UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Head Office: Università degli Studi di Padova

Department of Biology

Ph.D. COURSE IN: Biosciences
CURRICULUM: Biochemistry and Biotechnology
SERIES XXX

Characterization of Isoprene Synthase from *Arundo donax* L. in Relation to Abiotic Stress Tolerance

Thesis written with the financial contribution of China Scholarship Council.

Coordinator: Prof. Ildikò Szabò
Supervisor: Prof. Claudio Varotto
Co-Supervisor: Prof. Livio Trainotti

Ph.D. student : Jia Xu

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Riassunto

Isoprene (2-methyl-1,3-butadiene, C5H8) è un idrocarburo volatile con cinque atomi di carbonio, sintetizzato dalla via metabolica del MEP (metileritritolo fosfato) nei cloroplasti di varie specie di piante. È stato suggerito che l'isoprene possa aumentare la tolleranza delle piante alle condizioni di stress, perché la sua emissione potrebbe essere stimolata quando le foglie sono sottoposte a stress ambientali o ricuperano da essi. Le funzioni fisiologiche dell'emissione di isoprene sono state studiate per lungo tempo. Le ricerche precedenti hanno ben documentano che l'emissione di isoprene può aumentare la tolleranza termica o la resistenza allo stress ossidativo delle piante, ma è meno chiaro se l'emissione di isoprene può aumentare la tolleranza allo stress idrico, e i meccanismi molecolari delle piante che emettono isoprene in relazione alla siccità non sono mai stati investigati prima.

Nel presente lavoro, abbiamo caratterizzato la funzione fisiologica del primo gene *IspS* (*AdoIspS*) di una specie di monocotiledone, *Arundo donax* L., in relazione a differenti stress abiotici, tramite overespressione in *Arabidopsis thaliana*.

Le piante transgeniche hanno mostrato una maggiore produzione di semi rispetto a quelle non trasformate. Dopo aver confrontato il tempo di fioritura, la biomassa fogliare e la morfologia delle piante tra tipo selvatico (WT) Col-0 e linee *IspS*, i risultati suggeriscono che l'emissione di isoprene potrebbe fornire un vantaggio selettivo alle piante attraverso l'aumento del tasso di crescita delle piante.

Per investigare la funzione protettiva dell'emissione di isoprene in relazione a stress abiotici, in primo luogo, è stata esaminata la tolleranza termica tramite l'analisi comparativa dell'abilità di sopravvivenza tra piante *IspS* e Col-0. I risultati indicano che, dopo aver recuperato dallo shock termico, le piante *IspS*

hanno mostrato un più elevato tasso di sopravvivenza, contenuto di clorofilla e peso fresco in diverse fasi di sviluppo. Questo ha rivelato che la trasformazione del gene *IspS*, isolato da una specie di monocotiledoni, può conferire tolleranza termica a una specie che non emette isoprene, analogamente alle *IspSs* da dicotiledoni.

In secondo luogo, abbiamo investigato la sensibilità delle piante *IspS* alla somministrazione di acido abscissico esogeno (ABA), in quanto l'ABA è una molecola-chiave nella protezione delle piante sotto stress idrico, ed è prodotto a valle della via biosintetica del MEP. Le piante *IspS* hanno mostrato una ridotta sensibilità all'applicazione esogena di ABA sia in fase di germinazione sia in fase di semina. I risultati di qPCR indicano che, con il trattamento di ABA alla parte ipogea della pianta, la regolazione dei geni indotti da ABA e dei geni di biosintesi dell'ABA in radice non è influenzata dalla presenza di *IspS*. Tuttavia, nelle foglie transgeniche per *IspS*, l'espressione dei geni reattivi all'ABA viene indotta, mentre quella dei geni di biosintesi dell'ABA è repressa. Questi risultati supportano l'idea che l'emissione di isoprene potrebbe essere implicata nella biosintesi e accumulo fogliare di ABA. Pertanto, la presenza di *IspS* potrebbe influire/alterare ulteriormente il meccanismo della tolleranza alla siccità delle piante che non emettono isoprene, mediando i meccanismi dipendenti dall'ABA di protezione contro lo stress idrico.

Per verificare se ed in caso come la trasformazione di *IspS* altera la tolleranza alla disidratazione delle piante, nell'ultima parte del lavoro, le linee di Arabidopsis transgeniche per *IspS* sono state utilizzate nello studio di stress conseguente alla disidratazione. I risultati del test di perdita di acqua e l'analisi dell'apertura stomatica confermano che, le piante che emettono isoprene potrebbero richiedere una quantità minore di ABA per tollerare lo stress causato da disidratazione. Inoltre, il tasso di sopravvivenza, come anche il test di perossidazione dei lipidi e

l'espressione dei geni relativi alla disidratazione sono stati misurati per analizzare l'aumentata tolleranza alla disidratazione delle linee *IspS* a livello di intera pianta. I risultati indicano che la migliore tolleranza alla disidratazione mostrata in piante transgeniche per *IspS* forse risulta dal ridotto accumulo di specie reattive dell'ossigeno (ROS) in foglie che mettono isoprene.

Queste scoperte suggeriscono che il gene *IspS* ha un ruolo importante nella tolleranza delle piante a condizioni di stress abiotico. Comprendere i meccanismi regolatori del gene *IspS* in risposta allo stress abiotico potrebbe aiutarci a scoprire perché le piante che emettono isoprene superano meglio gli eventi di stress transitorio in natura.

Abstract

Isoprene (2-methyl-1,3-butadiene, C5H8) is a five carbon volatile hydrocarbon compound, which is synthesized through methylerythritol phosphate (MEP) pathway in the chloroplasts of many plant species. Isoprene has been suggested to enhance plants tolerance to stressful conditions because its emission could be stimulated when leaves are subjected to or recovering from environmental stresses. Physiological function(s) of isoprene emission has been studied for a long time, and previous studies well documented that isoprene emission can enhance plant thermotolerance and oxidative stress resistance; however, whether isoprene emission may increase plant tolerance to drought stress is less studied, and the molecular mechanisms of isoprene emitting plants in response to drought-related stress have never been investigated before.

In this study, we characterized the physiological function of the first *IspS* gene (*AdoIspS*) from a monocot species, *Arundo donax* L., in response to different abiotic stresses.

First of all, Phenotypic differences under normal growth condition between *AdoIspS* transgenic and Col-0 wild type plants were observed as follows: earlier flowering time, tendency to higher leaf biomass even though no significant difference, and a higher seed production derived from higher numbers of branching in *AdoIspS* transgenic plants.

The results suggested that isoprene emission may provide a selective advantage to plants by enhancing plant growth rate.

In order to investigate the protective function of isoprene emission in response to abiotic stresses, first, the enhanced thermotolerance was tested by analyzing the

survival ability between *AdoIspS* transgenic plants and Col-0. The results indicate that after recovering from the heat shock, *AdoIspS* transgenic plants exhibited a higher survival rate, chlorophyll content and fresh weight at different developmental stages, which revealed that the transformation of *AdoIspS* gene isolated from a monocot species can provide similar thermotolerance to isoprene non-emitter as *IspS*s from dicots.

Second, we investigated *AdoIspS* transgenic plants sensitivity to exogenous abscisic acid (ABA). *AdoIspS* transgenic plants showed a decreased sensitivity to exogenous ABA application at both germination and seedling stages. qPCR results indicate that under the treatment of ABA on root part, the regulation of ABA-induced genes and ABA biosynthesis genes in root is not affected by the presence of *AdoIspS*; however, in *AdoIspS* transgenic leaves, ABA-response mark genes are up-regulated and ABA biosynthesis genes are down-regulated, which supports the idea that isoprene emission may reduce ABA biosynthesis and accumulation in leaves. Therefore, the growth inhibition from exogenous ABA treatment decreased in *AdoIspS* transgenic plants.

To elucidate whether and how the dehydration tolerance was altered or not in later developmental stage of *AdoIspS* transgenic plants, *AdoIspS* transgenic Arabidopsis lines were further applied in dehydration stress studies. The results of water loss test and stomatal aperture assay consistently demonstrated that isoprene-emitting plants may have a reduced requirement for ABA to tolerant dehydration stress. Additionally, the survival rate, lipid peroxidation test and dehydration-related gene expression were measured to analyze *AdoIspS* transgenic plant tolerance to dehydration stress at the whole-plant level, and the results indicate that the better dehydration tolerance displayed in *AdoIspS* transgenic lines maybe due to the reduced ROS accumulation in isoprene-emitting

leaves.

These findings suggested that *AdoIspS* gene plays an important role in plant's tolerance to abiotic stress conditions. Understanding the regulatory mechanisms of *AdoIspS* gene in response to abiotic stresses could help us find out why isoprene-emitting plants cope better with transient stress events in nature.

1 Introduction

1.1 Isoprene

1.1.1 The production of isoprene

It has been 60 years since the first discovery of isoprene emission from plants (Sharkey & Monson 2017). This subject has been studied in a rich variety of scientific fields (Sanadze 2004).

Isoprene (2-methyl-1,3-butadiene, C5H8) is a five carbon volatile hydrocarbon compound, which is synthesized in the chloroplasts of some plant species, with a low solubility in water and a low boiling point (34 °C). Isoprene is the most abundant naturally emitted biogenic volatile organic compound (BVOC) (Brilli et al., 2007), which could be widely applied to industrial productions. For example, isoprene can be applied in the production of synthetic rubber, and as the fuel additive for gasoline and diesel (Bentley et al., 2014).

