Thermo Fisher Scientific) as a reference. The target fragment was purified with PureLink™ PCR Purification Kit according to the manufacturer's instructions.

The purified DNA was A-tailed using dATP and Euro Taq DNA Polymerase. The A-tailing products was ligated with the pGEM-T vector (Promega) using T4 DNA ligase at 4°C overnight. Then the ligation product was transformed into *E. coli* DH10B by electroporation. After cell growth at 37°C for about 1 h, the culture was plated on X-

Gal/IPTG LB Agar plates and inoculated at 37°C overnight. The white colonies on plates were picked and mixed with dNTPs, Euro Taq DNA Polymerase and pGEM-T vector primer pairs, 10 × PCR buffer and H_2O to conduct colony PCR. PCR program was set as: denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 90 s and the final elongation at 72°C for 5 min. The colony PCR products were analyzed in a 1% agarose gel electrophoresis. The positive colonies were cultured at 37°C and subjected to plasmid extraction using a PureLink™ Quick Plasmid Miniprep Kit. After sequencing confirmation, restriction digestion and purification, the target gene fragment was finally ligated with destination vector (pET-28b, pGEX-4T-1 and pQE-30 Xa) digested with same restriction enzymes, respectively. The recombinant plasmid was transformed into different *E. coli* strains including Rosetta(DE3), Rosetta(DE3)pLysS, BL21(DE3), BL21Star(DE3), BL21-CodonPlus by electroporation.

Table 2.3 primers used for IspS cloning into different expression vectors

Primer name	**Sequence (5' – 3')**
pET28b-*Chum/SminIspS_For*	GAATTCGATGGCAAGTCAAGTCCGTTCC
pET28b-*ChumIspS_Rev*	CTCGAGCTACACATTGCTGACCACAAACCT
pET28b-*SminIspS_Rev*	AAGCTTCTACACATTGCTGACCACAAACCT
pGEX4T1-*ChumIspS_For*	GAATTCATGGCAAGTCAAGTCCGTTCC
pGEX4T1-*ChumIspS*/*CpruIspS_Rev*	GCGGCCGCCTACACATTGCTGACCAC
pGEX4T1-*CpruIspS_For*	GAATTCATGGCAAGTCAAGTCCGC
pQE30Xa-*SminIspS_For*	GAGCTCATGGCAAGTCAAGTCCGT
pQE30Xa-*SminIspS_Rev*	CCCGGGCTACACATTGCTGACCAC
pET28b(no tags)-*ChumIspS_For*	CCATGGCAAGTCAAGTCCGTTCC
pET28b(no tags)-*ChumIspS_Rev*	CTCGAGCTACACATTGCTGACCACAAACCT

2.3 Recombinant protein expression of IspS in *E. coli*

A single colony of expression plasmid in *E. coli* was cultured in 3 mL of LB medium containing specific antibiotics (50 μg mL^{-1} kanamycin for pET-28b; 100 μg mL^{-1} Ampicillin either for pGEX-4T-1 or pQE-30 Xa) at 37°C overnight, then inoculated into 20 mL LB containing corresponding antibiotics. When the optical density of the culture was reached to 0.3, 0.6, 0.7, 0.9 or 1.2 at 600 nm (OD_{600}), the isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1, 0.2, 0.4, 0.8 or 2 mM to induce IspS expression by incubation for 3, 6, 12 or 15 h at 15, 20, 34 or 37°C.

To increase the solubility of IspS, different additives were added to the cultures after induction, including ethanol, $MgCl_2$ and Triton X-100 at final concentrations of 2%, 0.1 mM and 1%, respectively. For heat shock treatment, cells were cultured to an OD_{600} of 0.2 at 37°C. Then the culture temperature was changed to 42°C when the OD_{600} was reached to 0.4, followed by induction with 0.4 mM IPTG at 30°C for 15 h.

At the end of culture, cells of 5 mL culture were collected by centrifugation at 4°C (5000 g, 10 min) and resuspended in 500 μL lysis buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). The cell suspension kept on ice was lysed by sonication followed by centrifugation at 4°C (10000 g, 5 min). Afterwards the supernatant was collected, the pellet was resuspended in 500 μL lysis buffer. An aliquote of 50 μL supernatant or pellet was mixed individually with 50 μL 2× Laemmli buffer (45% (w/v) glycerol, 10% (w/v) SDS, 2% (w/v) bromophenol blue, 1 M DTT, 1 M Tris-HCl, pH 6.8) and boiled at 95°C for 5 min.

2.4 SDS-polyacrylamide gel electrophoresis

For preparation of 10% separating gel, a mixture of 1.25 mL Tris-HCl (1.5 M, pH 8.8), 1.79 mL distilled H_2O, 1.22 mL 40% (w/v) acrylamide, 0.67 mL 2% (w/v) bis-acrylamide, 50 μL 10% (w/v) sodium dodecyl sulphate (SDS), 25 μL 10% (w/v) ammonium

persulphate (APS) and 2.5 µL N, N, N', N'-tetramethylethylenediamine (TEMED) was added into fixed vertical glass plates, leaving approximately 2 cm at the top for the stacking gel and overlaid with ethanol.

After gel polymerization for around 30 min at room temperature, 4% stacking gel was prepared by mixing 0.25 mL Tris-HCl (1 M, pH 6.8), 1.42 mL distilled H_2O, 0.19 mL 40% (w/v) acrylamide, 0.10 mL 2% (w/v) bis-acrylamide, 20 µL 10% (w/v) SDS, 10 µL 10% (w/v) APS and 2 µL TEMED and added to the top. The comb was inserted into the stacking gel and the gel was kept at room temperature for another 30 min for complete polymerization.

The samples and protein molecular weight markers were centrifuged at 10000 g for 5 min and loaded into each well. The electrophoresis was performed at constant voltage (200 V) in cold running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) sodium dodecyl sulphate (SDS), pH 8.3) for approximately 50 min until the front dye reached the bottom of the gel. The protein bands were visualized after Coomassie-staining and de-staining. The staining solution was composed of 0.2% Coomassie Blue R250, 7.5% acetic acid, 50% ethanol. The de-staining solution contained 10% acetic acid, 30% methanol.

2.5 Agrobacterium-mediated transformation of *Arabidopsis thaliana*

Arabidopsis transformation was performed as previously described (Li et al., 2017). The target isoprene synthase gene was cloned into entry vector pENTR/D-TOPO and further recombined in the vector pK7WG2 by LR reaction with LR clonase II (Invitrogen). The positive clone was selected and used for plasmid purification which was further transformed into *Agrobacterium tumefaciens* strain GV3101-pMP90RK by electroporation. Then the transformants were mixed with 1 mL of YEB medium (1 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 5 g L^{-1} peptone, 5 g L^{-1} sucrose, 15 g L^{-1} agar, pH 7.2) and cultured at 28°C for 2 h. The culture was plated on YEB plates containing 50

mg L^{-1} spectinomycin and 100 mg L^{-1} Rifampicin.

The colonies picked from the plates were cultured in 50 mL fresh selective YEB liquid medium containing 50 mg L^{-1}spectinomycin, 100 mg L^{-1} Rifampicin and 2 mM Mg_2SO_4 and then mixed with the same 250 mL selective YEB liquid medium to continue culture at 28°C for 18 h.

300 mL *Agrobacterium* culture was continuously stirred and mixed with 300 mL 5% sucrose solution and 120 µL Silwet L-77. After immersing the inflorescences of *A. thaliana* in the *Agrobacterium* solution for about 15 s, the plants were covered with black plastic bags to protect from light for 22 h and then moved to the growth chambers in GMO greenhouse. After seeds harvesting, the seeds were sown onto MS plates containing 50 mg L^{-1} of kanamycin to screen for positive transgenic plants. The positive ones were green and strong, while the negative ones were yellow and tiny after seven-day growth.

2.6 Isoprene emission screening of transgenic Arabidopsis

Isoprene emission of transgenic Arabidopsis was screened by Proton Transfer Reaction–Mass Spectrometry (PTR-MS). Mature rosette leaves were detached from one-month-old plants. After weighing fresh weight, the leaves were transferred into a 20 mL glass vial containing 300 µL of distilled water. Before sealing, the vials were kept open for 30 min and then incubated at 30°C for 3 h under the light intensity of 130-150 µmol m^{-2} s^{-1}. Isoprene was determined with PTR-ToF 8000 apparatus (Ionicon Analytik GmbH, Innsbruck, Austria). The data was converted to parts per billion volume (ppbv) and the amount of isoprene emission for a single leaf was calculated by dividing the measured data by the fresh weight of corresponding leaf.

2.7 Mannitol treatment of Arabidopsis transgenic plants

2.7.1 Germination assay

The surface-sterilized seeds were sown on 1/6 MS solid medium supplemented with 200, 300 mM mannitol and grown for 7 days for seed germination rate and cotyledon greening rate assays. Seed germination was recorded when the radicle penetrated the seed coat completely, and the cotyledon greening was recorded when the cotyledon was open and green. Each biological replicate contained 40 seeds and four replicates were conducted.

2.7.2 Fresh weight measurement

For the initial genotype observation, the seeds of Col-0 and two *CpruIspS* transgenic lines were surface-sterilized and sown into square petri dishes in 1/6 MS medium containing 0, 250 mM, 300 mM, or 400 mM mannitol and grown vertically for 14 days. The plants of each genotype were picked carefully with forceps and the total fresh weight was measured. Each replicate contained 10 plants and 5 replicates were conducted.

2.7.3 Chlorophyll content determination

Seven-day-old Col-0 and two lines of *CpruIspS* transgenic seedlings growing on 1/6 MS medium were transferred to new plates with 1/6 MS medium containing 0 mM or 400 mM mannitol and grown vertically for another 10 days. The aerial parts were collected in liquid nitrogen and stored at -80° till usage. Chlorophyll contents were determined according to our previous method (Xu et al., 2020). Briefly, the samples were homogenized using a Tissue Lyser II (Qiagen) and resuspended with 800 µL of 80% ice-cold acetone. After centrifugation at 4600 rpm at 4°C for 13 min, the supernatant was collected, and the pellet was resuspended again with another 400 µL

of 80% ice-cold acetone and centrifuged as before. The absorbance of the combined supernatant was measured at 663 nm and 646 nm using an Ultrospec 3100 proUV/Visible Spectrophotometer (GE healthcare). According to Lichtenthaler and Wellburn (Lichtenthaler et al.,1983), the total chlorophyll content was calculated: C (μg mL^{-1}) = 7.18 A_{663} + 17.32 A_{646}. The final chlorophyll concentration (μg mg^{-1} of aerial part) of each sample was calculated by normalizing with the fresh weight. Four replicates were performed for this analysis.

2.7.4 RNA extraction and cDNA synthesis

Arabidopsis 10-day-old Col-0 and *CpruIspS* transgenic seedlings growing on 1/6 MS medium were transferred onto new plates with 1/6 MS medium containing 0 mM or 500 mM mannitol for 1 h or 3 h. Then the aerial part and root were separately collected. Total RNA was extracted with TRIzol reagent (Invitrogen) and treated with DNase I (Sigma) to eliminate genomic DNA contamination. Then 1 μg of total RNA was used for the first-strand cDNA synthesis with SuperScript™ III (Invitrogen).

2.7.5. Quantitative real-time PCR (qRT-PCR) analysis

The qRT-PCR was performed with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The 12.5 μL of reaction contained 6.25 μL of PlatinumSYBRGreenqPCRSuperMix-UDG, 1 μL of six-fold diluted cDNA, 0.25 μL of each primer (final concentration of 200 nM) and 4.75 uL of H_2O. The PCR program was set as: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. At least three technical and three biological replicates were conducted for each reaction. The housekeeping gene *AtEF1α* was selected as internal reference. The relative transcription levels of these genes were analyzed by the $2^{-\Delta\Delta Ct}$ method. The primers used for qRT-PCR are listed below:

Table 2.4 primers used for qRT-PCR

Primer name	**Sequence (5' – 3')**
EF1α-For	TGACTGTGCTGTGCTCATCA
EF1α-Rev	GTTGCAGCAGCAGATCATCT
P5CS-For	GAAGGATTACTTACAACGAGATGGA
P5CS-Rev	CTCTCCTCAAGTCTCAACCAAATAC
DREB2A-For	TCCAGCAGGTAGATCATCTCC
DREB2A-Rev	AGCAGGTTCGGTAATAGGCA
RAB18-For	GGAAGAAGGGAATAACACAAAAGAT
RAB18-Rev	GCGTTACAAACCCTCATTATTTTTA
COR15A-For	GATACATTGGGTAAAGAAGCTGAGA
COR15A-Rev	ACATGAAGAGAGAGGATATGGATCA

2.7.6 Water loss rate determination

Seven-day-old Col-0 and *CpruIspS* transgenic seedlings growing on 1/2 MS medium were transferred into commercial soil and cultivated in the same conditions mentioned above. After additional two-week growth, the mature rosette leaves were detached from plants, pooled and weighed immediately. The detached leaves were placed on trays under 40-50 % relative humidity at 23°C. Within 8 h, the fresh weight was measured and recorded every hour. For each genotype, five replicates were performed. Each replicate included 16 rosette leaves of similar size detached from eight plants.

2.7.7 Survival rate assay

For the survival rate determination, the *CpruIspS* transgenic lines and WT plants were grown under normal conditions as mentioned above for three weeks. Then the plants were deprived of watering until the soil humidity reached 20-25%, followed by re-watering for four days. Three replicates were performed for this analysis and each

replicate contained 20 plants.

2.8 Plasmid construction and transformation into *N. gaditana*

The destination vector used for gene cloning and the subsequent electroporation were provided by Prof. Tomas Morosinotto. The destination vector was constructed with the pBlueScript SKII as vector backbone. The gene of interest and the yellow fluorescent protein (YFP) coding sequence were linked by 2A self-cleaving peptide to form a single-cassette. The *Lhcx1* promoter and *FcpA* terminator were used to regulate the gene transcription.

The methods of IspS gene cloning and plasmid construction could be found in chapter 2.2. Briefly, two different lengths of cDNA fragments of *AdoIspS* (*AdoIspS*_short, *AdoIspS*_long) were amplified by PCR and cloned into the vector pENTR/D-TOPO, respectively. After restriction digestion, the recombinant plasmids were obtained by ligating the target *AdoIspS* fragments with the destination vector.

Table 2.5 primers used for IspS cloning into the vector in *N. gaditana*

Primer name	**Sequence (5' - 3')**
pENTR_*AdoIspS*_short_*For*	CACCACTAGTGAGCAGCGGCGTTCGGCGA
pENTR_*AdoIspS*_long_*For*	CACCACTAGTGTGATGGCGTCGAAGCAGC
pENTR_*AdoIspS*_short /long_*Rev*	GGATCCAAATATACATGGTTCCAAGAAAAGC

The recombinant plasmids were transformed into *N. gaditana* through electroporation as previously described (Hamed et al., 2017). Briefly, the *N. gaditana* cells were harvested at late log phase and washed three times with sorbitol. After cell resuspension, the mixture of cells and linearized DNA was subjected to electroporation. After recovery under dim light for 24 h, the cells were plated on plates with specific selection agents. After incubation at 25°C under constant light for 4-6 weeks, colonies were appeared and selected for flow cytometry analysis.

2.9 Flow cytometry assessment

The flow cytometry assay was conducted by Prof. Tomas Morosinotto's group. In brief, the colonies were grown for 10 days on non-selective plates and diluted to around $5*10^5$ cells/mL with deionized water individually. Then the cells were subjected to flow cytometry analysis. YFP fluorescence was detected with a FITC (fluorescein isothiocyanate) filter (excitation, 505 nm; emission, 530/30 nm). Signals for forward scatter (FSC), side scatter (SSC), FITC were recorded.

2.10 Isoprene emission screening

After the preculture of *N. gaditana* at 25°C for 48 h under continuous light, the cell culture was aliquoted into 20 mL vials with fresh F/2 medium to make the initial OD_{600} of 0.3-0.4, 0.6-0.8 or 0.5-1.0. Then the sealed culture was grown at different temperatures under continuous illumination of 130-150 μmol m^{-2} s^{-1} for a period of time (25°C for 42 h, 28°C for 42 h, 30°C for 25 h or 50 h). At the end of culture, the isoprene in the gas phase was determined with PTR-ToF 8000 apparatus (Ionicon Analytik GmbH, Innsbruck, Austria). The data was converted to parts per billion volume (ppbv) and the concentration of isoprene was calculated by normalizing the measured data with the corresponding OD_{600} value.

3 Results and discussion

3.1 Identification of novel isoprene synthases from palms and expression in *E. coli*

A variety of species from Arecaceae family are high isoprene emitters (Li et al., 2020b). Here, three independent isoprene synthase genes were isolated from the following species: *Chamaerops humilis*, *Sabal minor* and *Copernicia prunifera*. The diagnostic tetrad residues (FVFT) of Arecaceae IspS were found in these three IspS genes by sequence alignment. The amino acid sequence identity among the IspS proteins is around 89%–93%.

The heterologous expression and enzymatic characterization of several isoprene synthases have been achieved in *E. coli* (Miller et al., 2001; Schnitzler et al., 2005; Yeom et al., 2018; Li et al., 2019b; Oku et al., 2015). *E. coli* is one of the ideal hosts for expressing recombinant proteins due to its fast growth rate, continuous fermentation capacity, available molecular tools and high expression levels (Joseph et al., 2015). However, it also has some disadvantages. For example, rapid expression of heterologous proteins may lead to problems of unfolding/misfolding, and a lack of post-translational modifications may affect the activities of eukaryotic proteins (Francis et al., 2010).

To assess isoprene production in *E. coli*, we tried to express these three recombinant isoprene synthases in *E. coli* and optimize its soluble expression by using different expression vectors and *E. coli* strains and the induction conditions including post-induction temperature, cell density of induction (OD_{600}) and inducer (IPTG) concentration. In addition, different media additives (ethanol, Mg^{2+} and Triton X-100) and heat shock prior to induction were also tested.

3.1.1 IspS expression results under different post-induction temperatures

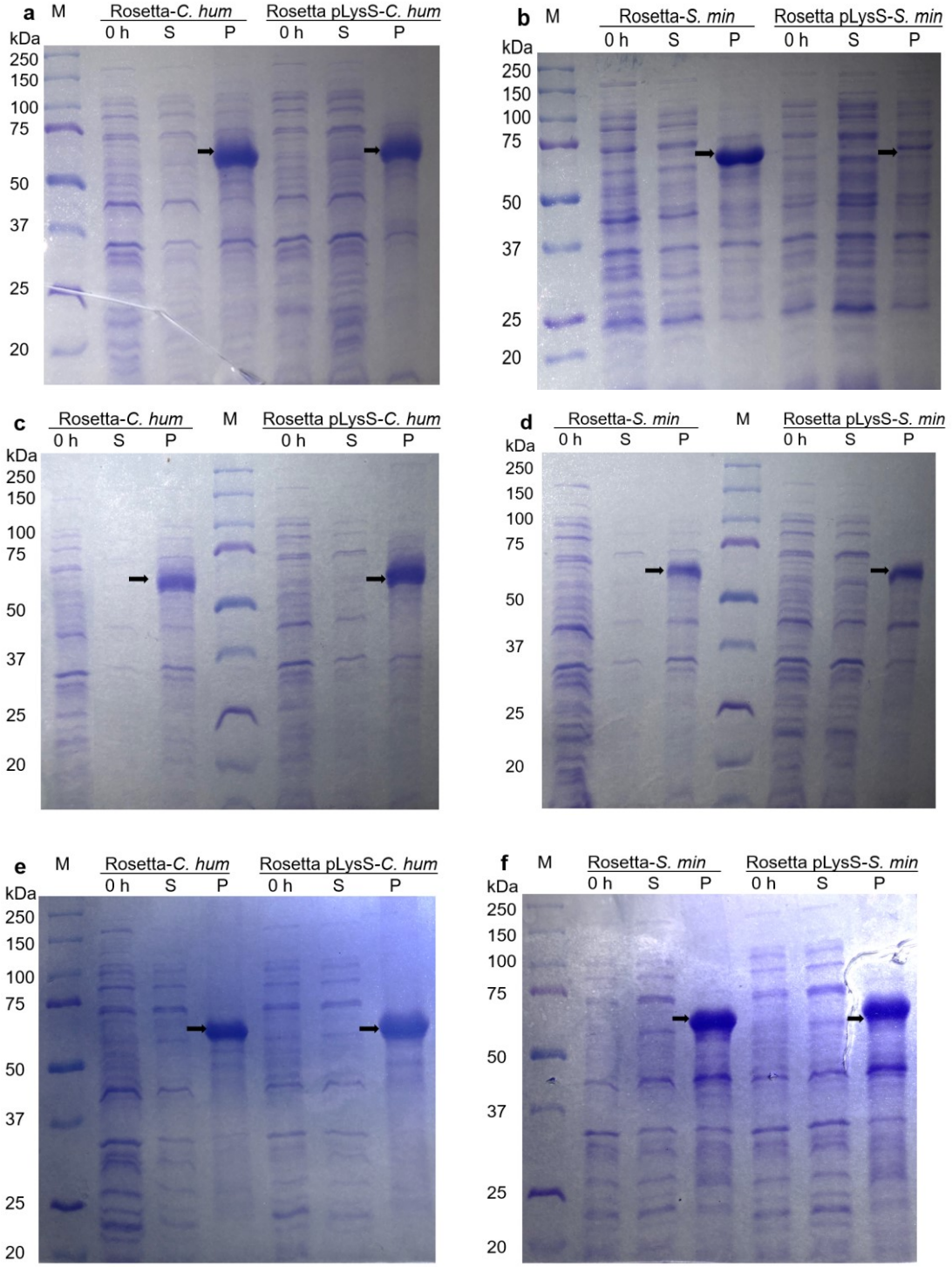


Fig. 3.1.1 SDS-PAGE analysis of the supernatant (S) and pellets (P) of Rosetta(DE3) and Rosetta(DE3)pLysS transformed with pET28b-*chumIspS* and pET28b-*SminIspS* at different temperatures. M: protein molecular weight markers. a) and b): cultures were inducted when

OD_{600} reached 0.6, with 0.2 mM IPTG, at 20°C for 12 h; c) and d): cultures were inducted when OD_{600} reached 0.8, with 2 mM IPTG, at 34°C for 3 h; e) and f): cultures were inducted when OD_{600} reached 0.06, with 0.1 mM IPTG, at 37°C for 6 h. IspSs were denoted by arrows.

Post-induction temperature is an important factor affecting heterologous gene expression in *E. coli* (Moradian et al., 2013). In general, higher post-induction temperature results in faster cell growth and higher protein expression, but may lead to protein aggregation (Hayat et al., 2018). Lower post-induction temperature can increase the solubility of recombinant protein, but as the temperature decreases, the cell growth rate reduces and correspondingly gene transcription and translation will also slow down, requiring longer induction time to obtain sufficient protein accumulation (Higuchi et al., 2020). Here, the post-induction temperature and induction time were as follows: 20°C for 12 h, 34°C for 3 h and 37°C for 6 h. The Rosetta(DE3) and Rosetta(DE3)pLysS strains were often used to enhance expression of eukaryotic proteins that contain rarely used codons in *E. coli* (Joseph et al., 2015). The pET-28b(+) was applied for expression of isoprene synthases according to the previous studies (Zurbriggen et al., 2012; Sharkey et al., 2013). Six consecutive histidine residues (His-tag) at N-terminus were fused with the recombinant isoprene synthase to improve the protein purity.

The recombinant *SminIspS* is about 65 kDa. As shown in the Fig. 3.1.1, the expression of *SminIspS* was significantly enhanced at 37°C in the Rosetta(DE3) and Rosetta(DE3)pLysS strains. But the target protein was insoluble as it was mostly found in the pellet when induced at 20°C, 34°C and 37°C. As expected, traces of target protein could be seen in the supernatant at 20°C indicating a little higher protein solubility. However, the effect was still not ideal, so we continued to optimize other induction conditions.

3.1.2 IspS expression under different cell densities of induction (OD_{600})

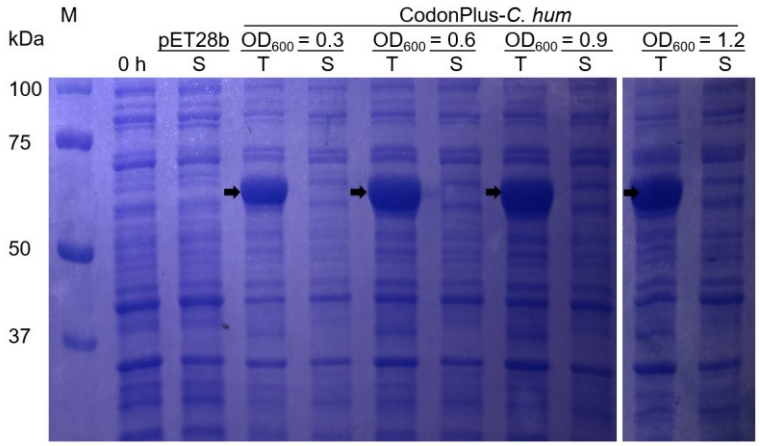


Fig. 3.1.2 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of BL21-CodonPlus transformed with pET28b-*ChumIspS* induced at different OD_{600}. Cultures were induced with 0.2 mM IPTG, at 15°C for 15 h. IspSs were denoted by arrows.

The cell density (OD_{600}) at induction phase also affected the protein expression in *E. coli* (Zhang et al., 2009). Here, the expression of the recombinant pET28b-Chum*IspS* in BL21-CodonPlus was induced when the OD_{600} reached 0.3, 0.6, 0.9 and 1.2 at 15°C. It could be seen that expression level of the target protein (~65 kDa) was obviously higher when the IPTG was added at OD_{600} higher than 0.6 (Fig. 3.1.2). While in all cases, the target protein expression in the supernatant was not evident when compared with the transformants containing empty vector pET28b. Thus, the OD_{600} (ranging from 0.3 to 1.2) didn't significantly affect the soluble expression of Chum*IspS*.

3.1.3 IspS expression under different concentrations of IPTG

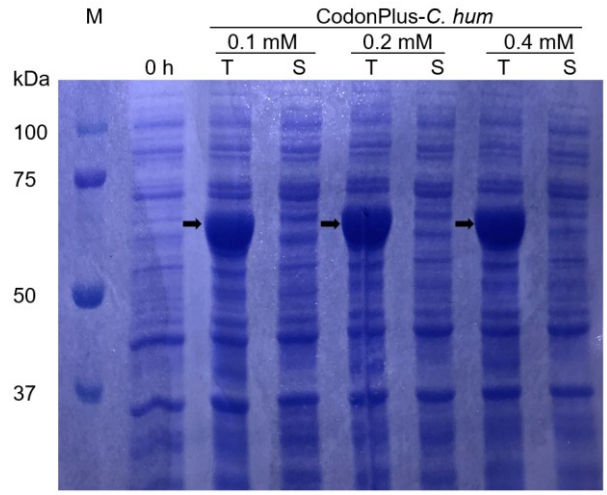


Fig. 3.1.3 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of BL21-CodonPlus transformed with pET28b-*ChumIspS* with different concentrations of IPTG. Cultures were induced when OD_{600} reached 0.6, at 15°C for 15 h. IspSs were denoted by arrows.

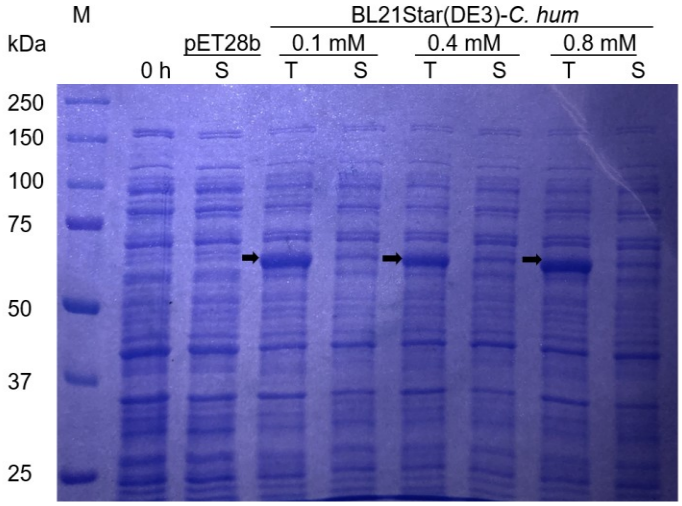


Fig. 3.1.4 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of BL21Star(DE3) transformed with pET28b-*ChumIspS* with different concentrations of IPTG. Cultures were induced when OD_{600} reached 0.6, at 15°C for 15 h. IspSs were denoted by arrows.

The concentration of IPTG affects soluble protein production (Francis et al., 2010). The expression of *ChumIspS* was induced with varying IPTG concentrations (0.1, 0.2, 0.4, 0.8 mM) when OD_{600} reached 0.6 at 15°C in BL21-CodonPlus (Fig. 3.1.3) and BL21Star(DE3) (Fig. 3.1.4). As shown in Fig. 3.1.3, the *ChumIspS* was highly induced

in all cases. Although the target band was pretty strong in the total lysate, the expression level in the supernatant was much less, indicating that the IPTG concentration was too high and the target protein was mostly insoluble.

Different expression responses to IPTG concentration were observed in BL21-CodonPlus and BL21Star(DE3) as the target band differed greatly under the same induction conditions. However, the expression optimization in BL21Star(DE3) was still ineffective because the intensities of target bands in the supernatant were not strong in pET28b-*ChumIspS* transformants compared with the empty vector transformants.

3.1.4 IspS expression using different vectors

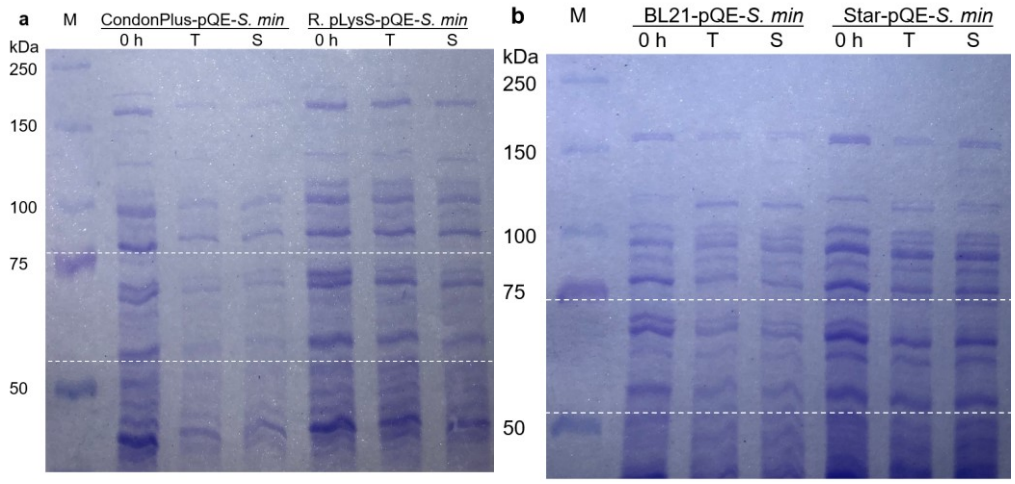


Fig. 3.1.5 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of the pQE30Xa-*SminIspS* transformants. Cultures were induced when OD_{600} reached 0.6, with 0.2 mM IPTG, at 15°C for 15 h. a): in BL21-CodonPlus and Rosetta(DE3)pLysS strains; b) in BL21(DE3) and BL21Star(DE3) strains. The target protein bands were supposed to appear in the area marked by the white dashed lines.

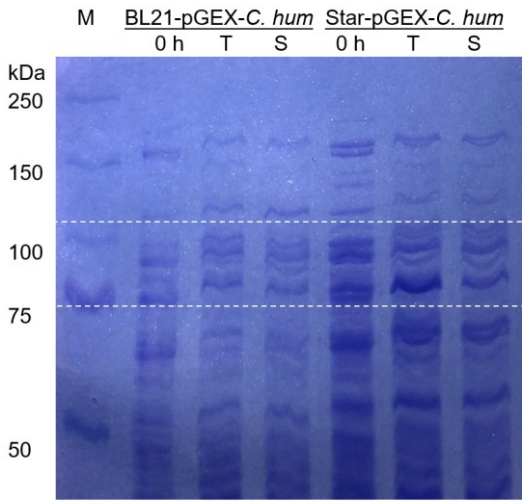


Fig. 3.1.6 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of BL21(DE3), and BL21Star(DE3) transformed with pGEX4T1-*ChumIspS*. Cultures were induced when OD_{600} reached 0.6, with 0.8 mM IPTG, at 25°C for 15 h.

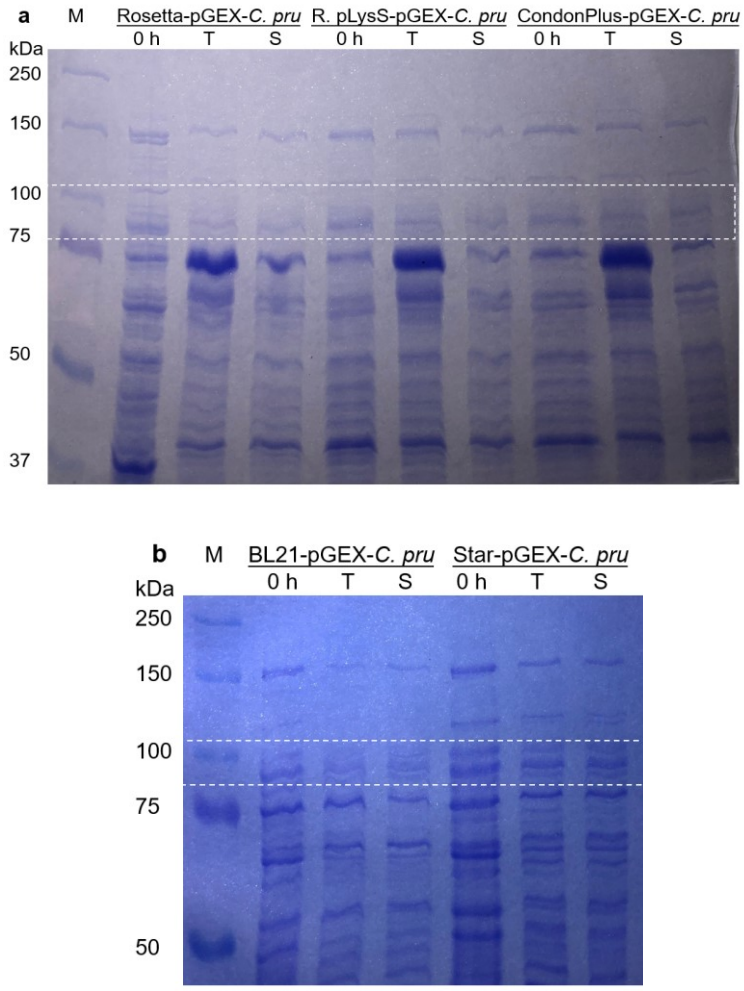


Fig. 3.1.7 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of the pGEX4T1-

CpruIspS transformants. Cultures were induced when OD_{600} reached 0.6, with 0.2 mM IPTG, at 15°C for 15 h. a): in Rosetta(DE3), Rosetta(DE3)pLysS and BL21-CodonPlus strains; b): in BL21(DE3) and BL21Star(DE3) strains.