Isoprene can be produced by a diverse group of organisms including plants, microbes, marine algae and animals, even human beings (Milne et al., 1995; Sharkey, 1996; King et al., 2012; Ye et al., 2016). But terrestrial vegetations are responsible for producing over 90% of atmospheric isoprene (Pacifico et al., 2009).

In plants, isoprene is synthesized through the methylerythritol 4-phosphate (MEP) pathway, directly from a prenyl diphosphate precursor, dimethylallyl diphosphate (DMADP) (Sharkey et al., 2008; Lichtenthaler, 1999). There are two pathways for DMADP production in plants, besides the MEP pathway, another one is the mevalonate (MVA) pathway. The MEP pathway is in the plastid and the other one

is in the cytosol (Vickers et al., 2009a; Logan et al., 2000; Kumari et al., 2013). (Fig. 1.1)

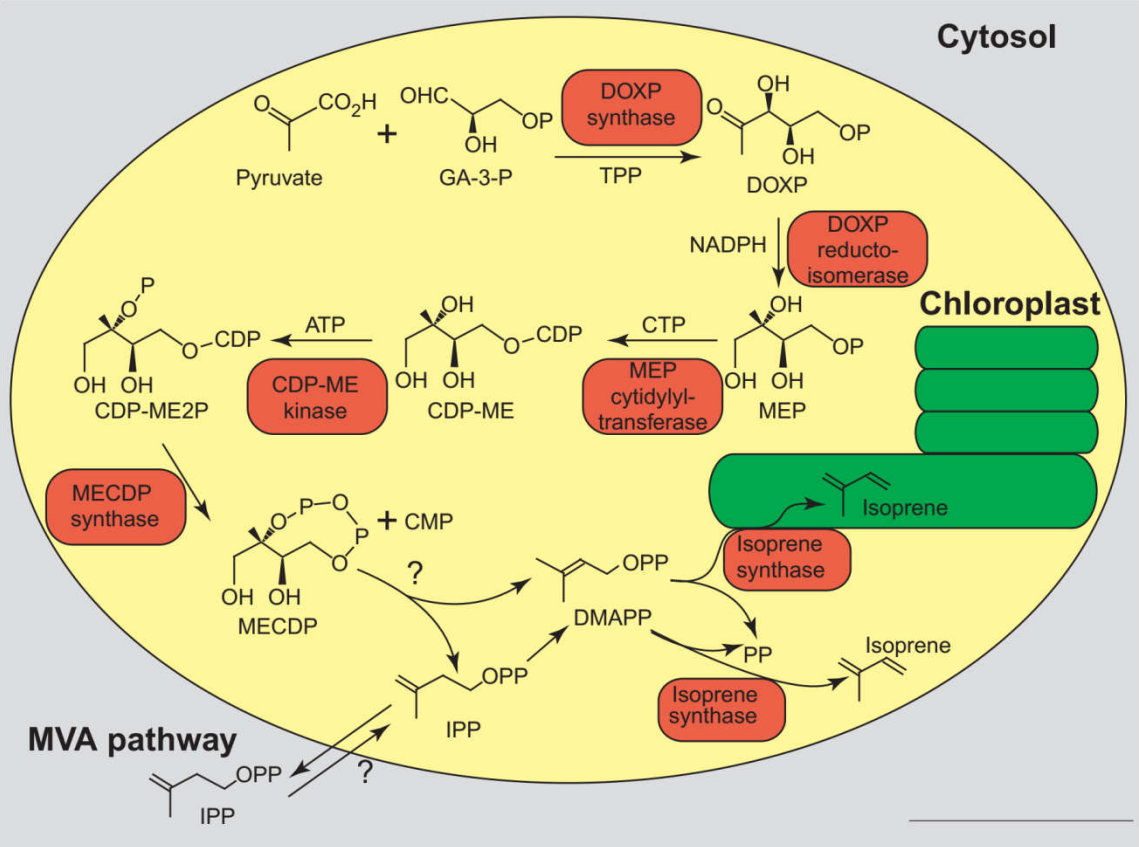


Fig. 1.1 A schematic depiction of the biosynthetic pathway that leads to isoprene formation in plants. (Logan et al., 2000)

In *E. coli*, after the concurrent over-expression of the MEP and MVA pathway, isoprene production can be largely improved due to the synergy of the dual pathway (Yang et al., 2016).

DMADP is converted into isoprene by Isoprene synthase (IspS). Therefore, isoprene production is determined by the size of DMADP pool and the activity of IspS (Wiberley et al., 2009; Bichele et al., 2010), which are in turn influenced by many factors, like the endogenous developmental stage of a leaf (Ahrar et al., 2015; Niinemets et al., 2015). Vickers *et al.* (2010) proposed that the control of isoprene production is complex. They proved that the availability of DMADP predominately controls isoprene emission rates in developed leaves. However, in young and developing leaves, in addition to DMADP level, the amount of IspS protein is the key regulator in controlling isoprene emission (Vickers et al., 2010).

Although IspS is the single enzyme needed to cause a non-emitting plant to emit isoprene (Sharkey 2013), the important role of DMADP availability in isoprene emission is surprisingly big which cannot be ignored. Rasulov *et al.* (2009) tested post-illumination isoprene emission to measure DMADP availability to isoprene synthase and suggested that, compared to changes in IspS enzyme kinetics, DMADP limitation is more related to the increased isoprene emission in response to short-term light, CO_2 and O_2 flux. This finding was supported by a previous report that in *Populus alba*, leaves developed under sun and leaves developed in the shade had the same amount of IspS protein, but their isoprene emission rates were totally different (Vickers et al., 2010). They also proved that the protein level of isoprene synthase didn't alter during heat and light stress, but as presented in many publications, isoprene emission always increases under heat and light stress. Therefore, they propose that DMADP availability should be the dominant factor controlling isoprene emission rates (Vickers et al., 2010).

After synthesized in the chloroplast, isoprene is immediately lost from the leaf. There is no known mechanism for the storage of isoprene (Sanadze, 2010). The carbon and energy losses from plants for isoprene production are very significant (Sharkey and Yeh, 2001; Zeinali et al., 2016; Velikova et al., 2015; Loivamäki et

al., 2007), especially when plants were exposed to stress conditions, up to 20% of recently fixed carbon can be directly utilized for isoprene synthesis (Harley et al., 1996; Vickers et al., 2009b). The reason why plants consume so much carbon and energy to produce isoprene is still ambiguous (Velikova et al., 2015). However, since the biosynthesis of isoprene is so costly, it is believed that this big investment certainly has relevant physiological and/or functional benefits in return (Fini et al., 2017).

Notably, there are only some certain plant species which are able to emit isoprene, while others are not. Isoprene emission could be detected across the whole plant kingdom, but isoprene emitting species are scattered across the phylogenetic tree (Monson et al., 2013; Dani et al., 2014). Isoprene is emitted by about one-fifth of the perennial plants in temperate zones and tropical areas of the world (Loreto & Fineschi 2015). The ability to emit isoprene is widespread in perennial and deciduous vegetations of temperate regions, and absent from C4 and annual plant life (Loreto & Fineschi 2015). Several views explaining such scattered phylogenetic occurrence of isoprene emission have been proposed based on the evolutionary pattern of IspS, which will be introduced later.

As mentioned above, isoprene production in plants is determined by both DMADP pool and the presence of IspS. Since IspS is the only enzyme needed to cause a non-emitting plant to emit isoprene (Sharkey 2013). Therefore, it is believed that IspS is responsible for the large amount of carbon loss from plants into the atmosphere (Vickers *et al.* 2010).

1.1.2 Atmospheric effects

Isoprene is the most important non-methane BVOC emitted by plants into the atmosphere, due to its impact on atmospheric chemistry which could influence air quality and climate (Morfopoulos et al., 2014; Pacifico et al., 2009).

Many plant species emit isoprene at high rates (Sharkey and Yeh, 2001; Loreto et al., 2014a). The estimated global isoprene emission ranges from 500 to 750 teragram per annum, which equals to 440 to 660 teragram carbon (Guenther et al., 2006). However, this number is possibly less accurate. Several models have been created to investigate isoprene emission from photosynthesizing leaves, and further to investigate the possible impact of isoprene on climate (Morfopoulos et al., 2014). The widely used models include algorithm developed by Guenther *et al.* (1993) (Guenther et al., 1993), Niinemets model (Niinemets et al., 1999) and MEGAN (Guenther et al., 2012). The latest one is a unifying model presented by Morfopoulos *et al.* (2014), which indicated that all the current isoprene emission models remain quite empirical, and there is plenty of scope for the improvement of the modeling of isoprene emissions by terrestrial plants at regional and global scales for future studies (Morfopoulos et al., 2014).

Isoprene is involved in atmospheric chemical and physical processes by affecting the production of tropospheric ozone (ground level), reacting with nitrogen oxides ($NOx = NO + NO_2$) and forming secondary organic aerosol (SOA).