Different vectors including pQE-30 Xa and pGEX-4T-1 were used to optimize isoprene synthase expression. The pQE-30 Xa allowed the recombinant expression of isoprene synthase with N-terminal fusion of 6×His-tag and the recombinant protein was around 65 kDa. As shown in Fig. 3.1.5, there was no significant target protein expression in both total lysate and supernatant among all strains used including BL21-CodonPlus, Rosetta(DE3)pLysS, BL21(DE3) and BL21Star(DE3), indicating that pQE-30 Xa was not suitable for effective expression of *SminIspS*.

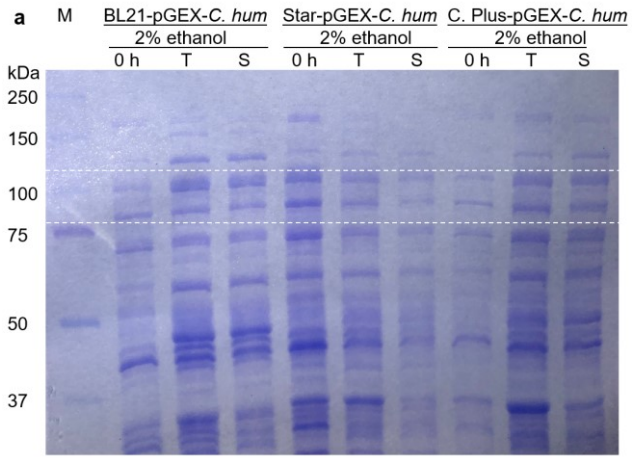
In some cases, the use of affinity tags such as maltose-binding protein (MBP) and glutathione *S*-transferase (GST) can effectively increase the solubility of recombinant proteins (Rabhi-Essafi et al., 2007; Hayat et al., 2018). Thus, pGEX-4T-1 was also used to express isoprene synthase in fusion with an N-terminal GST tag and the recombinant protein was around 91 kDa. As shown in Fig. 3.1.6, the expression optimization applying pGEX-4T-1 didn't work out with *ChumIspS* as there were no significant target bands in both total lysate and supernatant in all strains tested including BL21(DE3) and BL21Star(DE3). Similarly, for the recombinant pGEX4T1-*CpruIspS*, the target bands were not observed in both total lysate and supernatant in the strains of Rosetta(DE3), Rosetta(DE3)pLysS, BL21-CodonPlus, BL21(DE3) and BL21Star(DE3) (Fig. 3.1.7).

3.1.5 IspS expression using different additives or heat shock

The solubility of recombinant protein could be enhanced by utilizing the media additives such as NaCl, sorbitol, thiols and disulfides to help proteins fold properly, enhance proteins stabilities and prevent formation of inclusion bodies (Kaur et al., 2018).

For example, a certain amount of ethanol could enhance the soluble production of recombinant protein without structural damage by altering membrane properties and DNA synthesis resulting in gene amplification (Priyanka et al., 2022). The increased solubilities of target proteins were achieved by adding some metal ions especially Mg^{2+}, which might be related to the improvement of the cellular metabolic status (Yang et al., 2003; Sina et al., 2015). As a surfactant, Triton X-100 could affect lipid membrane structures and help to increase protein solubility subtly (Singh et al., 2017). Considering that membrane-bound isoprene synthase proteins were found in many species (Lehning et al., 1999; Schnitzler et al., 2005; Wiberley et al., 2005; Wildermuth et al 1998), the Triton X-100 might have positive effects on the soluble expression of isoprene synthases.

Besides, heat shock prior to induction was also an efficient way to enhance some recombinant protein solubilities (Chen et al., 2002; Kaur et al., 2018). Thus, we investigated the effects of different additives (2% ethanol, 0.1 mM Mg^{2+}, 1% Triton X-100) and heat shock on the soluble expression of isoprene synthases. It turned out that there was still no obvious expression of target recombinant proteins including pGEX4T1-*ChumIspS*, pQE30Xa-*SminIspS* and pGEX4T1-*CpruIspS* with or without 2% ethanol (Fig. 3.1.8). Similar results were observed in the transformants containing pGEX4T1-*ChumIspS* after addition of Mg^{2+} or Triton X-100 or heat shock treatment (Fig. 3.1.9).



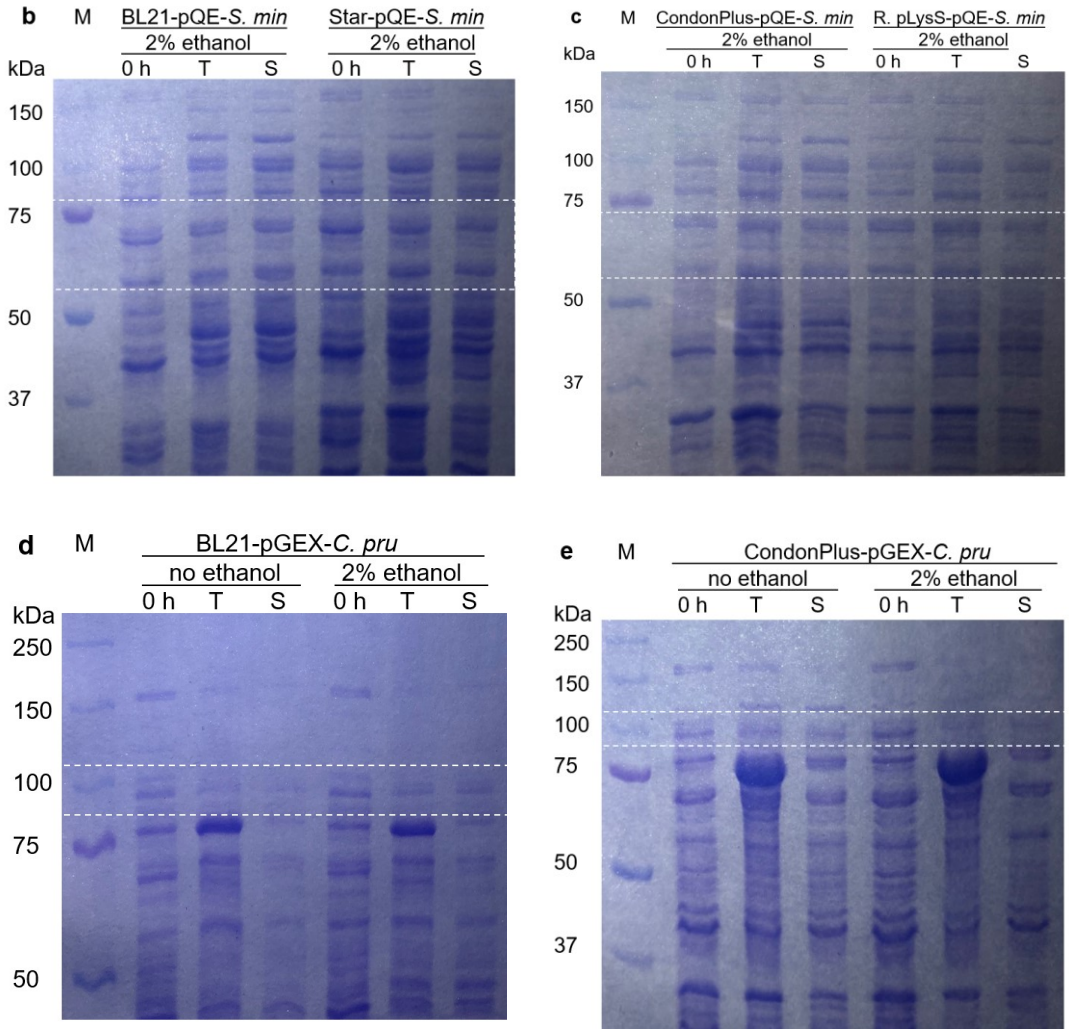


Fig. 3.1.8 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of cultures added with 2% ethanol. a): in BL21(DE3), BL21Star(DE3) and BL21-CodonPlus strains transformed with pGEX4T1-*ChumIspS*; b) and c): in BL21(DE3), BL21Star(DE3), BL21-CodonPlus and Rosetta(DE3)pLysS strains transformed with pQE30Xa-*SminIspS*; d) and e): in BL21(DE3) and BL21-CodonPlus strains transformed with pGEX4T1-*CpruIspS*. a), b) and c): cultures were induced when OD_{600} reached 0.6, with 0.8 mM IPTG, at 25°C for 15 h; d) and e): cultures were induced when OD_{600} reached 0.6, with 0.8 mM IPTG, at 30°C for 15 h.

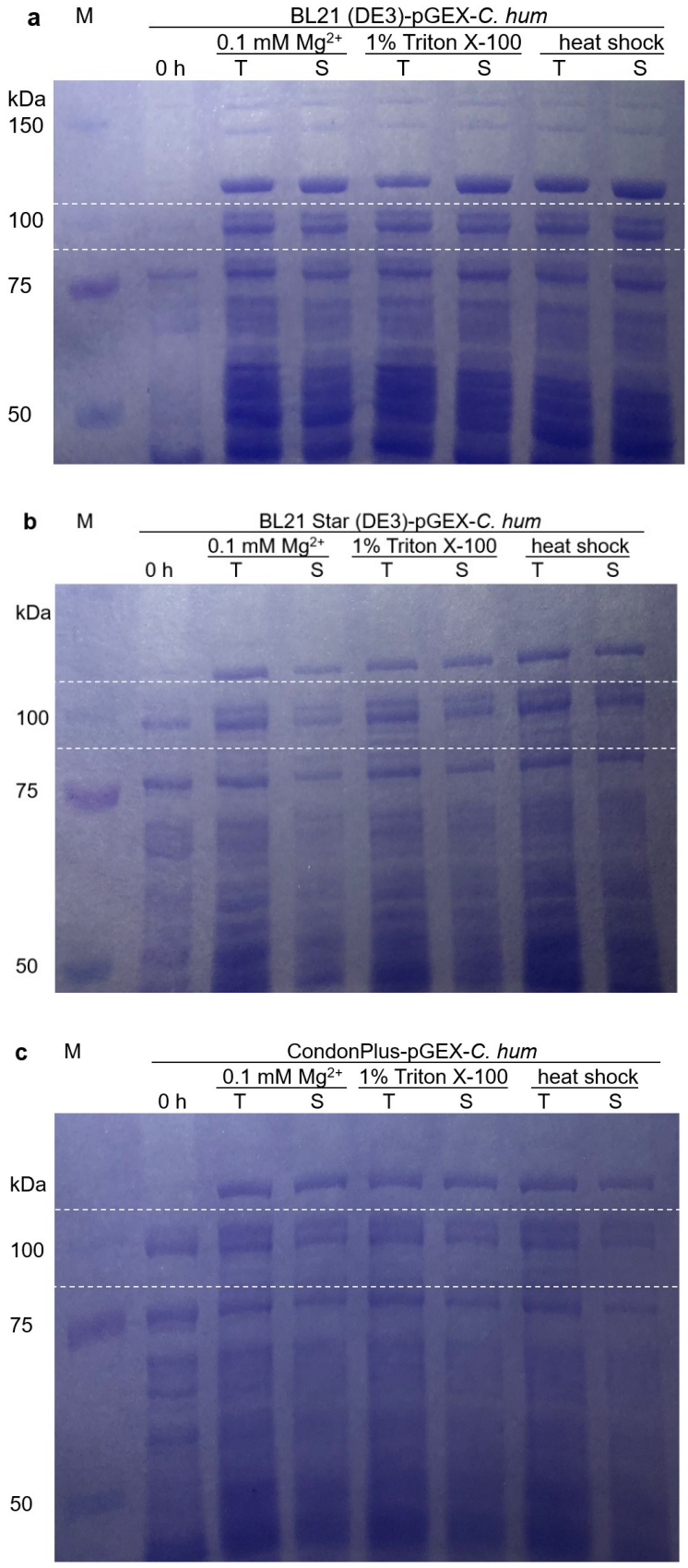


Fig. 3.1.9 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of the pGEX4T1-*CpruIspS* transformants cultures added with 0.1 mM Mg^{2+}, 1% Triton X-100 or treated with heat

shock. For addition of Mg^{2+} or Triton X-100, cultures were induced when OD_{600} reached 0.6, with 0.2 mM IPTG, at 15°C for 15 h. a): in BL21(DE3) strains; b): in BL21Star(DE3) strains; c): in BL21-CodonPlus strains.

3.1.6 IspS expression without any tags

According to the results described above, the recombinant proteins of *ChumIspS* and *SminIspS* in fusion with N-terminal 6×His tag were overexpressed leading to the large formation of inclusion bodies. The soluble expression was not improved by changing the host strains, induction temperature, cell density upon induction and IPTG concentrations.

Although the His tag was commonly used for efficient purification of heterologous protein, it might cause improper folding and reduced solubility of target protein, but the effects were target protein specific (Woestenenk et al., 2004; Tham et al., 2020; Li et al., 2009). What's more, the N- or C-terminal His-tag influenced the biochemical properties of isoprene synthase indicated in hybrid poplar (*Populus×canescens*) in terms of temperature, pH, and substrate dependence (Schnitzler et al., 2005).

Thus, we constructed the recombinant plasmids of pET28(no tags)-*ChumIspS* expressing target isoprene synthases without His tags. The recombinant proteins were expressed in different host strains shown in Fig. 3.1.10. The pET28(no tags)-*ChumIspS* was partially soluble in BL21Star(DE3) and Rosetta(DE3)pLysS as the target bands appeared in the supernatant. Then the soluble expression in Rosetta(DE3)pLysS were further optimized with different cell densities for induction (OD_{600}) (Fig. 3.1.11) and concentrations of IPTG (Fig. 3.1.12). As shown in Fig. 3.1.11, more target protein was expressed with OD_{600} increasing, indicating that the low protein production was probably related to the low growth rate. When the induction started at OD_{600} of 0.5, the target protein expression in the total lysate increased faster than the one in the supernatant, indicating that the high growth rate interfered the proper folding of target proteins.

Therefore, OD_{600} of 0.4 was selected for the initiation of induction, the concentration of IPTG was further optimized. According to Fig. 3.1.12, as the concentration of IPTG was increased from 0.05 mM to 0.2 mM, the increased expression of isoprene synthase could be observed in both total lysate and supernatant. But when the concentration of IPTG was increased from 0.2 mM to 0.8 mM, there were no significant changes of target bands in both total lysate and supernatant. Thus, the induction conditions (OD_{600} of 0.4, 0.2 mM IPTG, at 15°C for 15 h) were suitable for expressing the recombinant *ChumIspS* without his tags in Rosetta(DE3)pLysS.

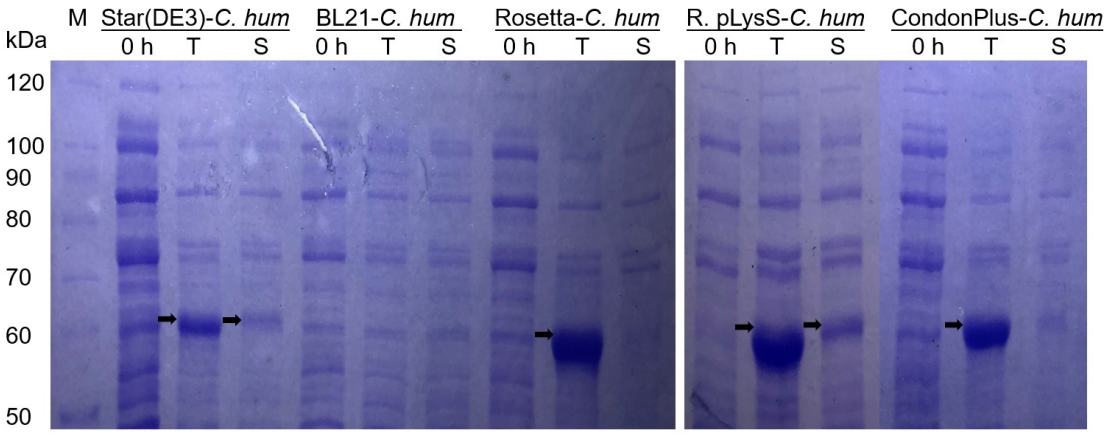


Fig. 3.1.10 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of the pET28(no tags)-*ChumIspS* transformants in different *E. coli* strains. Cultures were induced when OD_{600} reached 0.4, with 0.4 mM IPTG, at 20°C for 15 h.

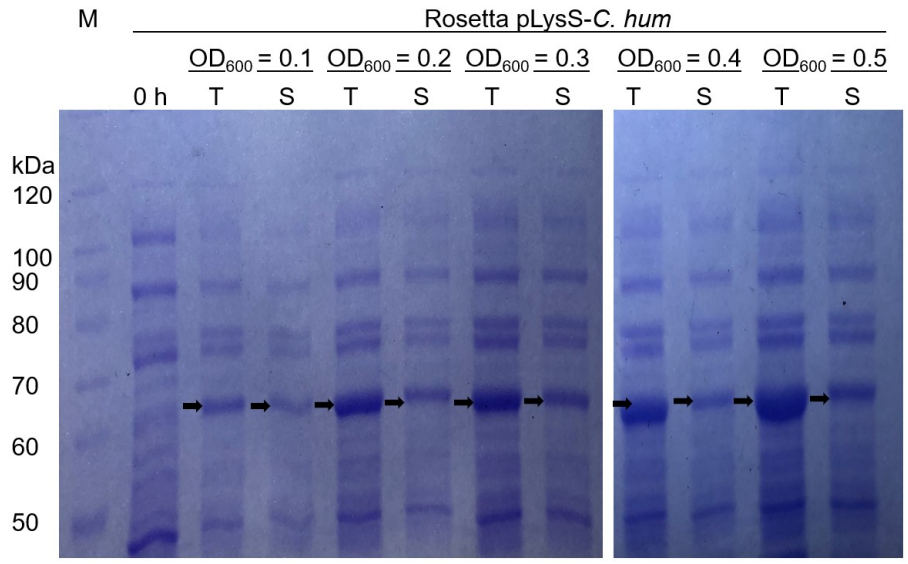


Fig. 3.1.11 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of the Rosetta pLysS transformed with pET28(no tags)-*ChumIspS* induced at different OD_{600}. Cultures were induced with 0.4 mM IPTG, at 15°C for 15 h.

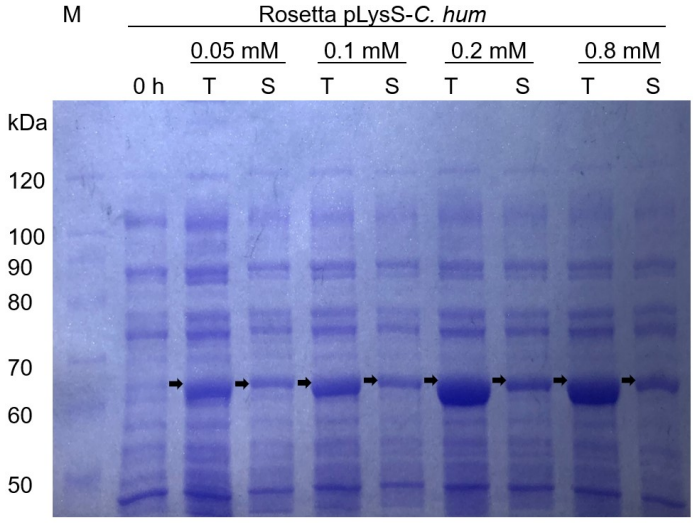


Fig. 3.1.12 SDS-PAGE analysis of the total lysate (T) and supernatant (S) of the Rosetta pLysS transformed with pET28(no tags)-*ChumIspS* with different concentration of IPTG. Cultures were induced when OD_{600} reached 0.4, at 15°C for 15 h.

3.1.7 Discussion

Heterologous expression of eukaryotic proteins in *E. coli* remains technical challenges in terms of expression levels, protein folding and post-translational modifications (Von Schaewen et al., 2018). In this study, the target isoprene synthases including

ChumIspS, *SminIspS* and *CpruIspS* were hardly expressed when cloned into the expression vectors such as pGEX-4T-1 and pQE-30 Xa. Heat shock prior to induction and different media additives including ethanol, Mg^{2+} and Triton X-100 were applied to optimize the expression, but few effects were observed.

When cloned into pET-28b(+), the recombinant *ChumIspS* and *SminIspS* were expressed in fusion with 6×His tags at N-terminus for simplifying purification. However, both the target isoprene synthases were over-expressed and mostly aggregated into inclusion bodies. Although the inclusion bodies could be solubilized and refolded to bioactive proteins, the recovery yield might be low and the protein integrity may get affected (Malekian et al., 2019; Tham et al., 2020). So, we focused on optimizing the soluble expression of isoprene synthases. Different induction conditions (temperature, cell density of induction and inducer concentration) and host strains were tested, while the target proteins were still hardly soluble. By cloning *ChumIspS* into pET-28b(+) without fusion of any His tags and optimization of induction-starting time and IPTG concentration, target bands were observed in the supernatant. It indicated that fusion of His tags with isoprene synthase could interfere the protein solubility. Besides, introducing affinity tags had a greater impact on the soluble expression of *ChumIspS* than optimizing induction conditions, so the vectors with different affinity tags should be chosen carefully.

Except for the *ChumIspS*, the soluble expression of *SminIspS* and *CpruIspS* were not achieved in *E. coli*. Thus, further protein purification and enzymatic characterization could not be achieved. It was therefore considered to use other models such as *Arabidopsis thaliana*, a widely used model of developmental and molecular plant biology, to carry out heterologous expression of isoprene synthases (Von Schaewen et al., 2018; Xu et al., 2020; Li et al., 2021).

3.2 IspS expression in *Arabidopsis thaliana*

3.2.1 Isoprene emission screening of IspS transgenic Arabidopsis

The isoprene emission abilities of T2 transgenic Arabidopsis with *ChumIspS*, *CpruIspS* or *SminIspS* were screened with 4-week-old rosette leaves by PTR-MS. For each construct, around 96 independent transgenic lines were used. As shown in Fig. 3.2.1, for the constructs of *ChumIspS* and *CpruIspS*, there were several transgenic lines with relatively high isoprene emission levels (>4 ppbv/mg), indicating that the IspS enzyme properly functioned in transgenic Arabidopsis.

For the construct of *SminIspS*, the majority of transgenic lines were found to emit a pretty low level of isoprene (<1 ppbv/mg), which might be related to the relatively low catalytic activity of *SminIspS* or poor transgene expression level due to position effects caused by random integration. For the Col-0 control, the isoprene emission level was less than 0.05 ppbv/mg. As there is no IspS gene in Arabidopsis, the limited isoprene emission of Col-0 might be from the non-catalytic lysis of DMAPP or other background noise.

Thus, we used the *ChumIspS* and *CpruIspS* transgenic Arabidopsis to further study the physiological responses of isoprene emitting plants under abiotic stresses. After screening for the T2 plants that carry a single copy of the integrated transgene, several single-copy transgenic lines with relatively higher isoprene emission were sown into soil to collect T3 seeds. Then the homozygous T3 seeds were used in the subsequent physiological studies.

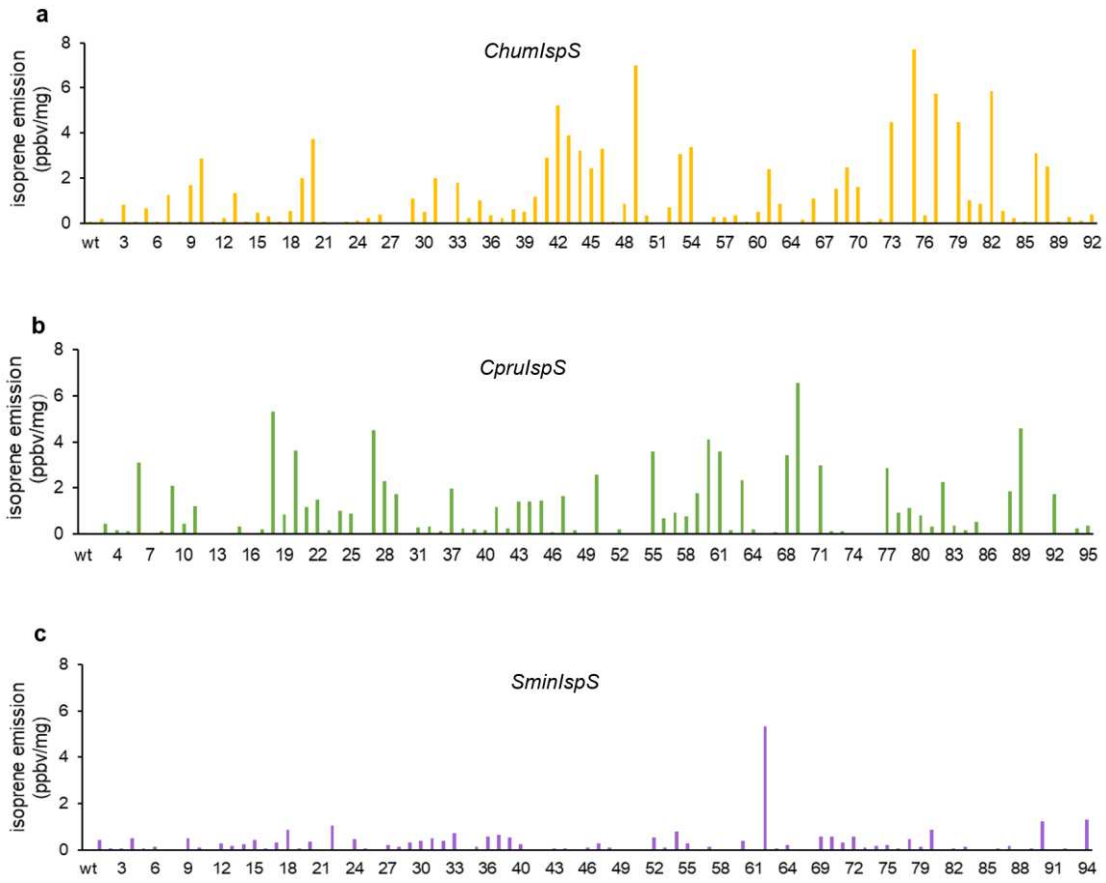


Fig. 3.2.1 Isoprene emission screening of independent T2 transgenic Arabidopsis overexpressing *ChumIspS* (a), *CpruIspS* (b), *SminIspS* (c) with wild type Col-0 (wt) as control.

3.2.2 No obvious phenotypic differences among wild type, *ChumIspS* and *CpruIspS* transgenic plants under salinity stress

The protective role of isoprene under abiotic stresses is widely accepted and a majority of studies focus on heat and oxidative stresses. In nature, plants are faced with more complicated conditions and environmental stresses such as salinity and drought stresses (Golldack et al., 2014). Salt stress causes severe problems to agriculture. For example, millions of hectares of land suffer from salinization, causing retarded plant growth and reduced production (Thi et al., 2017). With impending global warming, the frequency and duration of drought periods will increase, threatening the sustainable agriculture of the current century (Osmolovskaya et al., 2018). When plants face abiotic

stresses, abscisic acid (ABA) mediates plant responses as a chemical signal (Yang et al., 2018). In previous studies, *IspS* transgenic lines were more tolerant to dehydration by enhancing ABA sensitivity (Xu et al., 2020). However, further understanding is needed for the mechanism of isoprene-mediated ABA response and abiotic stress tolerance. Here we explored the isoprene emission effects under salinity, ABA and mannitol-induced drought stresses with *ChumIspS* or *CpruIspS* transgenic Arabidopsis.

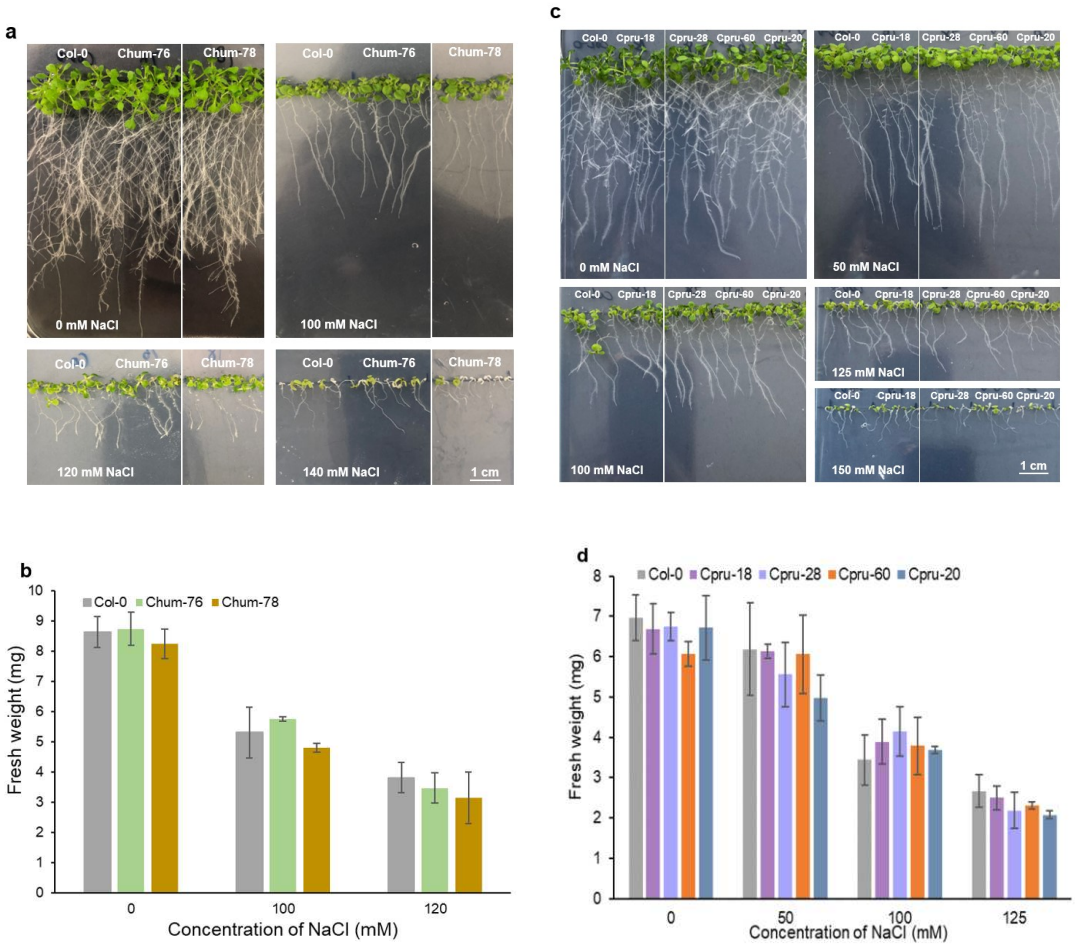


Fig. 3.2.2 Effects of NaCl on the phenotypes of Col-0 and *IspS* transgenic plants. a): images of Col-0 and *ChumIspS* transgenic plants growing vertically on the medium containing 0 mM, 100 mM, 120 mM or 140 mM NaCl for 15 days; b): corresponding individual fresh weight of Col-0 and *ChumIspS* transgenic plants; c): images of Col-0 and *CpruIspS* transgenic plants growing vertically on medium containing 0 mM, 50 mM, 100 mM, 125 mM or 150 mM NaCl for 11 days; d): corresponding individual fresh weight of Col-0 and *CpruIspS* transgenic plants.

Seeds germination and seedlings growth are widely used as indicators of abiotic stress

responses (Liu et al., 2015; Yang et al., 2019). Here the seedlings growth of *ChumIspS* and *CpruIspS* transgenic lines were compared with Col-0 under different concentrations of NaCl.

The seeds were sterilized and sown onto the medium and the phenotypes of Col-0 and *IspS* transgenic lines were compared after 11- or 15-day growth. Two replicates were conducted for each concentration as a test. As shown in Fig. 3.2.2a, there was no obvious phenotypic differences between Col-0 and *ChumIspS* transgenic lines (*Chum*-76 and *Chum*-78) under salinity treatment (0 mM, 100 mM, 120 mM, 140 mM NaCl). Correspondingly, the fresh weight of Col-0 and two *ChumIspS* transgenic lines were similar at 0 mM, 100 mM and 120 mM NaCl (Fig. 3.2.2b). When the concentration of NaCl was increased to 140 mM, the fresh weight determination was interfered with by the tiny and bleached plants, so it was not shown in the results.

Similar results were also observed between Col-0 and four *CpruIspS* transgenic lines (*Cpru*-18, *Cpru*-28, *Cpru*-60 and *Cpru*-20) in terms of phenotype (Fig. 3.2.2c) and fresh weight (Fig. 3.2.2d) under salinity treatment (0 mM, 50 mM, 100 mM, 125 mM, 150 mM NaCl).

3.2.3 No obvious phenotypic differences among wild type and *ChumIspS* transgenic plants under ABA treatment

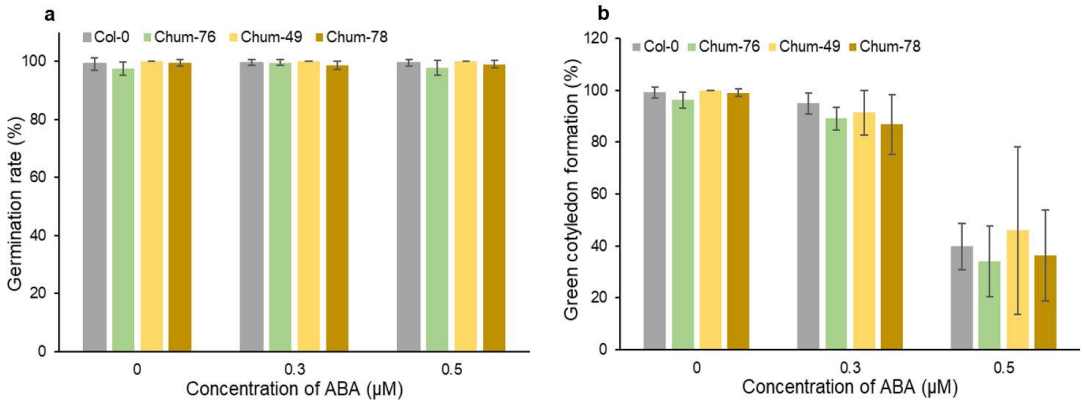


Fig. 3.2.3 Effects of ABA on seeds germination (a) and green cotyledon formation (b) of Col-0 and *ChumIspS* transgenic plants. Error bars correspond to the standard deviation of at least 4

replicates.

To evaluate the ABA effects at germination stage, seeds germination and green cotyledon formation of Col-0 and *ChumIspS* transgenic lines (*Chum*-76, *Chum*-49 and *Chum*-78) growing on the medium supplemented with ABA (0 μM, 0.3 μM, 0.5 μM) were recorded every day for 7 days. For germination rate, there was no significant difference among Col-0, *Chum*-76 and *Chum*-49 at the same ABA concentration every day. The germination rate of *Chum*-78 was significantly higher than that of Col-0 on the second day at 0.3 μM ABA and the second and third day at 0.5 μM ABA, while no significant difference was observed on the following days. For green cotyledon formation rate, there was no significant difference among Col-0 and three *ChumIspS* transgenic lines every day. After ABA treatment for 7 days, no significant difference in seeds germination rate (Fig. 3.2.3a) and green cotyledon formation rate (Fig. 3.2.3b) was observed among Col-0 and three *ChumIspS* transgenic lines.