Up in the stratosphere, ozone protects life on earth from the sun's ultraviolet rays by absorbing most of the ultraviolet radiation and preventing them from reaching the earth's surface (Allen et al., 1998; Kerr and McElroy C T., 1993). But in the troposphere, ozone (O_3) is harmful to human health and able to cause the most severe damage to plants (Wittig et al., 2009). O_3 is a greenhouse gas and a source of the hydroxyl radical (OH), with the third strongest radiative forcing on climate

between 1750 and 2005 (Fiore et al., 2002; Finlayson-Pitts and Pitts, 1997; Wittig et al., 2009; IPCC, 2007).

The increased tropospheric ozone level could cause astounding economic loss due to its damage to human and vegetation. In Europe, ground-level ozone causes about 22,000 deaths every year, in addition to crop yielding and biodiversity losses (Ashworth et al., 2013).

World Health Organization (WHO) recommended 60 ppbv as an upper limit of ozone level (Sanderson et al., 2003). Exposure to ozone above this level appears to cause respiratory and cardiac diseases, such as asthma, pneumonia and myocardial infarction (WHO, 2000).

One global three-dimensional general circulation model described the integrations for 100 years and coupled to the dynamic vegetation and chemistry model, in which the author suggested that the increase of ozone level is closely related to the increase of isoprene emission in most regions, for example, eastern Asia, eastern USA and Amazonia (Sanderson et al., 2003). The author also suggested that due to the increases of temperature, isoprene production could increase by 187 Tg per year, based on the calculation from the model mentioned previously, or 148 Tg per year if vegetation change has been included (mostly because of the decreased area of forest) (Sanderson et al., 2003).

People considered that isoprene would not increase atmospheric SOA at the beginning, because the oxidation products of isoprene, such as formaldehyde, glycolaldehyde, methyl vinyl ketone and methylglyoxal are highly volatile (Carlton et al., 2009). However, as concluded by Carlton *et al.* (2009), after extensive studies on SOA formation from isoprene oxidation products through the measurement of 1) those oxidation products in ambient particulate matter, 2) aerosol growth after the multistep oxidation process and 3) the low-volatility

species from the aqueous reactions of those products, isoprene's important role in increasing SOA formation in the atmosphere has been proved (Carlton et al., 2009).

As described in Surratt *et al.* (2010), isoprene oxidation initiates with atmospheric hydroxyl (OH) radical. The photooxidation of isoprene proceeds by formation of a hydroxy peroxy (RO_2) radical. In presence of high nitrogen oxides concentrations, RO_2 radicals react with NO to produce alkoxy radicals (RO) or organic nitrates ($RONO_2$). These RO radicals generated from isoprene will not produce big amounts of SOA, because normally they fragment into more volatile products. But under lower or non-nitrogen oxide conditions, RO_2 radicals react with HO_2 to produce less volatile oxidation products which result in high SOA yields (Surratt et al., 2010). The group of isoprene oxidation products with low volatility is the single largest source of atmospheric organic aerosol (Surratt et al., 2010). It is believed that more than one-fifth of SOAs are directly generated from isoprene (Kanawade et al., 2011). SOAs not only affect human health, but also cause visibility and climate problems (Tan et al., 2009; Volkamer et al., 2006).

As stated above, it is not surprising that isoprene has drawn a lot of attention in climate science (Morfopoulos et al., 2014).

1.1.3 Environmental constraints for isoprene emission

Isoprene emission from plants is strongly influenced by several environmental stimuli and constraints, such as temperate, light, ambient CO_2 concentration and abiotic stresses (e.g. drought and/or heat) (Loreto and Schnitzler, 2010; Sharkey and Monson, 2017).

In vivo, isoprene emission increases when leaf temperature rises. It was proposed that the temperature response of isoprene emission was likely linked to isoprene synthase activity (Sharkey and Monson, 2017). The optimal temperature of *in vivo* isoprene emission is normally below 40 °C, while the optimum temperatures for isoprene synthase were approximately 5 °C higher than that for the temperature dependence of isoprene emission, despite a large within and between species variability (Niinemets et al., 1999).

Isoprene emission from plants ceases under the dark condition, even with a proper temperature. Its emission rate increases when illumination intensity grows (Niinemets et al., 2015). Isoprene emitters tend to increase emission with increasing leaf energy status (Morfopoulos et al., 2014). Isoprene emission is sensitive to light and influenced by light use efficiency, which is likely linked to photosynthetic electron transport and energy dissipation in leaf (Sharkey and Monson, 2017; Morfopoulos et al., 2014; Behnke et al., 2010b; Niinemets et al., 1999).

High concentration of ambient CO_2 is also an influential factor affecting isoprene synthesis in plants. It is well known that the biosynthesis of isoprene is involved in recently assimilated CO_2 from photosynthesis (Monson et al., 2012; Potosnak et al., 2014). High concentrations of CO_2 stimulate plant photosynthesis, and inhibit isoprene emission as a consequence (Niinemets and Sun, 2015).

The inhibition of isoprene emission by high concentration of CO_2 can be lost at higher temperatures (Potosnak et al., 2014; Niinemets and Sun, 2015). The temperate range for this inhibition is 25-35 °C (Potosnak et al., 2014).

As isoprene emission sensitivity to high CO_2 concentration can be affected by leaf temperature, there is a multiple interaction between these three environmental factors (temperate, light intensity and CO_2 concentration) on isoprene emission from plants, as described by Niinemets and Sun (2015):

1. The optimum temperature of isoprene emission varied in dependence on light intensity and CO_2 concentration of plant's growth condition;

2. The light sensitivity of isoprene emission decreased with increasing temperature (from 35-50 °C);

3. The light sensitivity of isoprene emission was higher in plants which were grown under an elevated CO_2 condition (Niinemets and Sun, 2015).

Isoprene emission from plants is also affected by some environmental stresses, such as heat, drought and high light.

Isoprene emission rate increases under heat stress and high light stress conditions (Velikova and Loreto, 2005), and decreases only at severe water deficit conditions. In isoprene emitters, unlike photosynthesis, isoprene emission rates are not immediately affected by mild drought stress (Monson et al., 2007; Ryan et al., 2014). On the other hand, isoprene emission increased simultaneously when the leaf temperature of *Phragmites australis* was increased from 30 °C to 38 °C, while photosynthesis of the leaf decreased at the same time (Velikova and Loreto, 2005).

1.2 Isoprene synthase

1.2.1 General introduction

The IspS enzyme is localized in plant chloroplast, together with a large family of terpene synthases (Fortunati et al., 2008). Isoprene synthases are operationally soluble in some plant species (Schnitzler et al., 2005). The IspSs are active at a broad range of pH values and have their optimum pH between 7 and 10.5 (Lantz et al., 2015; Oku et al., 2015). This enzyme has requirements for Mg^{2+} or Mn^{2+} (to a lesser degree) for activity, the same as other prenyl diphosphate-utilizing enzymes (Sharkey and Yeh, 2001; Köksal et al., 2010; Lantz et al., 2015). The molecular weight of isoprene synthase in different plant species ranges from 58 to 95 KDa (Sharkey and Yeh, 2001). *In vitro* temperature for optimum activity ranges from 35 to 45 °C, and sometimes could reach 50 °C (Lantz et al., 2015).

Up to now, isoprene synthase has only been identified and characterized from a few plant families. Isoprene synthase sequences were first identified from hybrid poplar (*Populus alba* × *P. tremula*) using primers designed according to three polypeptides which were reported previously by Silver and Fall from University of Colorado in 1995 (Miller et al., 2001). Later, the *IspS* gene from kudzu (*Pueraria montana*) was isolated by screening the cDNA library with a probe of aspen isoprene synthase sequence (Sharkey et al., 2005).

Sharkey *et al.* (2013) identified nine novel isoprene synthase genes within the rosids, the clade from which the majority of *IspS* were obtained in recent years. By using a homology-based cloning approach they obtained isoprene synthase genes from a *Salix* and four *Populus* in the Salicaceae via primers which were designed based on *IspS* sequence from *Populus alba*, and isoprene synthase genes from *Robinia pseudoacacia* and *Wisteria* in the Fabaceae, by degenerate primers which were designed based on the amino acid sequence of *IspS* from kudzu

(Sharkey et al., 2013). The *IspS* sequences of *Eucalyptus globulus* and *Melaleuca alternifolia* in the Myrtaceae were identified by a homology based database searching (Sharkey et al., 2013). Recently, *IspS* genes were also identified and isolated from three tropical trees. The sequence of *IspS* from *Casuarina equisetifolia* shared less than 50% similarity with the others (*Ficus septica* and *Ficus virgata*) (Oku et al., 2015). Moreover, besides *IspS* sequences of these two Ficuses, all *IspS*s previously identified clustered in TPS-b clade II, but *IspS* sequence of *C. equisetifolia* is unusually positioned in TPS-b clade I. TPS-b clade II is the main *IspS* clade, which indicated structural similarities of IspS to monoterpene synthases (Oku et al., 2015).

To date, IspSs from about twenty plant species has been identified. But generally, the most extensive studies on IspSs were focused on poplar and kudzu (Ilmén et al., 2015), especially the IspSs from poplars, whose physiological functions on photosynthesis protection had been investigated intensively for decades (Jud et al., 2016; Loivamäki et al., 2007; Behnke et al., 2010b; Loreto et al., 2014b).