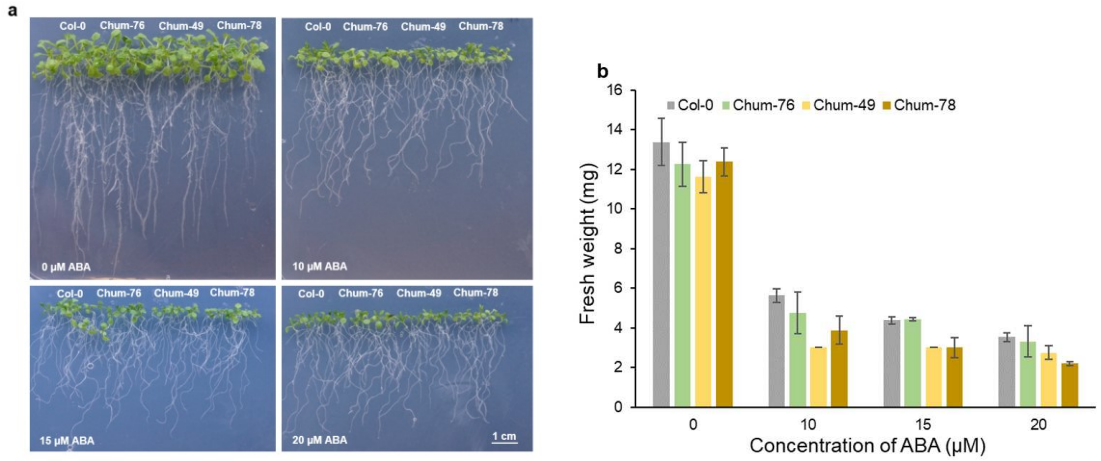


Fig. 3.2.4 Effects of ABA on the phenotypes of Col-0 and *ChumIspS* transgenic plants. a): images of Col-0 and *ChumIspS* transgenic plants transferred to the medium containing ABA at 3 days old stage and grown vertically for another 11 days; b): corresponding individual fresh weight of Col-0 and *ChumIspS* transgenic plants.

The ABA effects at post-germination stage were also studied. 3-day-old seedlings growing on 1/2 MS medium were transferred to the same medium containing ABA (0 μM, 10 μM, 15 μM or 20 μM) and grown for another 11 days. Two replicates were performed for each concentration as a test. As shown in Fig. 3.2.4a, there were no

apparent phenotypic differences between Col-0 and the three *ChumIspS* transgenic lines as they appeared similar in root length, aerial part size and color. At 0 μM and 20 μM ABA, the fresh weight of Col-0 and three *ChumIspS* was similar (Fig. 3.2.4b). At 10 μM and 15 μM ABA, the fresh weight of Col-0 and *Chum*-76 was similar and relatively higher than *Chum*-49, *Chum*-78.

3.2.4 Enhanced tolerance of *CpruIspS* transgenic plants to drought stress

3.2.4.1 Phenotypes comparison at germination stage

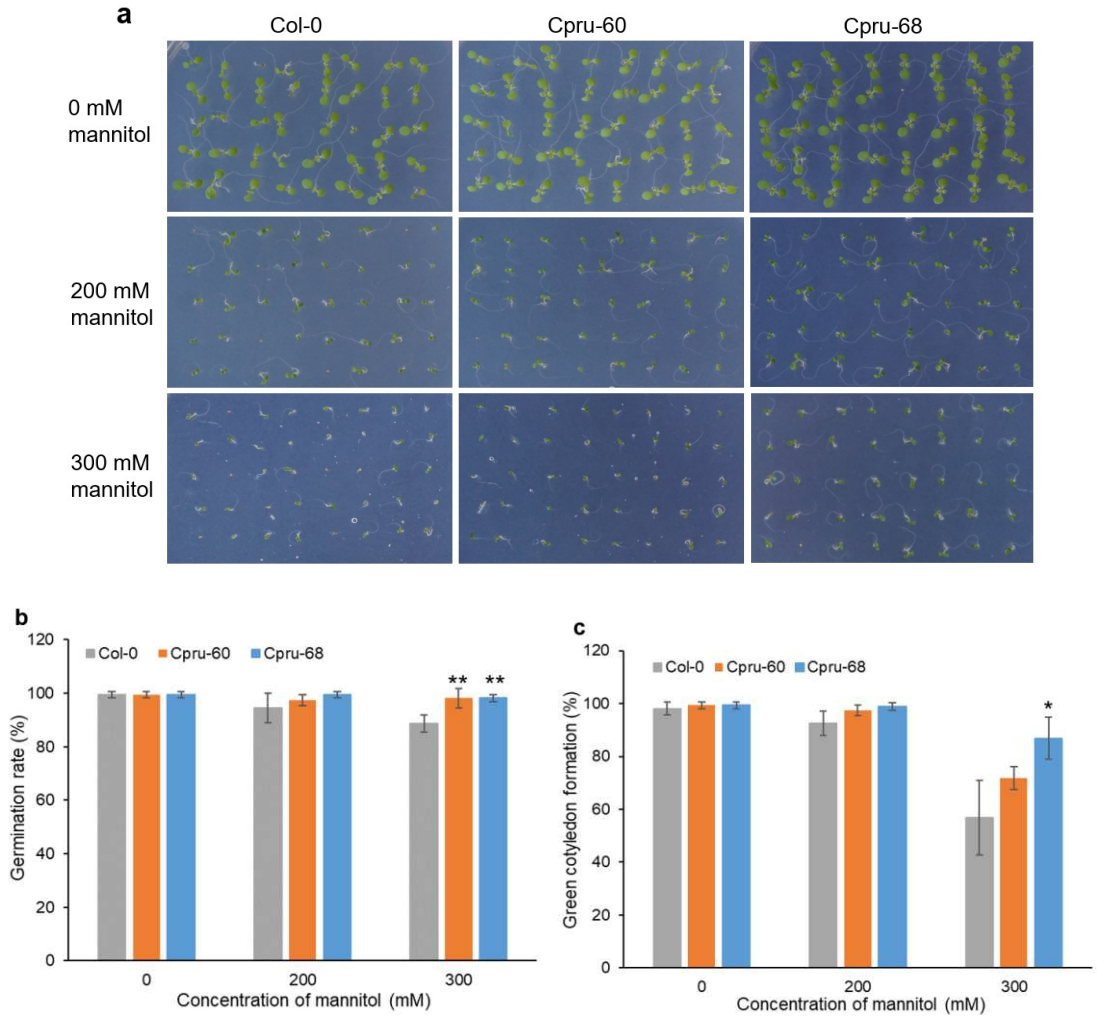


Fig. 3.2.5 Mannitol effects at the germination stage. a): images of Col-0 and *CpruIspS* transgenic plants growing on the medium containing mannitol (0 mM, 200 mM, 300 mM) for 7

days. Germination rate (b) and green cotyledon formation rate (c) of Col-0 and *CpruIspS* transgenic plants growing on the medium containing mannitol (0 mM, 200 mM, 300 mM) for 7 days. Asterisks indicate a statistically significant difference between transgenic line and Col-0 (**P < 0.01, *P < 0.05, Student's *t* test).

As an osmotic agent, mannitol can reduce the water potential of the medium, making it harder for plants to absorb water. Mannitol is often used in plant research to simulate drought stress (Wang et al., 2020). The mannitol-induced osmotic stress responses of *CpruIspS* transgenic lines were investigated at germination stage (Fig. 3.2.5). At 0 mM mannitol, there was no obvious difference in seeds germination rates of all lines each day. After 7-day growth on medium containing 200 mM mannitol, the germination rates of *Cpru*-60 (97.5%) and *Cpru*-68 (99.4%) were slightly higher than that of Col-0 (94.4%) (Fig. 3.2.5b). After 7-day growth on medium containing 300 mM mannitol, the germination rates of *Cpru*-60 (98.1%) and *Cpru*-68 (98.1%) were significantly higher than that of Col-0 (88.8%).

At 0 mM mannitol, green cotyledon formation rates of all lines were similar each day (Fig. 3.2.5c). At 200 mM mannitol, the green cotyledon formation rates of *Cpru*-60 (97.5%) and *Cpru*-68 (98.8%) were slightly higher than that of Col-0 (92.5%) after 7-day growth. At 300 mM mannitol, the green cotyledon formation rates of *Cpru*-60 (71.9%) and *Cpru*-68 (86.9%) were moderately or significantly higher than that of Col-0 (56.9%) respectively.

3.2.4.2 Phenotypes comparison and potential molecular mechanisms at post-germination stage

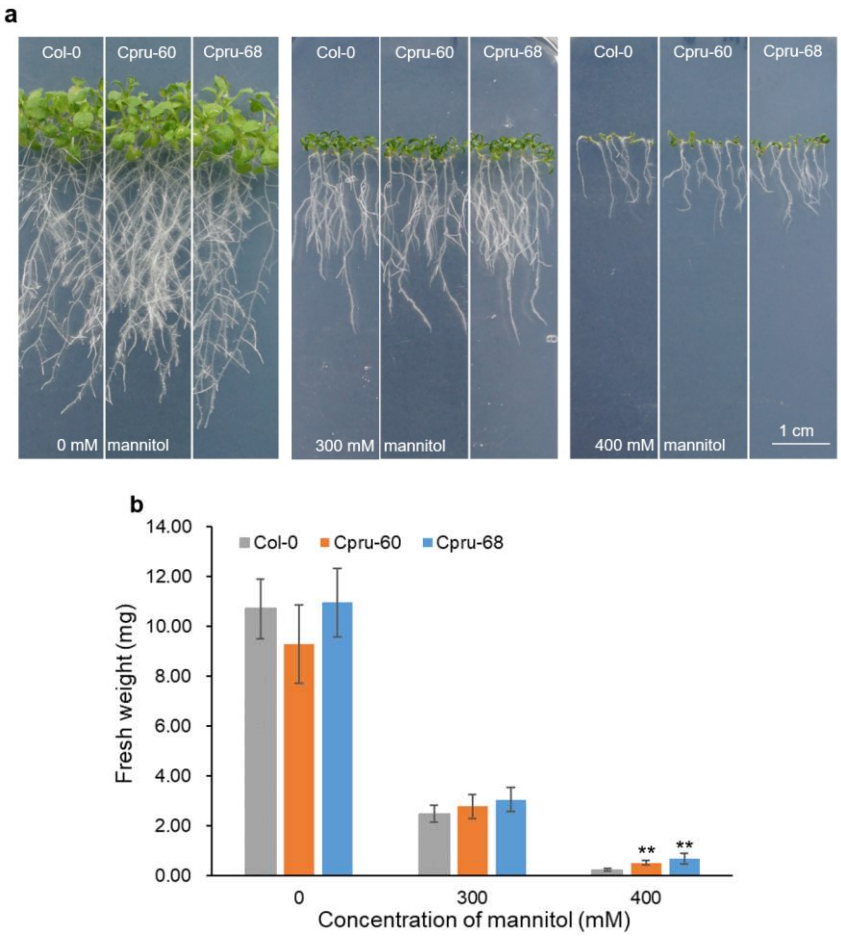


Fig. 3.2.6 a): images of Col-0 and *CpruIspS* transgenic plants growing vertically on medium containing 0 mM, 300 mM or 400 mM mannitol for 14 days; b): corresponding individual fresh weight of Col-0 and *CpruIspS* transgenic plants. Asterisks indicate a statistically significant difference between transgenic lines and Col-0 (**P < 0.01, Student's *t* test).

To explore the osmotic responses at post-germination stage, the phenotypes of *CpruIspS* transgenic lines and Col-0 were observed after vertically growing on medium with 0 mM, 300 mM, or 400 mM mannitol for 14 days. The plant growth was inhibited by mannitol in root elongation and aerial part growth (Fig. 3.2.6a). For the root elongation, there was no obvious difference between Col-0, *Cpru*-60 and *Cpru*-68 under any concentration of mannitol. For the aerial part, all three lines looked similar at 300 mM mannitol, while *Cpru*-60 and *Cpru*-68 had obviously stronger aerial part

than Col-0 at 400 mM mannitol, which was consistent with fresh weight. At 300 mM mannitol, Col-0, *Cpru*-60 and *Cpru*-68 had similar fresh weight (Fig. 3.2.6b). At 400 mM mannitol, the fresh weight of Col-0 was 0.23 g/plant. While the fresh weight of *Cpru*-60 and *Cpru*-68 were 0.51 g/plant and 0.69 g/plant respectively, more than twice that of Col-0. These results might be due to isoprene regulation of gene expression related to embryo growth, seed germination, and seedling development (Lantz et al., 2019b).

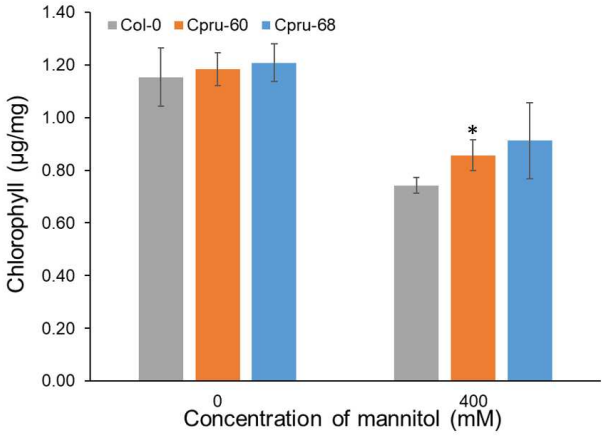


Fig. 3.2.7 Chlorophyll content of Col-0 and *CpruIspS* transgenic plants exposed to mannitol treatment (0 mM, 400 mM). An asterisk indicates a statistically significant difference between transgenic lines and Col-0 (*P < 0.05, Student's *t* test).

The chlorophyll content of leaves affects light absorption, which in turn affects photosynthesis efficiency (Ahammed et al., 2018). Generally, the chlorophyll content could be used as an indicator of drought stress responses (Wang et al., 2021; Li et al., 2006). In this study, the chlorophyll content of Col-0 (1.111 µg/mg), *Cpru*-60 (1.160 µg/mg) and *Cpru*-68 (1.180 µg/mg) were similar under control condition (0 mM mannitol). For treatment, 7-day-old seedlings were transferred to medium containing 400 mM mannitol and grown for another 10 days. The chlorophyll contents of *Cpru*-60 (0.857 µg/mg) and *Cpru*-68 (0.976 µg/mg) were significantly higher than that of Col-0 (0.743 µg/mg), indicating that isoprene might help to reduce damage to photosynthetic pigment.

The enhanced plant growth, greater fresh weight and higher chlorophyll contents

indicated that overexpression of *CpruIspS* in Arabidopsis might be related to alleviation of the osmotic stress at post-germination stage.

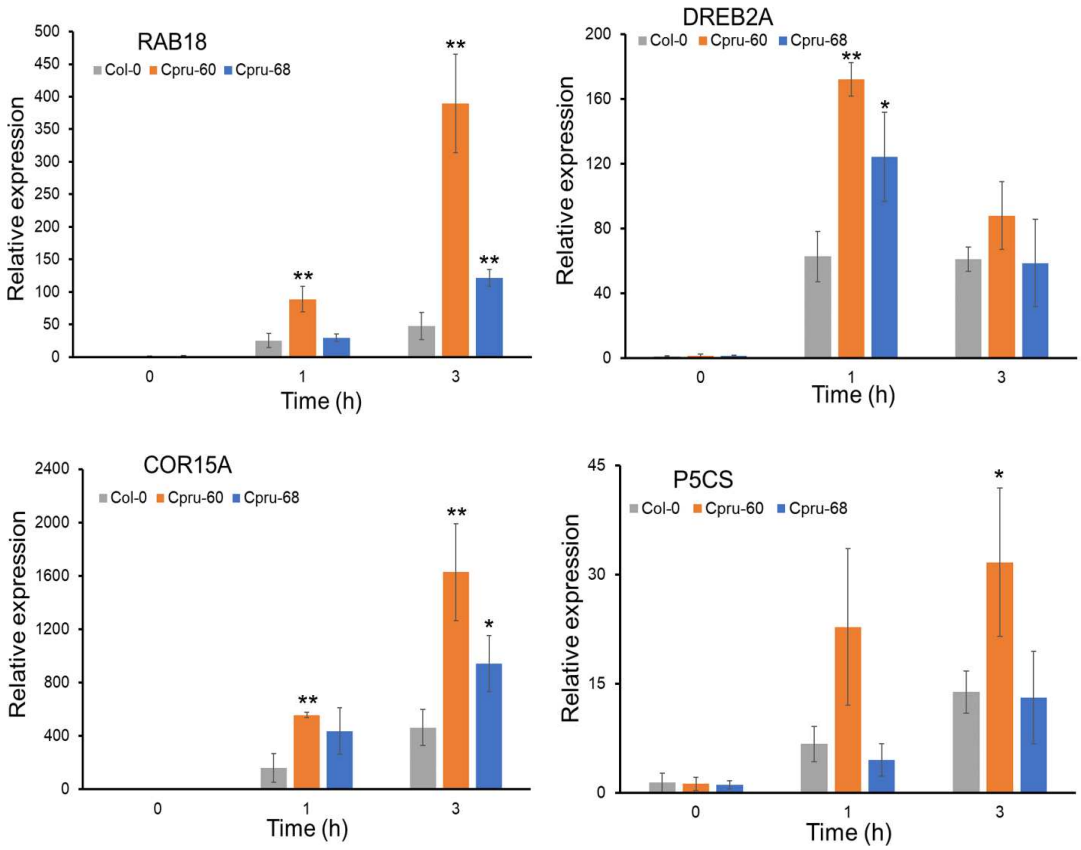


Fig. 3.2.8 Relative expression of stress-related genes (*RAB18*, *DREB2A*, *COR15A*, *P5CS*) in the aerial part of Col-0 and *CpruIspS* transgenic lines under mannitol treatment (0, 500 mM) for 1 h or 3 h using *EF1α* as a reference gene for normalization. Asterisks indicate a statistically significant difference between transgenic line and Col-0 (**P < 0.01, *P < 0.05, Student's *t* test).

To further understand the role of isoprene under osmotic stress at the molecular level, the expression of stress-related marker genes (*RAB18*, *DREB2A*, *COR15A* and *P5CS*) were analyzed in the aerial part and root by qRT-PCR with *EF1α* as the reference gene.

Under control conditions (0 mM mannitol), the expression of *RAB18*, *DREB2A*, *COR15A* and *P5CS* were similar among Col-0, *Cpru*-60 and *Cpru*-68 in both aerial part and root. After mannitol treatment (500 mM) for 1 h or 3 h, the expression levels of *DREB2A*, *RAB18* and *COR15A* in the aerial part of both transgenic lines were significantly higher than that of Col-0 (Fig. 3.2.8). For the expression of *P5CS* in the aerial part, there were no significant differences among Col-0 and two transgenic lines

except that a significantly higher level was found in *Cpru*-60 after mannitol treatment for 3 h.

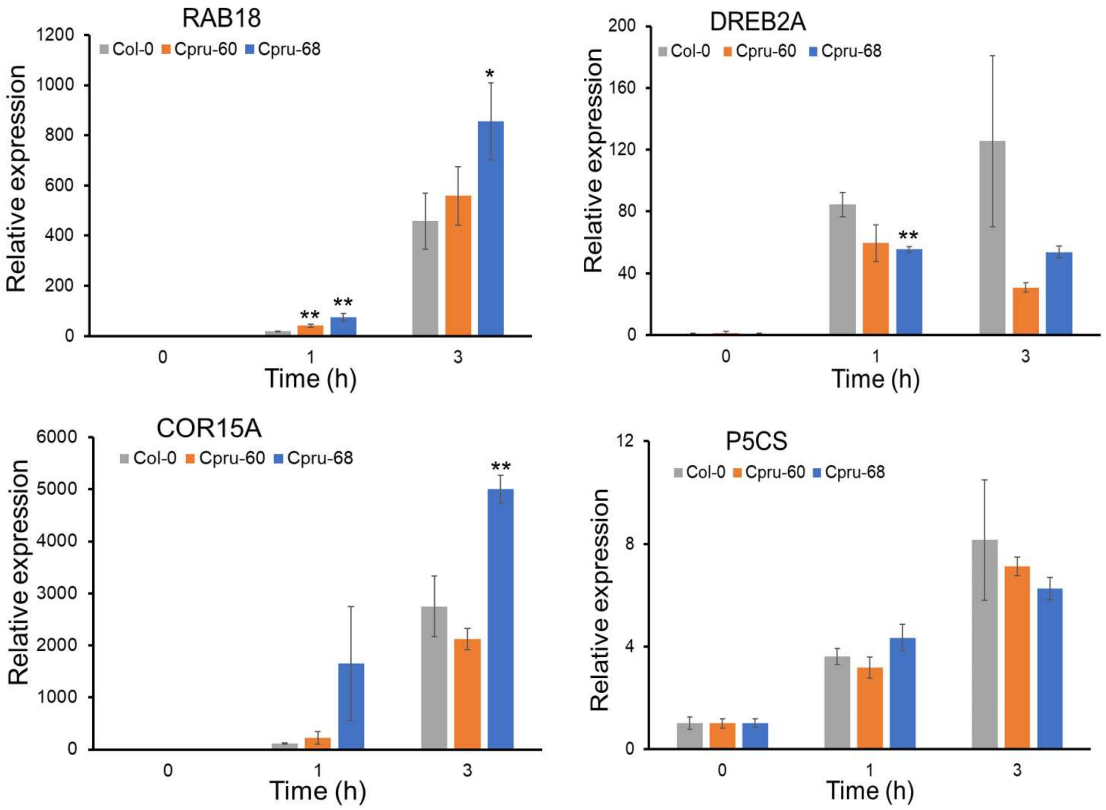


Fig. 3.2.9 Relative expression of stress-related genes (*RAB18*, *DREB2A*, *COR15A*, *P5CS*) in the root of Col-0 and *CpruIspS* transgenic lines under mannitol treatment (0, 500 mM) for 1 h or 3 h using *EF1α* as a reference gene for normalization. Asterisks indicate a statistically significant difference between transgenic line and Col-0 (**P < 0.01, *P < 0.05, Student's *t* test).

The gene expression patterns in the root were different from those in the aerial part. The expression of *RAB18* in the root of two transgenic lines was significantly higher than that of Col-0 after mannitol treatment for 1 h (Fig. 3.2.9). The relative expression of *DREB2A* in the root of two transgenic lines was moderately lower than that of Col-0, but without significant difference according to Student's *t* test. Except that *Cpru*-68 had a significantly lower relative expression of *DREB2A* than Col-0. In terms of *COR15A*, the relative expression of *Cpru*-68 seemed to be quite inconsistent with *Cpru*-60. The expression of *P5CS* was not differentially regulated among Col-0 and the two transgenic lines.

3.2.4.3 Phenotypes comparison at later developmental stage

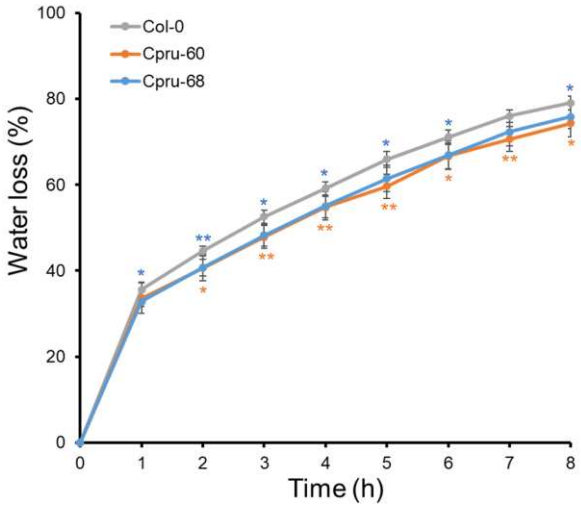


Fig. 3.2.10 Water loss rate of detached leaves of Col-0 and *CpruIspS* transgenic lines. Asterisks indicate a statistically significant difference between transgenic line and Col-0 (**P < 0.01, *P < 0.05, Student's *t* test).

To investigate the drought responses at the late developmental stage, the water loss rate (Fig. 3.2.10) and survival rate (Fig. 3.2.11) were determined using three-week-old plants. After being detached from plants, the water loss rates of mature rosette leaves of *Cpru*-68 and *Cpru*-60 (32.7%, 32.8%) were similar to that of Col-0 (35.6%) at the first hour. Since the second hour, the water loss rates of two transgenic lines were significantly lower than that of Col-0. After 8 h, the water loss rates of *Cpru*-60 and *Cpru*-68 were 73.0% and 73.9% respectively, while the one of Col-0 was 79.1%.

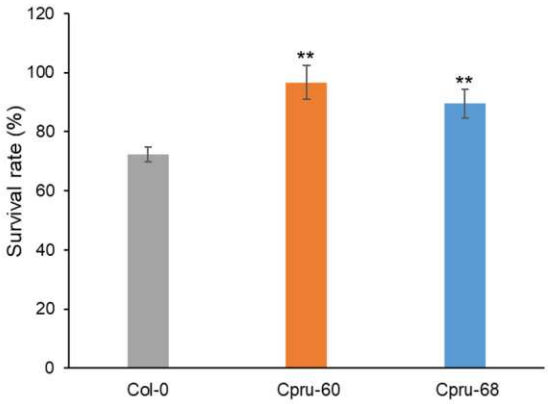


Fig. 3.2.11 Survival rates of Col-0 and *CpruIspS* transgenic plants after 4 days of re-watering. Asterisks indicate a statistically significant difference between transgenic line and Col-0 (**P <

0.01, Student's *t* test).

In the case of survival rate, after re-watering for 4 days, the survival rates of *Cpru*-60 and *Cpru*-68 were 96.7% and 89.5% respectively, significantly higher than that of Col-0 (72.4%). Therefore, corresponding to the higher water retention capacities, the survival rates of *CpruIspS* transgenic lines were also higher than that of Col-0, indicating enhanced drought stress responses of isoprene at the later developmental stage.

In summary, overexpression of the *isoprene synthase* gene from *Copernicia prunifera* in Arabidopsis helped to alleviate mannitol-induced osmotic stress throughout the plant life cycle. In the early developmental stage, *CpruIspS* transgenic lines had higher germination rates, larger green cotyledon formation rates and stronger seedlings. Besides, the results of chlorophyll content, water loss rate and survival rate further confirmed that isoprene played a positive role in drought resistance in the later developmental stage. At the molecular level, our study indicated that isoprene might stimulate the expression of drought-responsive genes to provoke protective measures such as stomatal closure to reduce water loss and enhancement of membrane integrity to protect the photosynthetic apparatus.

3.2.5 Discussion

Isoprene emission comes at the expense of high energy consumption (20 ATP and 14 NADPH per isoprene molecule) and carbon lost (typically 2% of photosynthesis) (Lantz et al., 2019b). But isoprene emission could be enhanced under adverse environmental conditions, suggesting that the benefits of isoprene may outweigh the costs (Sharkey et al., 2008; Hanson et al., 2001). Under non-stressful conditions, expression changes of many classes of genes were observed by isoprene fumigation of non-emitting Arabidopsis for 24 h, including the induction of chloroplast, phenylpropanoid biosynthesis and translation machinery genes (Harvey et al., 2016). These results indicated for the first time that isoprene could also play a role as a general signaling

molecule in non-emitting plants.

However, unlike the volatile isoprenoids, such as monoterpenes, the physiological function of isoprene in plant defense and communication with other organisms is more elusive (Pollastri et al., 2021). The unclear evolution and uneven pattern of isoprene emission in plants increase the difficulty of exploring its physiological function (Pollastri et al., 2021).

The thermo-protective and redox-protective effects of isoprene in plants have been widely demonstrated (Velikova 2008). For example, under moderately high temperature treatment, the photosynthesis of isoprene-emitting *Phragmites australis* leaves was reduced, but to a significantly lower extent than that of isoprene-inhibited leaves (Velikova et al 2005). The isoprene-emitting plants had much less H_2O_2 content, significantly reduced foliar lesions and photosynthetic damage than non-emitting plants (Vickers et al., 2009b). But apparent effects of isoprene emission under other environmental stresses have only been observed in some studies and little is known about whether and how isoprene alleviates abiotic stresses (Lantz et al., 2019b).

Salinity stress is a serious problem affecting one third of the world's irrigated land and it is caused by the accumulation of large amounts of soluble salts in the soil as irrigation water evaporates (Qados 2011; Abdel-Farid et al., 2020). Salinity affects seeds germination and seedlings growth, disrupts plant osmosis and imposes ion toxicity, thereby affecting plant growth and crop productivity (Dehnavi et al., 2020; Shrivastava et al., 2015).

It has been found that the expression of genes involved in isoprenoid biosynthesis was induced under long-term salinity stress, probably as a self-defense response to cell damage (Basyuni et al., 2011). Isoprene emission of *Eucalyptus globulu*s leaves was not severely affected by salinity stress and burst after the stress relief, suggesting that isoprene emission was resistant to salinity stress in natural isoprene emitters (Loreto et al., 2000). For the transgenic isoprene emitters, there is no available data on

isoprene emission changes under salinity stress. According to Behnke et al., although isoprene emission slightly increased under salinity stress, it did not make a difference in coping with salt as the isoprene-emitting and transgenic non-isoprene emitting plants had similar net CO_2 assimilation and transpiration rates (Behnke et al., 2013). At the moment, whether and how isoprene affects salinity resistance is still unclear.

In our study, we did not see obvious difference in seedlings growth of Col-0 and *ChumIspS* and *CpruIspS* transgenic Arabidopsis during the early growth stage under salinity stress. In a previous report exploring the protection mechanism of isoprene, no obvious difference was observed between wild-type and *IspS* transgenic Arabidopsis under salinity treatment (Sasaki et al., 2007). It indicated that isoprene might not alleviate the inhibition of plant growth under salinity stress. To figure out whether isoprene is involved in salinity responses in other aspects, there are still many things to explore. For example, measuring the isoprene emission changes may help better understand whether and how isoprene emission responds to salinity stress.

The phytohormone abscisic acid (ABA) regulates plant growth and development in many respects, such as embryo maturation, germination, cell division and elongation, and mediation of environmental stress responses (Finkelstein 2013). In leaves, both isoprene and ABA are synthesized through the MEP pathway (Marino et al., 2017). Isoprene emissions correlated with ABA levels in leaves (Barta et al., 2006) and fruits (Eccher et al., 2013), suggesting a possible coordinated regulation of production (Tattini et al., 2014).

The first study about isoprene effects on exogenous ABA stress found that *AdoIspS* transgenic Arabidopsis exhibited reduced growth inhibition compared to Col-0 (Xu et al., 2020). It did not explore whether isoprene emission responds to exogenous ABA treatment, but it suggested that isoprene decreased ABA sensitivity in germinating seeds and roots and the effects varied at tissue, spatial and temporal scales.

However, in our study, no significant difference in seeds germination and seedlings

growth was seen between *ChumIspS* transgenic lines and Col-0 under ABA treatment. Due to the limited reports of exogenous ABA treatment, it is difficult to explain the different phenotypes. The lack of phenotypic differences in this study may be due to the low isoprene emission of used *ChumIspS* transgenic lines, approximately 39%-52% of the emission of *AdoIspS*-79. It is also unsure whether the isoprene effects on exogenous ABA responses are enzyme-specific.

Drought stress is one of the most serious environmental stresses affecting plant growth, metabolism and yield, and its severity and duration are critical (Seleiman et al., 2021). Plants have a series of drought-responsive mechanisms. The isoprenoids biosynthetic routes belong to the metabolic pathway in response to water shortage (Loyola et al., 2012). For example, carotenoids contents gradually increased with decreasing relative water content and were associated with cytoprotective effects by reacting with reactive oxygen species (ROS) (Munné-Bosch et al., 2000). However, there is no consensus on the role of isoprene under drought stress.

There were no consistent results for the effect of drought stress on isoprene emission, which means that isoprene emission could increase, decrease or remain unchanged under drought treatment. The isoprene emission of *Xerophyta humilis* was stimulated by drought and peaked at 80% relative water content (RWC) (Beckett et al., 2012). According to Brilli et al., isoprene emission was not affected when fractions of transpirable soil water (FTSW) exceeded 60%, then slightly decreased as FTSW reduced. When the water stress became serious (FTSW< 30%), isoprene emission was severely inhibited (Brilli et al., 2007). In another study, the mild drought treatment (lack of irrigation for 7-8 days for 20°C-grown plants) did not change isoprene biosynthesis of date palm (Arab et al., 2016). This may be related to the degree of drought, as a life-threatening drought can trigger different responses from short-term mild water stress (Claeys et al., 2013).

The enhanced drought resistance was observed in some studies of natural isoprene emitters. The isoprene-emitting *Arundo donax* showed enhanced drought resistance

in terms of lower non-photochemical quenching (NPQ) and reduced lipid peroxidation, as well as better recovery after rehydration than the non-emitting *Hakonechloa macra* (Doneva et al., 2017; Velikova et al., 2016). The isoprene-mediated drought stress resistance has also been found in transgenic isoprene emitters. Isoprene emission appeared to have a more rapid and stronger protective response to mild drought stress, while isoprene synthesis could enhance non-volatile isoprenoids production to further protect leaves under long-term drought stress (Tattini et al., 2014). A conservative water-use strategy with earlier stomatal closure and enhanced sensitivity of transpiration to drought conditions was observed in isoprene-emitting Arabidopsis (Faralli et al., 2020).

However, these studies mainly focused on investigating the physiological responses of natural or transgenic isoprene emitters at a certain stage of plant growth. There is limited information about the isoprene-related morphological and physiological changes at different developmental stages. In this study, we observed enhanced drought resistance of isoprene throughout the whole plant life cycle of *CpruIspS* transgenic Arabidopsis.

Drought stress inhibits seeds germination, shoot growth and biomass production (Shahverdikandi et al., 2011). Alleviation of drought stress was observed in seeds germination rate, leaf tissue growth and chlorophyll content of *CpruIspS* transgenic plants at the germination stage and post-germination stage. However, no obvious difference was observed in terms of root length, which was consistent with the root length results of *AdoIspS* transgenic plants under polyethylene glycol (PEG)-induced osmotic stress (Xu et al., 2020), indicating that isoprene might not be involved in alleviation of root growth inhibition.

The expression level analysis of stress-responsive genes indicated that isoprene might act as a priming agent to regulate subsequent defense mechanisms. In the aerial part, the expression of *RAB18*, *COR15A* and *DREB2A* were up-regulated in *CpruIspS* transgenic lines compared to Col-0 under mannitol treatment. Among these genes,

RAB18 is involved in ABA regulation which can be induced by various abiotic factors including ABA, drought and cold stress (Ciereszko et al., 2002; Wang et al., 2020). *COR15A* is an ABA-dependent cold/osmotic-responsive gene (Seok et al., 2018). The mature *COR15A* protein participates in the protection of chloroplasts membrane (Liu et al., 2014b), which is consistent with higher chlorophyll content of *CpruIspS* transgenic plants. *DREB2A* is a well-known transcription factor in the drought response pathway and can regulate the expression of a subset of downstream genes in an ABA-independent way (Sakuma et al., 2006; Yang et al., 2018; Li et al., 2020c). The results indicated that isoprene might regulate the osmotic stress responses in ABA-dependent and ABA-independent ways.

The gene expression regulation by isoprene was tissue-specific as only the expression of *RAB18* was obviously up-regulated in the root of *CpruIspS* transgenic lines. This may be due to the predominantly active transcription of IspS in leaves and the leaf-specific function of IspS (Cinege et al., 2009). The expression of *P5CS* did not differ significantly in *CpruIspS* transgenic lines and Col-0 in both aerial part and root. *P5CS* is related to proline accumulation which regulates the osmotic balance (Ju et al., 2020; Amini et al., 2015; Li et al., 2020c), and its effect is not dominant in this study.