The characterization of IspS from bryophyte is in its beginning stage. Recently the first moss IspS protein has been isolated and characterized from *Campylopus introflexus* (Lantz et al., 2015), although the purification and the cDNA sequence clone of this IspS enzyme are still under way (Lantz et al., 2015).

1.2.2 Identification studies of isoprene synthase

Isoprene emission species are scattered across the phylogenetic tree in the angiosperm lineage, although it could be detected across the whole plant kingdom (Monson et al., 2013; Dani et al., 2014). The *IspS* genes are present only in some plant genera (Dani et al., 2014). Besides this sparse presence of *IspS* gene in taxonomic distribution in plants, it is also hypothesized that *IspS* gene function may has been gained and lost many times independently during the evolution of plants (Monson et al., 2013).

The evolutionary pattern of IspS has been proposed to explain such sparse phylogenetic occurrence of isoprene (Li et al., 2017). The debate focused on whether IspS appeared and was lost multiple times independently or appeared only once in the early evolution of terrestrial plants (Hanson et al., 1999; Harley et al., 1999; Lerdau and Gray, 2003; Sharkey et al., 2005; Li et al., 2017). It is now clear that, the isoprene emission trait must have evolved independently by parallel evolution in the major lineages of plants (Sharkey et al., 2013), reflecting the general patterns of evolution of terpene synthases (Chen et al., 2011). However, whether this model applies also to lower taxonomic levels is still debated. One view proposes that a single gain of isoprene synthase function happened early during the radiation of rosids, and then followed by multiple losses (Sharkey et al., 2013; Sharkey, 2013). Another view proposes that due to the hypothetically low number of amino acidic mutations, isoprene emission trait could repeatedly undergo gain and loss at the family or genus level (Monson et al., 2013; Li et al., 2017).

It is now more clear how plant IspSs originated across the evolutionary history due to the knowledge obtained after decades of intensive studies on the identities of the ancestral enzymes from which IspS likely evolved and the identification of

a diagnostic tetrad for IspS genes(Monson et al., 2013; Sharkey, 2013; Sharkey et al., 2013; Li et al., 2017; Sharkey and Monson, 2017).

In angiosperms, isoprene synthases are phylogenetically closely related terpene synthases (Sharkey et al., 2013). The analysis of poplar IspS crystal structure and alignment of isoprene synthase proteins from rosids identified a core of four conserved amino acids in IspS (Li et al., 2017).

While screening for enzymes having exclusive isoprene synthase activity from sequence identity, the residues F338 and F485 have been considered as essential markers, because these two residues were suggested to constrain the binding pocket size of the DMADP substrate diphosphate anion moiety, even though this hypothesis for long time was not experimentally tested (Gray et al., 2011; Sharkey et al., 2013; Li et al., 2017). Another two amino acids, S446 and N505, are preferentially found in isoprene synthases, but display some degree of variation in some IspS (Ilmén et al., 2015; Oku et al., 2015). *IspS* genes so far have only been identified in rosids, except one from asterids (Ilmén et al., 2015).

A recently published work from our group presented the isolation and functional validation of the first isoprene synthase gene, *AdoIspS*, from a monocotyledonous species (*Arundo donax* L., Poaceae). The *IspS* gene from *A. donax* filled a large gap in the taxonomic sampling and to date it is the single-product *IspS* gene from the most taxonomically divergent lineage with respect to the main IspS rosids clade. In this study, maximum likelihood phylogenetic reconstruction was carried out on a large set of class b terpene synthases (TPS-b), and confirmed that *AdoIspS* is at the base of TPS-b Clade 2 (Li et al., 2017). (Fig. 1.2)

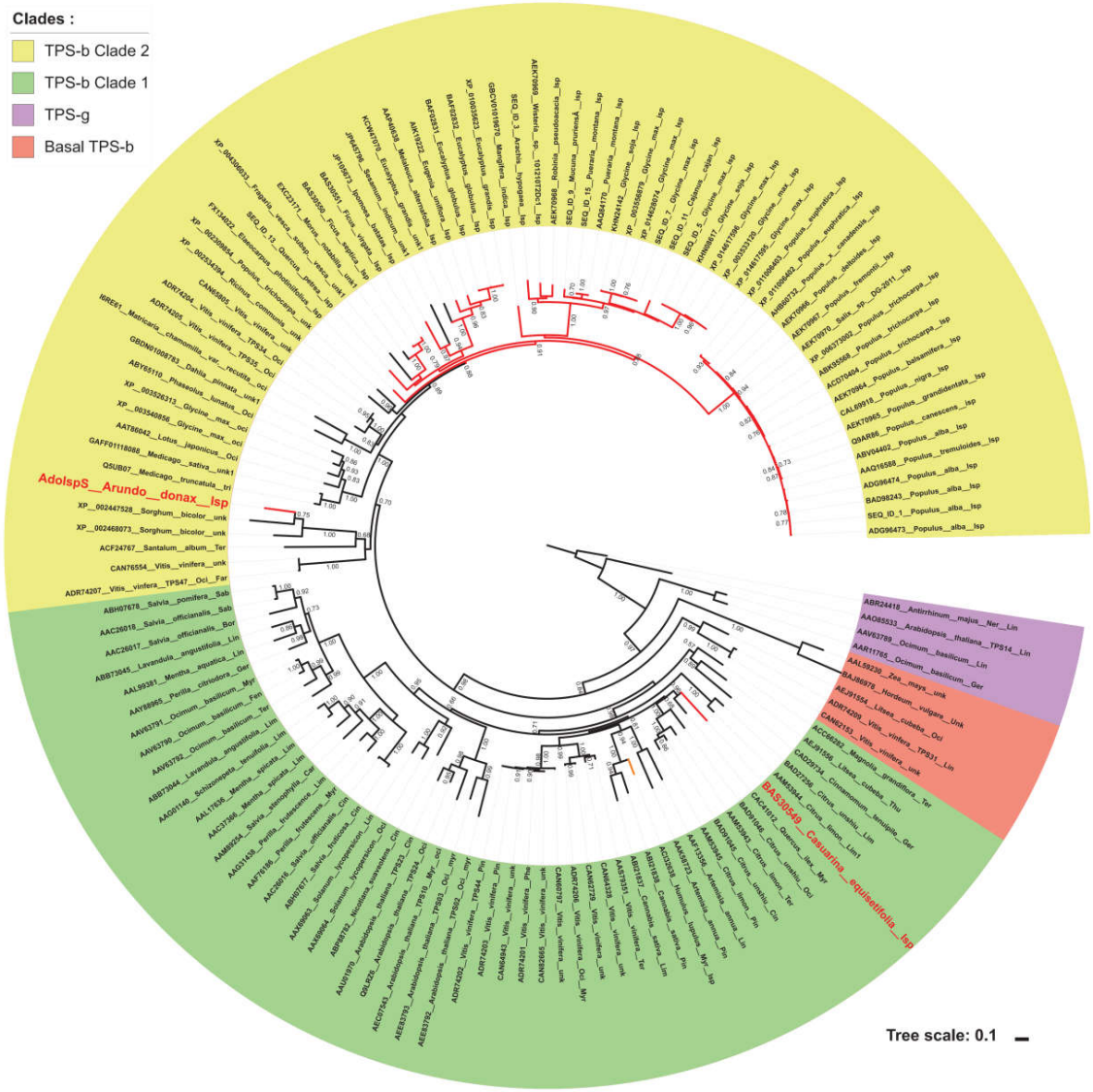


Fig. 1.2 Phylogenetic reconstruction of class b terpene synthases and some representatives from class g with maximum likelihood. (Li et al. 2017)

The functional and evolutionary relevance of the residues considered diagnostic for IspS function was demonstrated by site-directed mutagenesis. A pivotal role of F310 was found in the evolution of short-chain terpene synthases (TPS) (Li et al., 2017). (Fig. 1.3)

Previous studies predicted that both F310 and F457 residues could determine isoprene production instead of monoterpene synthesis from geranyl pyrophosphate (Sharkey et al., 2005; Köksal et al., 2010). Our results demonstrated in vivo that only position 310 plays a fundamental role in *AdoIspS* substrate specificity determination by active site size modulation. The tolerance of isoprene activity to amino acid replacements at position 310 and 457 is similar. F457, however, is basically unable to promote significant *in vivo* neofunctionalization. The different plasticity of the two residues (Yoshikuni and Keasling, 2007), indicates that compared with appearance of F457, acquisition of an F at position 310 most likely constituted a more relevant constraint in the evolution of IspS activity (Li et al., 2017). Our results also indicated that ocimene synthases is the most likely ancestral TPS from which IspS evolved by active site size reduction following acquisition of F at position 310 (Gray et al., 2011).

The region homologous to *AdoIspS* S417-L422 has been showed to contribute to product specificity in monoterpene synthases (Kampranis et al., 2007), and S477 may be part of a mechanism of substrate sensing and conformational remodeling, associated to transition to closed conformation, ancestrally shared by several TPS enzymes (Whittington et al., 2002).

For clarity, the four amino acids corresponding to the "IspS diagnostic tetrad" (F338, S446, F485 and N505 of PcIspS) as previously identified by Sharkey *et al*. (2013) are F310, S418, F457 and S477 in *AdoIspS*.