However, the expression levels of *COR15A* and *P5CS* in *AdoIspS* transgenic lines were downregulated compared to Col-0 under PEG-mediated osmotic stress in a previous study (Xu et al., 2020), different from our results. This may be related to the used material as whole seedlings were used in the previous study rather than separated aerial and root parts. In addition, the difference may be caused by enzyme specificity or treatment intensity. The reasons are uncertain due to insufficient understanding of the mechanism of isoprene-mediated drought responses and its elucidation will be object of future studies.

The benefits of isoprene at later development stage were reflected in the water loss rate and survival rate. Reducing water loss is an important strategy for plants to respond to drought conditions (Ghirardo et al., 2021). ABA-mediated stomatal closure

to reduce transpiration and minimize water loss is a rapid plant response (Wang et al., 2021; Cruz De Carvalho 2008). Overexpression of *IspS* in *A. thaliana* has been reported to promote ABA-induced stomatal closure by enhancing the expression of ABA-signaling gene *RD29B*, thereby reducing water loss rate under short-term water stress (Xu et al., 2020). Similarly, we observed up-regulation of *RAB18* involved in stomatal-specific ABA signaling pathway in *CpruIspS* transgenic plants, as well as significantly lower water loss rate of detached leaves. The results indicated that isoprene might be involved in ABA-dependent regulation of stomatal closure, thereby enhancing drought tolerance.

Plants were re-watered when soil water content reached 20-25% to reduce environmental disturbance and better determine survival rate. Significantly higher survival rates of *CpruIspS* transgenic plants were observed, suggesting that isoprene emission conferred enhanced drought tolerance.

The morphological, physiological and molecular responses indicate that isoprene is involved in alleviation of drought stress in this study. However, the mechanism of isoprene-mediated abiotic stress tolerance needs to be further explored. It has been hypothesized to be related to membrane stabilization, direct reaction with ROS and/or indirect effects on the oxidative state (Vickers et al., 2009a). Recent studies suggest that a direct effect of isoprene is impractical or not important for plants because the intramembrane isoprene concentration is too low to modify thylakoid membrane fluidity and ROS-quenching by isoprene is extremely costly (Harvey et al., 2015). It seems to be more likely that isoprene acts as a signal molecule or priming molecule to alter gene expression, proteome and metabolome (Lantz et al., 2019b; Zuo et al., 2019; Vanzo et al., 2016; Pollastri et al., 2021).

3.3 IspS expression in *Nannochloropsis gaditana*

N. gaditana is a unicellular marine alga exhibiting an autotrophic lifestyle (Cecchin et al., 2020). *N. gaditana* has a relatively small diameter of 2-4 μm (Hu et al., 2013). The *N. gaditana* cell is spherical to slightly oval with a rugged cell wall, a thylakoid-stacked chloroplast and large lipid droplets (Fig. 3.3.1) (Parthasarathy et al., 2014).

N. gaditana is well-known for high content of lipids such as myristic acid, palmitic acid and oleic acid (Ren et al., 2014). Besides, it is also a good source of high-value pigments such as chlorophyll, violaxanthin, zeaxanthin and astaxanthin (Liu et al., 2017a; Macías-Sánchez et al., 2005). Currently, *N. gaditana* is mainly used in biofuels and food areas (Simionato et al., 2011; Camacho-Rodríguez et al., 2015). For example, the eicosapentaenoic acid extracted from *N. gaditana* is a good source of biofuels.

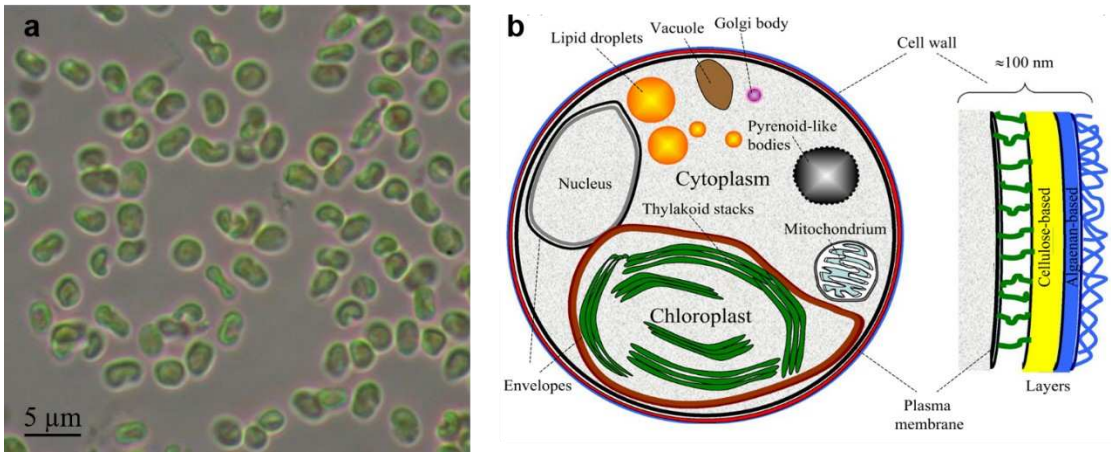


Fig. 3.3.1 Morphology of *Nannochloropsis gaditana* (a)(Hamed et al., 2017) and schematic diagram of the cellular structure (b)(Zhang et al., 2019).

Advantages

As photosynthetic organisms, microalgae utilize solar energy, H_2O and CO_2 to satisfy growth needs (Brennan et al., 2010). Therefore, *N. gaditana* could in principle be applied for the renewable synthesis of heterologous compounds and contribute to reduced carbon emissions. *N. gaditana* has high adaptability to the environment and can grow in the wastewater providing environmental benefits (Li et al., 2019a; Devasya

et al., 2021). Besides, rapid growth and high biomass yield make *N. gaditana* an ideal candidate for industrial exploration. Although the large energy consumption and capital investment required for large-scale cultivation, harvesting and extraction limit the current industrial application of microalgae, the production of high-value compounds such as isoprene can improve the economic availability of algal production systems (Chen et al., 2019).

N. gaditana has a haploid and compact genome estimated to be 29 Mb (Jinkerson et al., 2013). The availability of nuclear, mitochondrial and plastid genomes sequences has promoted the commercial potential of *N. gaditana* by genetic engineering (Radakovits et al., 2012). Applications in non-consumption fields such as isoprene production are less risk to human health. The outdoor growth trial suggested that the genetically modified microalgae didn't have significant ecological impacts and were indistinguishable from wild type strains (Shurin et al., 2016).

Compared with *E. coli* and yeast, which have been widely used in heterologous fermentation production, eukaryotic microalgae may provide more favorable cellular environments for isoprene biosynthesis as the cell structures of eukaryotic microalgae are similar to that of plants, and the precursors of plastidic isoprenoids such as carotenoids, sterols, prenyl side-chains of chlorophylls are synthesized through MEP pathway in chloroplasts (Lauersen 2019; Schwender et al., 1996). Moreover, eukaryotic microalgae have efficient pathways to synthesize isoprenoid pigments (e.g., chlorophylls and carotenoids) and electron carriers (e.g., plastoquinone) that are indispensable for the structure of photosynthetic apparatus and function of photosynthesis (Lohr et al., 2012). Thus, eukaryotic microalgae may have great potential for isoprene synthesis due to efficient IPP/DMAPP supply.

In addition, the microalgal production of isoprene also has benefits in production separation (Melis 2012). Because the volatile isoprene naturally released by the cells can be recovered by sequential activated carbon absorption, desorption and condensation steps (Zou et al., 2017). It is not affected by the technical difficulties of

cell harvesting, lysis and product extraction in microalgae (Bhave et al., 2012).

Difficulties

Although the regulation of synthesized lipids composition of *Nannochloropsis* has been achieved through gene overexpression or knockout (Zienkiewicz et al., 2017; Dolch et al., 2017; Ajjawi et al., 2017), the transgene transformation efficiency needs to be further improved (Li et al., 2014). The small cell size, chloroplast surrounded by four membranes and the lack of efficient plastid genetic tools in *N. gaditana* make gene transformation into the plastid genome more difficult (Sproles et al., 2021; Cui et al., 2021).

The relatively successful method is by transformation into the nuclear genome. However, the random integration may cause position effects leading to highly variable gene expression levels among different transformant strains and even gene silencing (Ryu et al., 2021; Südfeld et al., 2022). Therefore, efficient and high-throughput screening methods for positive transformants are to be developed.

Although the research on microalgae has made great progress in the past decade, the understanding of the basic biology of microalgae is still limited, which is a major bottleneck in microalgal bioproduction (Chen et al., 2019). *N. gaditana* relies only on the MEP pathway operating in chloroplasts for isoprenoid production as the MVA pathway is absent (Han et al., 2020). The incomplete understanding of metabolic processes and regulatory networks increases the difficulty of bioproduction in *Nannochloropsis* (Tran et al., 2016).

Objectives

The objectives of this study were to explore the potential of *N. gaditana* as a platform for isoprene bioproduction by heterologous expression of isoprene synthases and culture conditions optimization. The study tasks include construction of IspS-2A-YFP plasmid, plasmid transformation into the nuclear genome by electroporation,

fluorescence determination with flow cytometry and isoprene determination with PTR-MS and cultivation conditions optimization.

3.3.1 Construction of IspS-2A-YFP plasmid and electroporation into *N. gaditana*

In the study of Miller et al., the endogenous chloroplast transit peptide (CTP) coding sequence was not necessary for the heterologous expression of *IspS* from *Populus alba* × *Populus tremula* and it could even inhibit isoprene release in transgenic *E. coli* (Miller et al., 2001). Two different lengths of *AdoIspS* fragments were used here. The target *Ado*IspS without the putative CTP consisting of 16 amino acids which were analyzed by the software (ChloroP-1.1) was named *AdoIspS*_long. With reference to the plastidic leader of *IspS* from *Populus alba* × *Populus tremula* in the previous study (Miller et al., 2001), the other target *Ado*IspS without putative CTP consisting of 25 amino acids was named *AdoIspS*_short by sequence alignment.

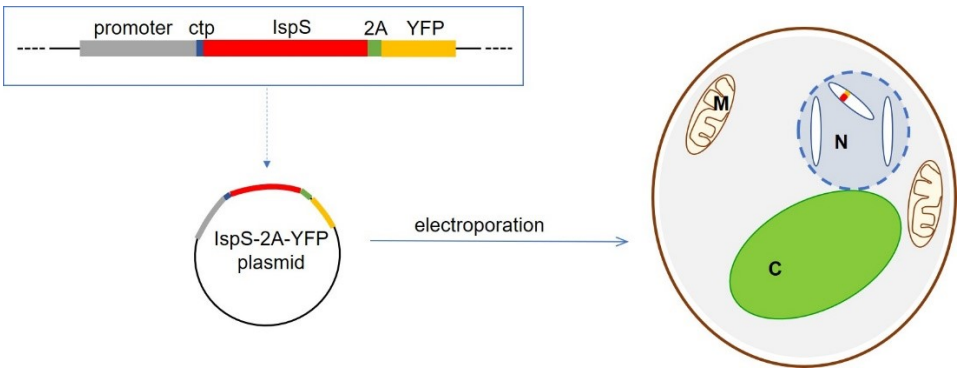


Fig. 3.3.2 Schematic diagram of recombinant plasmid and plasmid transformation into the nuclear genome of *N. gaditana*. The pBluescript SK II vector region of the plasmid is depicted with the dashed line. The linear DNA fragments in the box encode light-harvesting complex protein (Lhcx1) promoter, chloroplast transit peptide (ctp), isoprene synthase (IspS), 2A peptide and yellow fluorescent peptide (YFP) in order. The abbreviations used: C, chloroplast; N, nucleus; M, mitochondria.

The schematic diagram of the recombinant IspS-2A-YFP plasmid is shown in Fig. 3.3.2. The *Lhcx1* promoter was used to regulate the expression of IspS and YFP. The putative

CTP-encoding sequence of light-harvesting complex protein from *N. gaditana* was used for targeting the expressed IspS from nucleus to chloroplast. The *Ado*IspS and YFP were expressed together by linking with self-cleaving 2A peptides.

The 2A peptides were used to obtain unfused and monomeric isoprene synthase, thus avoiding changes in catalytic properties caused by fusion with other proteins (Rasala et al., 2013). During translation, the ribosome recognizes the highly conserved sequence of 2A and skips the formation of a glycyl-prolyl peptide bond, termed “ribosome skipping”, resulting in simultaneous expression and cleavage of two proteins (Wang et al., 2015; Liu et al., 2017b). Application of 2A sequences between co-expressed genes could increase the transformation efficiency by reducing vector size and achieve equivalent expression levels of proteins (Koh et al., 2018).

The downstream YFP was used as a marker for efficient positive transformants screening through fluorescence determination. YFP can fluoresce without addition of other compounds and is one of the brightest and most widely-used genetically encoded probes (Shaner et al., 2007; Harms et al., 2001).

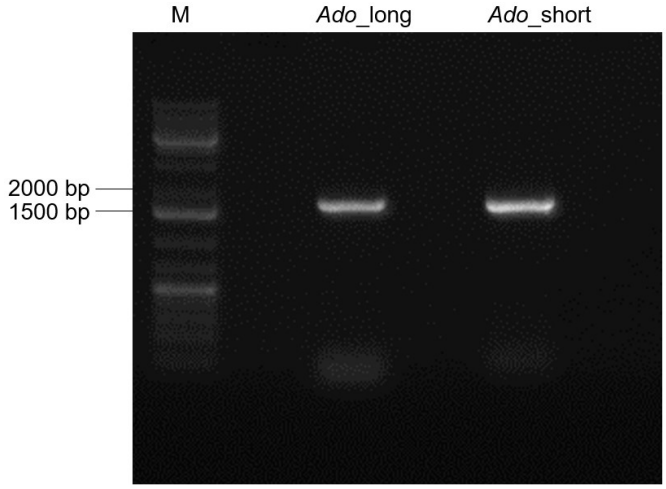


Fig. 3.3.3 Agarose gel electrophoresis of PCR products of *AdoIspS*_long and *AdoIspS*_short. M, GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific).

The target fragments, *AdoIspS*_long of 1647 bp and *AdoIspS*_short of 1620 bp, were

amplified with the corresponding primers listed in table 2.5 and analyzed with 1% agarose gel (Fig. 3.3.3). After purification, the target fragments were ligated with the vector pENTR/D-TOPO and transformed into *E. coli* DH10B. The transformants containing the target fragments were identified by colony PCR. The plasmid was extracted and digested with SpeI and BamHI to obtain fragments of *AdoIspS*_long and *AdoIspS*_short. The digested fragments were ligated with destination plasmid and transformed into *E. coli* DH10B. The positive transformants were identified by colony PCR (Fig. 3.3.4). For the construct of *AdoIspS*_long, positive transformants were colonies 4, 5, 7, 8 (Fig. 3.3.4a). For the construct of *AdoIspS*_short, positive transformants were colony 1, 2, 3, 4 (Fig. 3.3.4b). Then the plasmids were extracted and transformed into *N. gaditana* by electroporation.

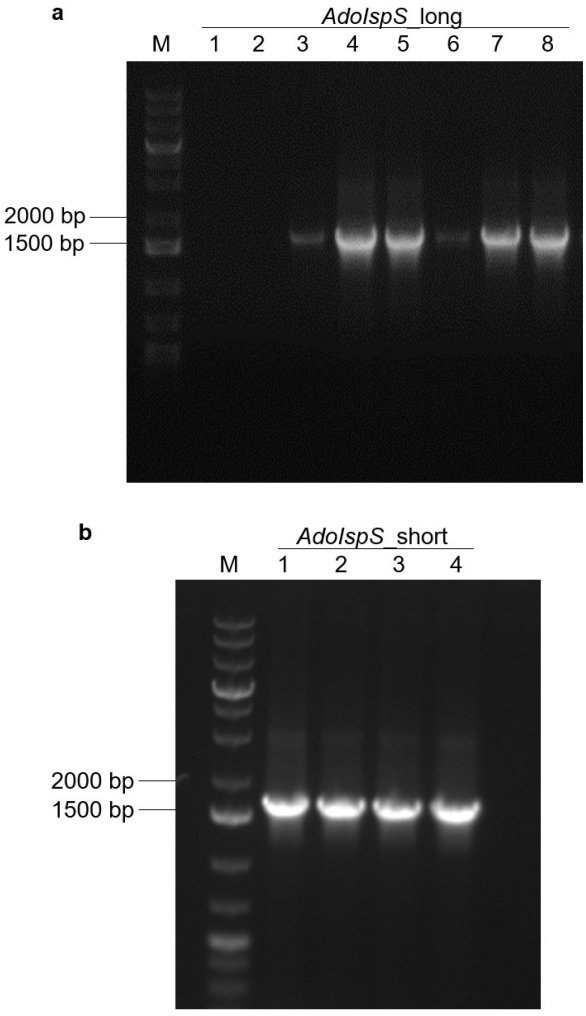


Fig. 3.3.4 Agarose gel electrophoresis of colony PCR products of transgenic *E. coli* with a

construct containing *AdoIspS*_long (a) and *AdoIspS*_short (b). M, GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific). Lanes 1-8 (a) and lanes 1-4 (b), colony PCR products of independent colonies.

3.3.2 Flow cytometry analysis results

The fluorescence of a population of transformants was determined by flow cytometry to identify the cells with a high potential for isoprene production, assuming that the metabolite accumulation was correlated with gene expression (Yu et al., 2021).