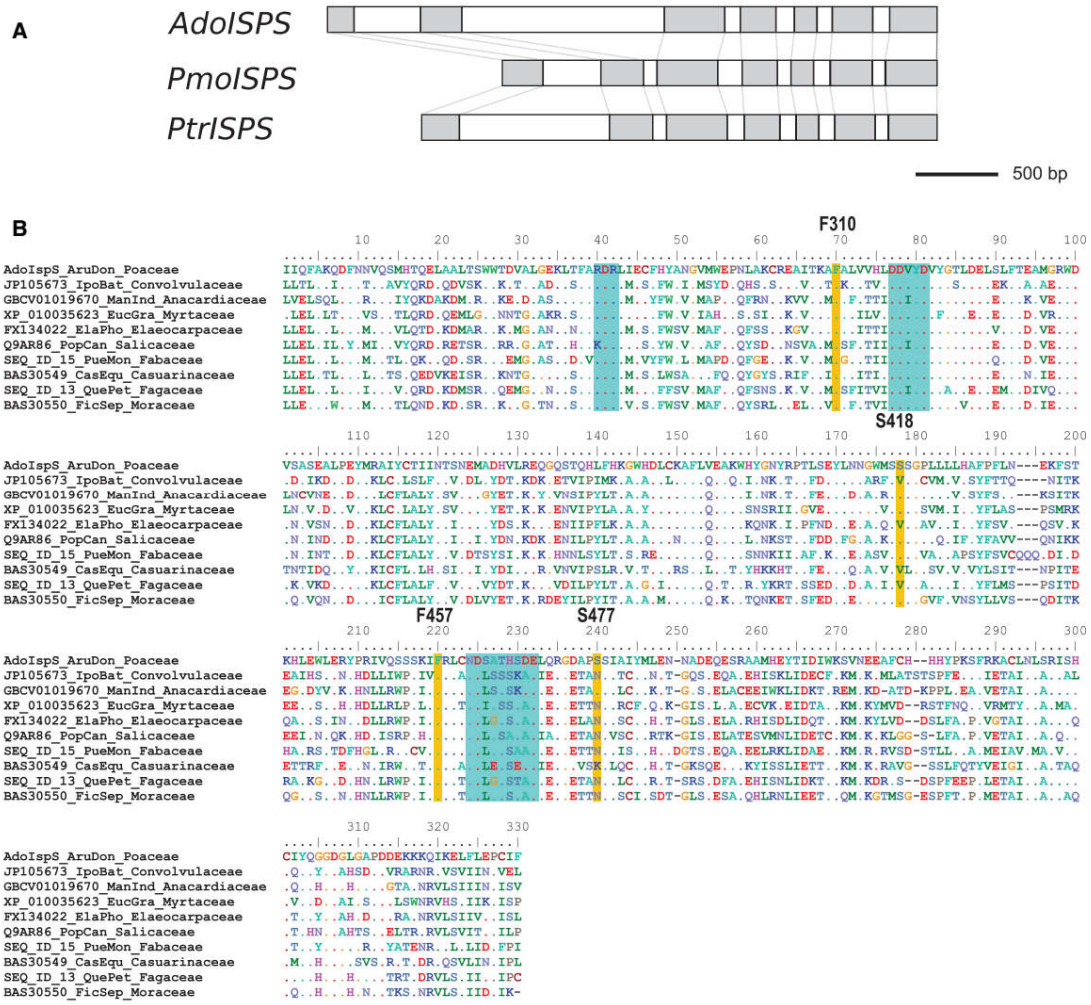


Fig. 1.3 Comparison of *AdoIspS* to selected isoprene synthases from dicots. (Li et al. 2017)

This study also provided a unified model for IspS evolution (Fig 1.4) and suggested that 1) primary acquisition of IspS activity seems to be a relatively complex and rare event, 2) reduced hydroxylation ability due to lack of the cavity necessary for water coordination at the bottom of the active site is ancestral for isoprene as well as ocimene synthases and 3) all rosids and possibly fabids IspSs are the likely result of a single, primary gain of IspS function (Li et al., 2017).

As Fig 1.4 shows, there may be an evolutionary link between the structural and functional bases of isoprene emission, parallel evolution of IspSs may be driven by relatively simple biophysical constraints (Li et al., 2017).

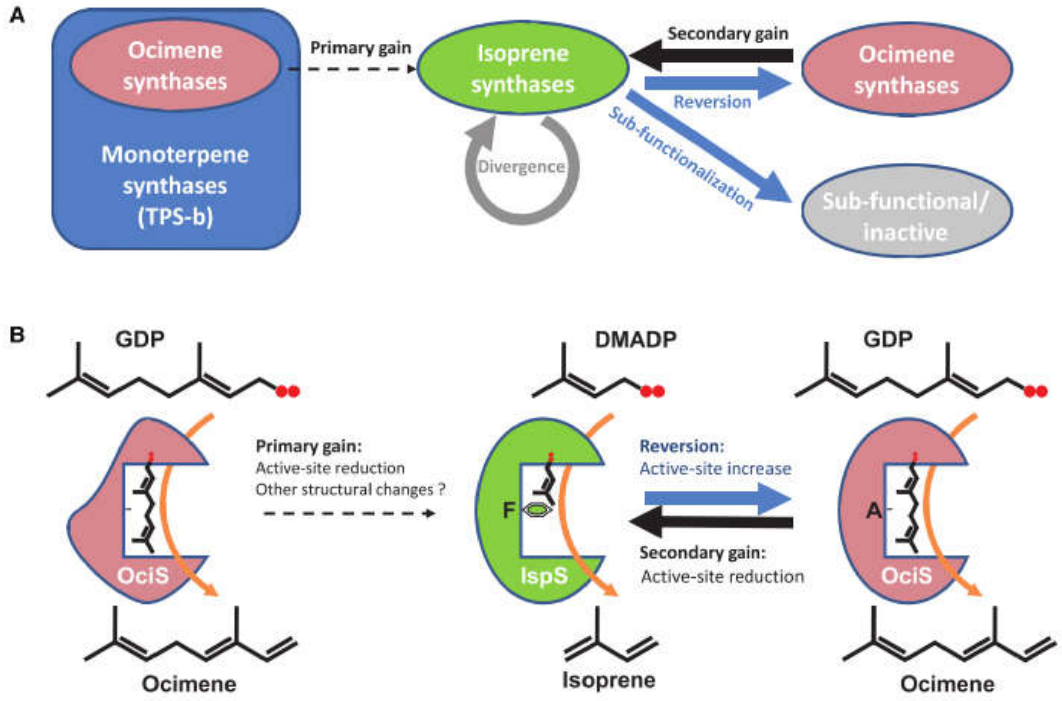


Fig.1.4 Model of gain/loss of isoprene synthase function in angiosperms. Solid arrows indicate experimentally supported processes, dashed arrows postulated ones. (A) IspS genes are proposed to have evolved multiple times from ocimene synthases (TPS-b clade), but the rate of first recruitment from the pool of pre-existing ocimene synthases is currently unknown. It is postulated that primary gains are relatively rare (dashed black line), but once an *IspS* gene has been acquired in a clade, single mutations can switch the enzyme substrate specificity back and forth between isoprene and ocimene synthase by maintaining active site overall structure, potentially leading to repeated losses and secondary gains of the trait in the same clade (blue arrow "Reversion" and black arrow "Secondary gain"). Additional mutations can lead to either divergence or can hit functionally fundamental amino acids leading to sub-functionalization (or complete protein inactivation). (B) Molecular mechanism for the evolution of isoprene synthases supported by the results of this work. Both primary and secondary gains of isoprene emission are associated with a mechanism of active site reduction (depicted as a shallower cavity in the enzyme) by replacement of the seventh position before the first Dof the DDXXD motif (*AdoIspS* position 310) with the bulky phenylalanine residue (F). The reduction of the size of active site prevents the efficient use of geranyl pyrophosphate (GDP) as substrate for the production of ocimene, causing instead the conversion of dimethylallyl pyrophosphate (DMADP) to isoprene. Additional mutations leading to putative structural changes may be required for the first recruitment (dashed arrow) of an Ocimene synthase (OciS) from the pool of TPS-b genes present in each species. Enzymatic activity can potentially revert to ocimene synthesis by a second substitution to the same position with a smaller residue, which is preferentially alanine (A) in *AdoIspS* (Li et al., 2017).

The insights provided in this study could be a good starting point, even though the selective drivers underlying evolution of the trait are still unknown (Sharkey and Monson, 2017). To dissect the details of lineage-specific conservation of isoprene emission, the identification of the selective drivers is therefore necessary (Li et al., 2017).

As thoroughly discussed in the study mentioned above, isoprene synthases were most likely originated by parallel evolution from TPS-b monoterpene synthases (Li et al., 2017). However, the biochemical properties of isoprene synthase are different from most of the known monoterpene synthases (Schnitzler et al., 2005). Firstly, although both isoprene synthase and monoterpene synthase are strongly temperature-dependent, isoprene synthase has a much higher temperature range of optimal enzyme activity. Secondly, compared to monoterpene synthase, isoprene synthase has a much higher and boarder range of Michaelis constants (Km), from 0.5 mM to 8 mM (Schnitzler et al., 2005).

Since AdoIspS is the first isoprene synthase identified from monocots, its biochemical properties are possibly different from other known isoprene synthases, and this could be a future research direction.

1.2.3 *IspS* gene expression in relation to stress conditions

In plants, isoprene emission could be further stimulated when leaves were subjected to or recovering from environmental stresses (Brilli et al., 2007; Harrison et al., 2013; Velikova and Loreto, 2005; Centritto et al., 2014).

Our group found that *AdoIspS* expression was mainly responsive to heat stress, it is reported that the largest transcriptional response of *AdoIspS* was caused by heat shock, at all time points tested (Li et al., 2017). This temperature dependency of *IspS* expression also exists in isoprene synthase from poplar (Vickers et al., 2010). To a lower extent, *AdoIspS* expression was also responsive to drought and heavy metal stresses, which matches isoprene emission in response to environmental stresses (Loreto and Schnitzler, 2010).

1.3 Photoprotective functions of Isoprene emission

1.3.1 Physiological effects of isoprene emission

-Heat stress

The thermotolerance increased observed in isoprene-emitting plants has been investigated for two decades (Vanzo et al., 2016). The fact that isoprene plays a protective role in plant photosynthesis under high temperature stress has been evidenced by many experiments (Vickers et al., 2009a).

It is believed that isoprene emitting plants are better fit to future climate, because isoprene-emitting leaves have a better tolerance to heat, their photosynthesis recovered more quickly after thermal stress than leaves in which isoprene emission was inhibited by fosmidomycin (Velikova and Loreto, 2005).

It has been proved that compared to isoprene emitting leaves, in isoprene-inhibited leaves, chloroplasts released more energy as heat, thus impaired the photosynthetic apparatus, and the thylakoid membranes of isoprene-inhibited leaves appeared leakier at an elevated temperature (Pollastri et al., 2014; Velikova et al., 2011), additionally, a considerably increased non-photochemical quenching (NPQ) of fluorescence and a lower photochemical quenching also appeared in these non-emitting plant leaves (Pollastri et al., 2014). These results indicate that isoprene emitting leaves could maintain photosynthetic apparatus under high temperate, thus have a better tolerance to heat stress.

These results were corroborated with studies using transgenic non-emitting poplars whose isoprene emission was inhibited by RNA interference (Behnke et al., 2007). Besides above mentioned reduced photo-protective traits, non-emitting poplars also produced more antioxidants under non-stressed condition, which

indicated that even in the absence of stress, isoprene-repressed poplars still need to cope with an enhanced level of oxidative stress (Behnke et al., 2007). Although the photosynthetic electron transport and net assimilation rates were similar between isoprene emitting and non-emitting poplars under normal growth condition, these traits were affected more severely under heat stress in non-emitting plants (Behnke et al., 2007).

Therefore, thanks to a lower need for heat dissipation in photosynthetic membranes and higher PSII efficiency even under physiologically high temperatures, the capability to emit isoprene will be a growth advantage for natural isoprene-emitting species to face a frequent and transient temperature change (Velikova et al., 2011; Pollastri et al., 2014; Velikova and Loreto, 2005; Behnke et al., 2007).

Transgenic plants over-expressing the *IspS* gene from poplar in Arabidopsis showed a striking thermotolerance with a much higher survival rate than wild type plants (Sasaki et al., 2007). In the leaf surface of transgenic plants, the high isoprene emission was concurrent with a decreased leaf temperature, which indicated that isoprene emission has an important role in protecting plants against heat (Sasaki et al., 2007).

Furthermore, the lost thermoprotection in these non-emitting leaves could be recovered by exogenous isoprene treatment (Sharkey et al., 2001, 2008). This is due to the fact that isoprene is a compound with double bonds (alkenes) which could enhance plants thermal protection. This is a general effect, which isoprene shares with some monoterpenes (Sharkey et al., 2008).

Therefore, based on above mentioned experimental evidences, isoprene emission enhances plants' tolerance to heat stress, as confirmed by 1) chemically-inhibiting isoprene emission from a natural isoprene emitter, 2) RNA interference to

suppress isoprene emission, 3) transforming *IspS* into non-emitting species to emit isoprene and 4) using exogenous isoprene to treat non-emitting plants (Sharkey et al., 2008).

-Sun fleck

Plants emitting isoprene also display an enhanced tolerance to sunflecks. Sunfleck is a transient exposure to a combination of high temperature and high light (Behnke et al., 2013), which can cause dramatical leaf temperature fluctuation (Vickers et al., 2009a).

Under sun-flecking conditions, isoprene emission plays a crucial role in maintaining photosynthesis. Compared to non-emitting plants, isoprene emitting plants exhibited less impaired electron transport rates and net CO_2 assimilation (Behnke et al., 2010a, 2013). Besides, isoprene emitting plants also showed a higher accumulation of phenolic compounds when exposed to sunflecks, which could improve plants defences against stress conditions (Behnke et al., 2010b). Therefore, transgenic plants engineered to reduce the emission of isoprene are probably not able to resist high-temperature periods (Behnke et al., 2013).

Besides, isoprene has also been considered to enhance tolerance to high light, since rapidly increased leaf temperature can also be induced by light flecks (Rasulov et al., 2015; Niinemets and Sun, 2015).

Ultraviolet-B light (UV-B) is the primary cause of photodamage to photosystem II (PSII) under natural sunlight (Takahashi and Badger, 2011). When exposed to UV-B light, isoprene emitting and non-emitting plants showed similar PSII performance and photosynthesis, suggesting that during the UV-B light treatment, isoprene emission did not reduce the rate of photodamage in plants. But unlike non-emitting plants, in isoprene emitting plants, photosynthesis and PSII, together

with isoprene biosynthesis, recovered after the treatment (Centritto et al., 2014). These results indicate that isoprene-emitting plants not only have a better tolerance to sunfleck, but also recover better after strong sunlight exposure. This could be a selective advantage in the future climate changes due to the reducing ozone in the stratosphere.

-Oxidative stress

Isoprene emission has long been reported to participate in antioxidative defence directly and indirectly.

Ozone

The inhibition of isoprene emission from a natural isoprene emitter increased leaves sensitivity to ozone stress (Loreto and Velikova, 2001). Compared with a natural isoprene emitter, grey poplar, RNA-interfered non-emitting poplar plants exhibited a strong and steep increase of nitric oxide (NO) and the S-nitrosylation levels after the exposure to ozone (Vanzo et al., 2016). The generation of NO, as well as ROS, is a general response from plant to many abiotic stresses (Vanzo et al., 2016). S-nitrosylation is the covalent binding of NO, and plays a pivotal role in NO signaling. S-nitrosylation modulates ROS production in cell, while S-nitroso-proteome in isoprene emitting plants was less susceptible during acute ozone exposure (Vanzo et al., 2016). The molecular dialogue between isoprene and NO, reduced nitrosative pressure in isoprene emitting plants and suggested that isoprene may indirectly regulate the status (e.g. extent and velocity) of nitrosative stress and ROS production in cell (Vanzo et al., 2016).

Compared to control azygous plants (non-isoprene emitter), transgenic isoprene-emitting tobacco was more resistant to ozone damage (Vickers et al., 2009b). Non-emitting azygous plants showed significantly reduced

photosynthesis and elevated foliar damage from the very beginning of the stress. On the contrary, the photosynthesis in isoprene emitting plants was less affected by ozone fumigation (Vickers et al., 2009b). The accumulation of reactive oxygen species (ROS) in isoprene emitting plants was also far less than that in non-emitting plants, as well as the requirement of antioxidant capacity, which was also reduced in isoprene emitting plants (Vickers et al., 2009b). Plenty of visible foliar lesions were formed on non-emitting leaves due to the high accumulation of H_2O_2 caused programmed cell death but not on isoprene-emitting leaves (Vickers et al., 2009b). These results clearly showed that the presence of isoprene could protect plants against oxidative stress (Vickers et al., 2009b).

Singlet oxygen

It has been proposed that the primary role of isoprene in plants is to remove the singlet oxygen (Zeinali et al., 2016). Singlet oxygen is the most prominent ROS which causes severe damage to leaves (Zeinali et al., 2016). A significant decrease in leaf carbon assimilation and increase of internal isoprene concentration can be induced by singlet oxygen which was generated by Rose Bengal (RB) (Affek and Yakir, 2002). RB-treated isoprene-emitting leaves maintained photosynthesis rate and repressed H_2O_2 and malondialdehyde (MDA) content under different light intensities except the highest one (Velikova et al., 2004). MDA is an indicator of membrane damage and lipid peroxidation under oxidative stress (Vickers et al., 2009a; Behnke et al., 2010b; Loreto and Velikova, 2001). However, compared to isoprene-emitting leaves in the same set of stress conditions, photosynthesis rate was dramatically reduced in non-emitting leaves, followed by the concomitant increase of H_2O_2 and MDA content (Velikova et al., 2004). These results indicated that isoprene emission could protect plants against singlet oxygen. The application of exogenous isoprene gas reconstituted the isoprene pool in leaf and protected photosynthesis in non-emitting leaves, which again demonstrated that isoprene

provides effective protection for plants against singlet oxygen (Affek and Yakir, 2002; Velikova et al., 2004).