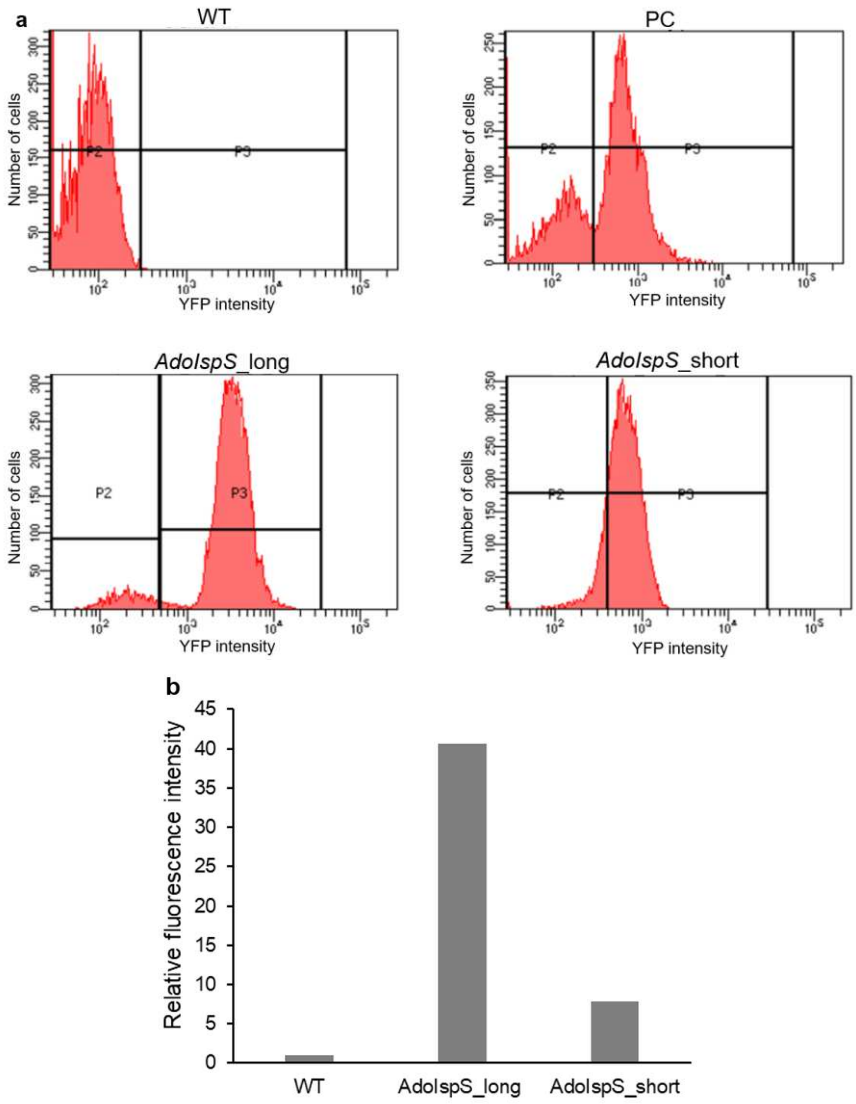


Fig. 3.3.5 Flow cytometry analysis. a): YFP intensity in cell population of WT (wild type), PC (positive control), *AdoIspS*_long positive transformant and *AdoIspS*_short positive transformant; b): relative fluorescence intensity of WT (wild type), *AdoIspS*_long positive transformant and

*AdoIspS*_short positive transformant. Relative fluorescence intensity was calculated as the ratio of mean emission intensity in the samples to that in the wild type.

For each construct of *AdoIspS*_long and *AdoIspS*_short, 200 colonies were picked and subjected to flow cytometry analysis. Only one positive transformant was obtained for each construct, indicating a relatively low transformation efficiency (~0.5%).

The YFP intensities of the cell population are shown in Fig. 3.3.5a. For wild type, a majority of cells (86.5%) were in the P2 subset with low YFP intensity. The cells overexpressing YFP were used as the positive control (PC). For the PC, most cells (67.7%) were in the P3 subset with high YFP intensity. For the positive transformant of *AdoIspS*_long, 90.8% of cells were in the P3 subset with much higher YFP intensity. For the positive transformant of *AdoIspS*_short, 81.6% of cells were in the P3 subset.The fluorescence intensity of the positive transformants of *AdoIspS*_long and *AdoIspS*_short were 40.6 and 7.8 relative to WT, indicating the successful expression of the transgene (Fig. 3.3.5b).

3.3.3 Isoprene emission under different cultivation conditions

Many studies have investigated the effects of culture conditions on microalgal biomass and productivities to maximize target products yields (Camacho-Rodríguez et al., 2015; Fakhry et al., 2015; Sandnes et al., 2005). Here we explored isoprene emission levels of the transformants under different culture conditions (temperature, cultivation time and initial OD_{600}). The isoprene emission abilities of two positive transformants (*AdoIspS*_long and *AdoIspS*_short), two corresponding negative transformants and wild type were determined by PTR-MS.

Considering that the preferred cultivation temperature of *Nannochloropsis* is about 25°C (Han et al., 2020), the isoprene emissions of transgenic *N. gaditana* under different temperatures (25°C, 28°C and 30°C) were investigated. As shown in Fig. 3.3.6 and Fig. 3.3.7, the isoprene emission levels increased as temperature increased.

However, the isoprene emission levels of two positive transformants were similar to that of negative transformants and wild type.

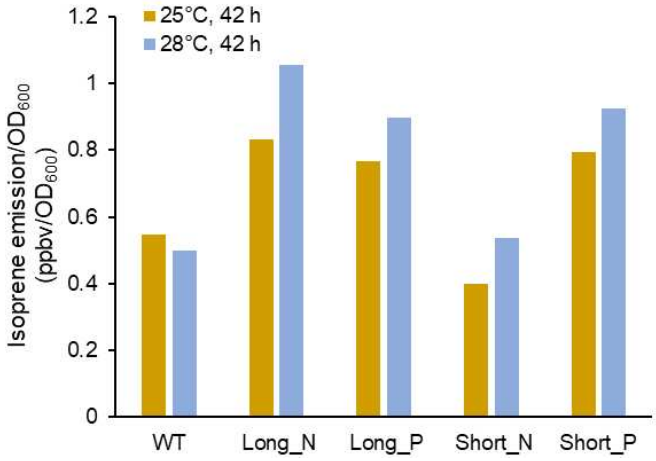


Fig. 3.3.6 Isoprene emission level of transgenic *N. gaditana* cultured at 25°C or 28° for 42 h with initial OD_{600} of 0.5-1.0. Abbreviations are: WT, wild type; Long_N, negative transformant of *AdoIspS*_long construct; Long_P, positive transformant of *AdoIspS*_long construct; Short_N, negative transformant of *AdoIspS*_short construct; Short_P, positive transformant of *AdoIspS*_short construct.

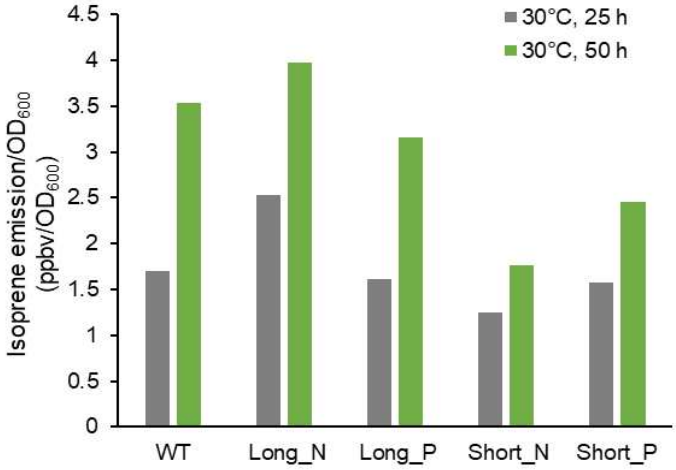


Fig. 3.3.7 Isoprene emission level of transgenic *N. gaditana* cultured at 30°C for 25 h and 50 h with initial OD_{600} of 0.3-0.4.

Although the isoprene emission levels of all transformants and wild type after 50-hour cultivation were higher than those after 25-hour cultivation, the isoprene levels of two positive transformants were similar to that of negative transformants and wild type (Fig.

3.3.7).

Since the cell density affected the production of microalgae (Richmond et al., 2001; Quinn et al., 2012), the effect of initial OD_{600} was also tested. As shown in Fig. 3.3.7 and Fig. 3.3.8, after culture at 30°C for 24-25 h, cells with an initial OD_{600} of 0.6-0.8 released a higher amount of isoprene than that with an initial OD_{600} of 0.3-0.4. However, for the culture with an initial OD_{600} of 0.6-0.8, the isoprene emission levels were quite similar among all transformants and wild type (Fig. 3.3.8).

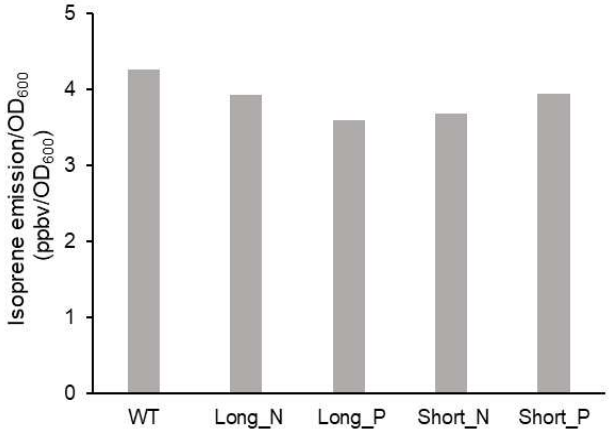


Fig. 3.3.8 Isoprene emission level of transgenic *N. gaditana* cultured at 30°C for 24 h with initial OD_{600} of 0.6-0.8.

In conclusion, heterologous isoprene production was not achieved in *N. gaditana*. The positive *N. gaditana* transformants with *AdoIspS*_long or *AdoIspS*_short, the negative transformants and wild type were all found to emit isoprene at a pretty low level under different culture conditions (temperature, cultivation time and initial OD_{600}). In addition, there were no obvious differences in isoprene emission among different transformants and wild type. As there has been no endogenous isoprene synthase identified in *N. gaditana* so far and the high sensitivity of PTR-MS to parts per billion by volume level (ppbv) of volatile compounds (Soukoulis et al., 2013), the low levels of isoprene detected might be from an unknown source of DMAPP hydrolysis.

3.3.4 Discussion

In principle, the heterologous production of isoprene in *N. gaditana* could be achieved by introducing *IspS* gene as DMAPP is a critical metabolite in all living organisms. However, in our study, we did not observe significant isoprene emission in transgenic *N. gaditana*. Negative results concerning heterologous expression in algae have been reported in other studies. For example, the heterologous expression of acetyl-CoA carboxylase (ACCase) in *Navicula saprophila* and heterologous expression of FatB1 thioesterase in *Chlamydomonas reinhardti* did not enhance fatty acid biosynthesis as expected (Blatti et al., 2013). The outcomes of genetic engineering in microalgae are considered to be unpredictable because of limited transformation tools and insufficient understanding of microalgal metabolism and regulatory networks (Blatti et al., 2013; Kang et al., 2017).

Specifically, there are some possible reasons for our failure. Firstly, the insignificant emission of isoprene may be an individual effect as there is only one positive transformant of each construct. The extensive variability of the target character is often observed between transformants with the same transgene construct (Butaye et al., 2005). The random integration makes it impossible to obtain genetically identical transformations. That's why populations of transformants are usually screened to obtain stable individuals with desired gene expression levels (Úbeda-Mínguez et al., 2017). For this problem, it is recommended to apply a transformation method with higher efficiency and screen more transformants to obtain several transgenic lines with ideal expression levels for further study. Besides, proper regulatory sequences, mainly promoters and terminators, are also important for transgene expression (Schroda 2019). For example, an approximately 40-fold difference in transgene expression can be achieved by simply using different terminators in *Chlamydomonas reinhardtii* (Kumar et al., 2013). The fact that YFP was properly expressed, however, seem to exclude this possibility.

Secondly, the expressed isoprene synthase might be inactivated. One possible reason is the protein misfolding caused by codon bias (Wu et al., 2015). Synonymous codon usage preferences vary widely between species, which is called codon bias (Plotkin et al., 2011). It has been found that even a single synonymous codon substitution can significantly affect gene expression levels, protein folding and protein cellular function (Gustafsson et al., 2004; Angov 2011). In this case, the codon optimization based on the codon bias of *N. gaditana* genome can be applied to promote proper protein folding and catalytic function.

Thirdly, the translated IspS in the cytoplasm might not be targeted into the chloroplast to function correctly. As the MVA pathway is lost in *N. gaditana*, DMAPP is synthesized through the MEP pathway in chloroplast. Unlike the chloroplast of plants, the chloroplast of *Nannochloropsis* is surrounded by another pair of membranes (forming chloroplast endoplasmic reticulum) that originates from the secondary symbiosis of a red algal ancestor (Koh et al., 2019). Thus, nuclear-expressed gene, which are then translated into the cytoplasm, need to pass through four membranes to localize in chloroplast, which increases the difficulties of correct localization and proper function of heterologous proteins in *Nannochloropsis*. In the study of Koh et al., the successful delivery of heterologous proteins into the chloroplast of *Nannochloropsis salina* was achieved by adding signal and transit peptide sequences (Koh et al., 2019). The first 15 amino acids of ctp used in our study is a potential signal peptide analyzed by SignalP-4.1, while the delivery efficiency of the signal and transit peptide may not be ideal due to a lack of deep understanding of genetic information. The delivery efficiency of ctp could be evaluated by fluorescence assays displaying cellular localization if the YFP is fused to ctp (Moog et al., 2015).

Lastly, the synthesis of isoprene might be inhibited by unknown metabolic control within *N. gaditana*. Although the genome drafts have been obtained, the metabolism and regulatory networks of *N. gaditana* are still poorly understood at the moment, which is a major bottleneck in the genetic manipulation and bioproduction of microalgae

(Corteggiani et al., 2014; Alboresi et al., 2016; Chen et al., 2019). However, it cannot rule out the feasibility of efficient biosynthesis in *N. gaditana*. With the development of metabolic flux analysis, further understanding of the enzymes and regulatory networks of complete metabolic pathways could facilitate efficient biosynthesis and industrial-scale applications of microalgae.

To realize the great potential of *N. gaditana* as a model system for isoprene production, there are many aspects to optimize. In terms of IspS, it could be achieved by the discovery of novel IspS with high catalytic activity or enhancing catalytic activity through directed evolution or rational design. The direct evolution requires high-throughput screening methods such as fluorescent protein-based expression screening (Kim et al., 2018). The rational design needs more information of the structure-function relationship of IspS. In terms of metabolic pathways, maximal flux to isoprene synthesis could be achieved by overexpressing enzymes of MEP pathways, especially the rate-limiting ones. For example, overexpression of heterologous 1-deoxy-D-xylulose 5-phosphate synthase (DXS) resulted in higher CO_2 absorption capacity, increased biomass and lipids production in *Nannochloropsis oceanica* (Han et al., 2020). Besides, as we learn more about the metabolism of *N. gaditana*, more DMAPP can be directed to isoprene production by deleting or down-regulating pathways that consume DMAPP.

4 Conclusion and outlook

In this study, three novel isoprene synthase genes were isolated from palm species including *Chamaerops humilis*, *Sabal minor* and *Copernicia prunifera*. The novel IspS diagnostic tetrad from Arecaceae family was identified by sequence alignment and functionality was validated by isoprene emission in *IspS* transgenic *Arabidopsis thaliana* (Li et al., 2021). It provided potential biocatalysts for efficient isoprene production in heterologous systems.

For IspS expression and catalytic ability assays, the expression plasmids were transformed into *Escherichia coli* at first. However, it turned out that the three isoprene synthases were either not expressed or mostly aggregated as inclusion bodies after optimization of expression conditions including post-induction temperatures, cell densities of induction, IPTG concentrations, expression vectors and so on. This may be related to the limits of expression levels, protein folding and post-translational modifications that widely observed in heterologous expression of eukaryotic proteins in *E. coli* (Von Schaewen et al., 2018).

Physiological responses of isoprene emitter in response to salinity, ABA and mannitol-induced osmotic stresses were studied by over-expression of *ChumIspS* or *CpruIspS* in *Arabidopsis thaliana*. Under salinity stress, seedlings growth of *ChumIspS* or *CpruIspS* was similar to that of wild type. Under ABA treatment, seeds germination and seedlings growth of *ChumIspS* were similar to those of wild type. Therefore, *Arabidopsis* transgenic plants overexpressing *ChumIspS* or *CpruIspS* did not respond to salinity or ABA treatment during seeds germination and post-germination stages in this study.

Positive morphological and phenotypical responses under osmotic stress during different developmental stages were observed in *CpruIspS* transgenic plants, suggesting that isoprene was involved in alleviation of osmotic stress. Besides, relative expression levels of drought-responsive genes indicated that isoprene might mediate

drought responses through ABA-dependent and ABA-independent pathways. For some isoprenoid emitters, isoprenoid emissions were directly related to ABA content in leaves (Barta et al., 2006). Thus, the enhanced isoprene emission under drought stress may imply an increase in ABA biosynthesis, which regulates stomatal aperture in response to changes in water availability (Barta et al., 2006; Sharkey et al., 1993). A recent study indicated that isoprene enhanced ABA-induced stomatal closure (Xu et al., 2020). While, the cross-talk between isoprene and ABA under drought stress needs to be further explored. Functional genomics and metabolomics methods could help to understand the role and related mechanisms of isoprene under abiotic stresses, and further explore the impact of climate changes and future isoprene emission on atmospheric chemistry.

To test the potential of sustainable isoprene production, the isoprene synthase gene was introduced and expressed in *Nannochloropsis gaditana*. The positive *IspS* transgenic *Nannochloropsis* were obtained by screening fluorescence intensity, while their isoprene productions measured with PTR-TOF-MS were as low as that of wild type even after culture conditions optimization. The insufficient isoprene production was probably due to low level expression, substrate limitation or unknown metabolic control within *N. gaditana*. Although our attempts do not achieve ideal results, *Nannochloropsis* could be promising microalgal cell factories for isoprene production with the development of more efficient gene expression vectors and transformation methods. Moreover, *Nannochloropsis* is well-known for high lipids production, which might be possible for efficient isoprene production through metabolic modification.

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