-Drought

Unlike other abiotic stresses, the biological function(s) of isoprene emission in protecting plants from drought stress were much less investigated. As reviewed previously, a unified idea cannot be derived from previous reported studies on how isoprene emission reacts in response to drought (Ryan et al., 2014). During the past ten years, only three studies used transgenic plants overexpressing *IspS* to investigate whether isoprene emission could protect plants under drought stress. Although the number of studies is limited, the opinions are still diverse.

The first study on transgenic plants overexpressing *IspS* to test the tolerance of isoprene emitting plants to drought stress reported that transgenic plants have no enhanced tolerance to drought stress, because the survival rate of isoprene emitting plants showed no clear difference from wild type plants after withholding water for 3 weeks (Sasaki et al., 2007). This result is reasonable as, after all, there is still no evidence that isoprene emitting plants perform better than non-emitting plants in any kind of long term abiotic stresses up to now (Vickers et al., 2009a). Furthermore, compared to wild type plants, transgenic tobacco plants showed a significantly lower shoot biomass after seven-day deficit irrigation period, which is also reasonable, since the biosynthesis of isoprene could divert carbon and energy from photosynthetic components (Ryan et al., 2014). But a single shortcoming cannot offset the selective advantage provided by isoprene emission to plants (Ryan et al., 2014).

The studies from two independent groups proved that isoprene emission from transgenic tobacco could protect plants by maintaining photosynthesis and whole-plant water use efficiency (WUE) under drought stress (Ryan et al., 2014;

Tattini et al., 2014). Moreover, the ROS content was also lower in *IspS* transgenic plants, with a lower lipid peroxidation level (Ryan et al., 2014). Thus, isoprene emitting plants could recover better and faster than non-emitting plants after the stressful period, which makes them more tolerant to water-deficit conditions (Ryan et al., 2014).

Besides, under water-limited conditions, natural isoprene emitters often maintain a high level of isoprene emission and increase the isoprene concentration inside the leaf, indicating the important role of isoprene in protecting plants against drought stress (Brilli et al., 2007; Marino et al., 2017).

-Biotic stress

Isoprene emission also affects herbivores feeding preference and insects attraction for leaves (Loivamaki et al., 2008; Laothawornkitkul et al., 2008). *In vivo* and *in vitro* tests showed that isoprene had a negative impact on caterpillars' (*Manduca sexta*) feeding decision as it deterred caterpillars from feeding (Laothawornkitkul et al., 2008). However, isoprene does not cause a general negative impact on all herbivore species. The feeding preference of *Pieris rapae* and *Plutella xylostella* was not affected by isoprene emission. Meanwhile, their enemy and a bodyguard of trees, the parasitic wasp (*Diadegma semiclausum*) could be interfered by isoprene and lost its attraction to volatiles from herbivore-infested plants (Loivamaki et al., 2008). Similar to herbivores, the behavior of some parasitic wasps will not be changed by the presence of isoprene, because isoprene cannot be perceived by those insects' antennae, for example *Cotesia rubecula* (Loivamaki et al., 2008). Therefore, attention should be paid to herbaceous plants which are close to natural high isoprene emitters to avoid isoprene deterring their bodyguards (Loivamaki et al., 2008).

1.3.2 Hypotheses about the role of isoprene in abiotic stress tolerance

Isoprene is a well-studied BVOC, improving plant tolerance to abiotic stress, although its protective mechanisms are still not fully understood (Harvey and Sharkey, 2016), several hypotheses have been put forward to explain the role of isoprene emission in plant abiotic stress tolerance.

The major hypotheses about how the protective mechanisms of isoprene emission improve plants tolerance against biotic and abiotic stresses include membrane stabilization (Sharkey and Singsaas, 1995; Velikova et al., 2015), direct reactions with reactive oxygen and/or nitrogen species (Affek and Yakir, 2002; Velikova et al., 2004, 2012) and indirect alteration of ROS signaling (Vanzo et al., 2016; Vickers et al., 2009a).

Therefore, Vickers *et al.* (2009a) summarized and proposed a unified mechanism and explained that the presence of antioxidant behavior of isoprene improves a variety of abiotic stresses resistance in isoprene emitting plants by 1) directly reacting with oxidants, 2) indirectly intermediating ROS signaling, and/or 3) stabilizing lipid membranes (Vickers et al., 2009a). As reviewed previously, under ozone stress, isoprene emitting plants showed a lower requirement for antioxidants since isoprene emission reduced the oxidizing burden inside cells (Vickers et al., 2009b). The ROS-scavenging ability of isoprene can be activated on the leaf surface and inside plants as well. *In planta*, the ROS detoxification by isoprene is mainly relying on the conjugated double bounds (delocalized π-electrons) in the isoprene molecule (Vickers et al., 2009a).

-ROS scavenger

In plants, quite few responses are stress-specific (Vickers et al., 2009a). Most forms of biotic or abiotic stresses elicit an enhanced production of reactive oxygen species (ROS) as a generic response (Mittler et al., 2004; Vickers et al., 2009b). ROS are important signaling molecules involved in plant defense response, not only serving to respond to abiotic stresses (Apel and Hirt, 2004; Suzuki et al., 2012), but also controlling pathogen defense process and initiating programmed cell death (Apel and Hirt, 2004; Mittler et al., 2004). ROS signaling is required by plant cells under normal growth condition as well (Vickers et al., 2009a). The cellular balance of ROS production and scavenging is normally kept under tight control (Suzuki et al., 2012; Van Breusegem and Dat, 2006). However, the redox balance is hard to maintain under stress-induced growth-limiting environmental conditions, thus enhanced ROS production possibly leads to a significantly high ROS accumulation which causes damage to plant cells (Van Breusegem and Dat, 2006; Vickers et al., 2009b).

Plants prevent ROS excess by direct reactions (ROS quenching and removing) (Mittler et al., 2004; Apel and Hirt, 2004) and indirect actions (via actions of hormones and crosstalk between signaling pathways) (Apel and Hirt, 2004). Oxidative stress occurs when the antioxidant defense network is overloaded. Antioxidant network includes antioxidant compounds (low molecular-weight antioxidants and secondary metabolites) and enzymes (Fini et al., 2011; Brunetti et al., 2015). Antioxidant enzymes and low molecular-weight antioxidants are primary ROS detoxifying agents, and may be depleted under a severe excess of radiant energy. Consequently, secondary metabolites complement the functions of primary antioxidants, effectively countering the photo-oxidative damage (Brunetti et al., 2015).

Isoprene is a secondary metabolite, whose functions as antioxidants have been extensively investigated in the past (Brunetti et al., 2015; Loreto and Velikova, 2001; Loreto, 2001). Isoprene biosynthesis is widespread among perennial and deciduous species (Loreto and Fineschi, 2015). In plants, its emission will be further stimulated when leaves are subjected to or recovering from environmental stresses (Velikova and Loreto, 2005; Centritto et al., 2014). These findings suggest that plants in favor of emitting isoprene because isoprene emission is capable of responding to many stresses, thus providing benefits to plants in their response towards a vast array of adverse environmental conditions (Vickers et al., 2009a; Loreto and Fineschi, 2015).

-Membrane stabilization

The hypothesis about isoprene enhancing photosynthetic membrane properties to help plants cope better with thermal stress has been proposed more than twenty years and its mechanisms are still not well understood (Sharkey and Singsaas, 1995). Photosynthetic processes in leaves always exhibit a reduced efficiency when plants are exposed to heat stress, because membranes turn into a more fluid structure under high temperature (Vickers et al., 2009b).

RNA interfered non-emitting plants had a changed lipid composition of thylakoid membranes (Velikova et al., 2015). The photochemical efficiency could be negatively affected in non-emitting plants, due to a significantly reduced unsaturated fatty acids level and decreased thylakoid membrane fluidity, which negatively related to nonphotochemical quenching (NPQ) and energy-dependent quenching (qE) (Velikova et al., 2015). Compared to isoprene emitting plants, the chloroplast ultrastructure was also altered in non-emitting plants, with a reduced amount of grana stacks and increased length of stroma thylakoids (Velikova et al., 2015). A rearrangement of the chloroplast protein profile can also be triggered by the suppression of isoprene synthase by RNA interference (Velikova et al., 2014).

Due to the absence of isoprene emission, the levels of photosynthesis-related proteins were decreased, associated with increased levels of metabolism-related proteins (Velikova et al., 2014). These results strongly supported that isoprene emission functionally associates with membrane structural organization, and improves thylakoid membrane structure (Velikova et al., 2015, 2014). Up to now, the thylakoid membrane structure has not been studied in *IspS* over-expression plants. The leaf growth of isoprene-emitting Arabidopsis plants was significantly faster than wild type plants (Loivamäki et al., 2007), which suggested the presence of isoprene facilitated photosynthetic processes (which are membrane-associated) (Vickers et al., 2009a). Therefore, it is possible that *IspS* overexpression plants also have an altered membrane structure. Additionally, the expression of gene(s) related to membrane integrity in isoprene emitting plants in response to abiotic stress has not been reported yet. Clearly, this subject is interesting and may need further investigation.

A theoretical model proposed that isoprene participates into the phospholipid membranes through physical stabilization and enhancement of hydrophobic interactions under heat stress (Siwko et al., 2007; Velikova et al., 2011; Vickers et al., 2009a). But this mechanistic theory still hasn't been tested *in vivo*. Exogenous isoprene fumigation can improve chemically inhibited isoprene-emitting plant's tolerance to heat (Sharkey et al., 2001; Velikova et al., 2011), but it didn't enhance the thermal tolerance of isolated membranes (Logan et al., 1999; Vickers et al., 2009a). A reasonable explanation is that, unlike intact plant membranes, isolated membranes cannot physiologically respond to isoprene (Vickers et al., 2009a). Besides, it is unlikely that isoprene protects plants only by means of the antioxidant and membrane stabilizing potential mechanisms, because isoprene has already been proposed as a signaling molecule which can affect phenylpropanoid metabolism by exogenous supplementation (Harvey and Sharkey, 2016).

-Signal

Isoprene has been suggested as a general signal which influences stress perception and signaling cascades in all plant species (Behnke et al., 2010a; Harvey and Sharkey, 2016). The application of exogenous isoprene to Arabidopsis altered the expression of many genes in this natural isoprene non-emitter under non-stressful conditions (Harvey and Sharkey, 2016). These expression patterns changed through a conserved mechanism within isoprene emitting plants and non-emitting plants, since the changes in Arabidopsis happened so rapidly, which means the altered expression pattern was not affected by plant acclimation to the absence of isoprene (Harvey and Sharkey, 2016). Phenylpropanoid biosynthesis in plants is a general response to many different environmental stresses, and phenylpropanoid biosynthetic genes could be down-regulated by the suppression of isoprene synthase and up-regulated by isoprene fumigation (Behnke et al., 2010a; Harvey and Sharkey, 2016). In addition, isoprene fumigation could induce a network with about sixty ERF and WRKY transcription factors in total (Harvey and Sharkey, 2016). The WRKY family (comprising 74 members) and AP2/ERF superfamily (comprising 145 members) are widely involved in responses to abiotic stresses as well as plant development (Li et al., 2016; Liu et al., 2012a; Stepanova, 2005; Harvey and Sharkey, 2016). Therefore, isoprene fumigation under environmental stress conditions may also improve plant tolerance through the regulation of signaling transduction.

It has been suggested that under drought stress there may be a potential cross-talk between isoprene and abscisic acid (ABA) (Marino et al., 2017), because ABA can also be produced through the MEP pathway (Marino et al., 2017; Tattini et al., 2014). ABA is a plant hormone and a pivotal molecule which participates in drought perception and signal transduction pathways in drought stress protection (Jensen et al., 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Tattini et al.,

2014). Drought-induced ABA biosynthesis initiates ABA relocation and accumulation in guard cells, causing a loss of guard cell turgor pressure which results in stomatal closure (Desikan et al., 2004).

Isoprene biosynthesis increases in plants under mild water stress, concomitant with an enhanced level of free ABA and a decreased ABA-glucoside/free ABA ratio (Marino et al., 2017). These results supported the idea that the *de novo* ABA biosynthesis through the MEP pathway can be induced by drought stress. However, it has also been proposed that isoprene biosynthesis competes with non-volatile isoprenoids produced through the MEP pathway (Ghirardo et al., 2014).

Under severe drought stress, isoprene emission declined in transgenic tobacco overexpressing *IspS*, and associated with an increased foliar ABA content with respect to non-emitting tobacco (Tattini et al., 2014). However, by using the same transgenic tobacco plants, it has been reported that isoprene emission has no effect on foliar ABA concentration under water deficit (Ryan et al., 2014). Apparently, this subject still needs further investigation and proper experimental validation.

Isoprene may also react with NO and interfere with the signaling pathway activated by NO-ROS interactions, and indirectly modulate the production of ROS (Vanzo et al., 2016; Velikova et al., 2005). NO signaling regulates many plant development processes, hormonal signaling, as well as biotic and abiotic stress responses (Desikan et al., 2004; Yan et al., 2007; Vanzo et al., 2016). The presence of isoprene reduced NO emission and altered S-nitrosylation level during ozone treatment (Vanzo et al., 2016), as reviewed previously. Therefore, isoprene may modulate plant physiological status in response to abiotic stress by modulating the production of NO and ROS signaling molecules in cells (Vanzo et al., 2016).

1.4 Climate change

1.4.1 Global climate change

General concerns about the potentially serious effects of global temperature increase have been raised for years (Iba, 2002). High temperature, as a result of global warming, damages plant development and growth, which could cause crop failures and may lead to widespread famine (Bita and Gerats, 2013). For this reason, scientific researchers have made efforts to promote the adaptability of plants to the high temperature environment for a long time (Sharkey, 2000; Wahid et al., 2007; Hemmati et al., 2015). Global warming also affects global mean aridity trend by decreased precipitation and/or increased evaporation (Dai, 2013). Drought is an extreme climate event, for instance, agricultural drought caused by precipitation shortage also leads to crop production reduction as well as high temperature (Dai, 2011). Plants are vulnerable to environmental changes because they are sessile organisms and are susceptible to adverse climatic effects (e.g. high temperature and drought) (IPCC, 2007). Temperature and precipitation extremes strongly affect plant growth and survival conditions globally (Reyer et al., 2013). Moreover, a recent study predicted that if droughts occur under the condition of raised temperature from global warming, the water scarcity will set in more severely and rapidly (Trenberth et al., 2013), which is likely to be a greater challenge for plants. Therefore, IPCC (2014) called climate change as one of the most significant global impacts on agriculture. Climate change could be divided in two different types: one is the variation of the average temperature and/or the average precipitation; the other one is the change of temperature and/or precipitation variability (Reyer et al., 2013). Plants are more vulnerable to the change of the latter (Reyer et al., 2013). In these circumstances, it is reasonable to suppose that obtaining a quick strategy to cope with short-term climate extremes is an adaptive advantage for plant species.

1.4.2 Isoprene emission in response to climate change

It has been well documented that isoprene emission protects plants against transient temperature stress and helps plants recovering faster after stress (Velikova and Loreto, 2005; Behnke et al., 2007, 2010b; Loreto and Fineschi, 2015; Vickers et al., 2009b). The natural isoprene emitters have been proved being distributed more widely in fast-growing species to avoid long-term stress (Dani et al., 2014; Loreto and Fineschi, 2015). It has been hypothesized that isoprene emission was originally developed since plants left aquatic environment, and acted as a rapid method to help plants survive in terrestrial transient temperature changes (Vickers et al., 2009a).

Besides increasing water scarcity, global warming is also accompanied by an increasing oxidative stress environment, which could lead to high oxidative properties in the forthcoming atmospheric changes (Lerdau, 2007; Vickers et al., 2009a).

Therefore, as reviewed previously, due to the rapid and primitive responses to environmental stress (e.g. heat, high light, ozone and drought), isoprene emitters would have a selective advantage under approaching climate extremes (Lerdau, 2007; Pollastri et al., 2014).

1.5 Giant reed

1.5.1 A bioenergy crop

A. donax is a high-yielding, perennial and fast-growing weed, which can grow up to 6–8 m in height (Angelini et al., 2009; Cosentino et al., 2016; Pompeiano et al., 2016). This plant species is widespread around the Mediterranean region, while it is native from eastern Asia (Cosentino et al., 2016). *A. donax* has been listed as an invasive species in United States because it has greatly expanded its native range, even though it is sexually sterile and only reproduces vegetatively (Mariani et al., 2010; Nackley et al., 2014). The ability of *A. donax* to invade and naturalize in marginal land caught attention of engineers and scientists (Cosentino et al., 2016; Nackley et al., 2014; Pompeiano et al., 2016).

In fact, *A. donax* has been planted for many years as an erosion control remedy, due to its permanent and deep root system which could extend at most 1.2 m downwards, thus effectively prevent soil from erosion (Nackley et al., 2014; Pompeiano et al., 2016).

It is also an excellent energy crop and biomass feedstock (Fu et al., 2016; Nackley et al., 2014). *A. donax* may produce more than 35 tons ha^{-1} of dry biomass annually with a minimal agronomic investment, and the related bio-ethanol yield may reach 50% of the dry matter (Angelini et al., 2009; Li et al., 2017).

1.5.2 A high resistance to environmental stress

A. donax has a high resistance to biotic and abiotic stresses (Mariani et al., 2010). Besides, *A. donax* is highly tolerant to heavy metal conditions (Barbosa et al., 2015; Sablok et al., 2014). Previous study reported that net photosynthesis, fresh weight and dry biomass of *A. donax* were not affected by high concentrations of

Cd and Ni (Papazoglou et al., 2005). Due to the high phytoextraction and phytostabilization, *A. donax* can be used to absorb heavy metal contaminants from the soil, thus help to remediate and revegetate heavy metal-contaminated bare areas (Papazoglou et al., 2005; Barbosa et al., 2015).

Water shortage impairs the growth of *A. donax*. Compared to other environmental stress, soil water deficiency reduces *A. donax* growth more than all the others combined (Pompeiano et al., 2013, 2016).

1.5.3 An isoprene emitter from monocots

A. donax belongs to Arundinoideae, which is a small subfamily of the Poaceae. The majority species of Arundinoideae were found to emit isoprene (Hewitt et al., 1990; Kesselmeier J, 1999; Ahrar et al., 2015). Despite not being the most highly emitting species among them, *A. donax* emits on an absolute basis the largest amounts of isoprene due to its widespread distribution and extremely fast rate of biomass accumulation.

A recent study showed differences of isoprene emission rate present between two *Arundo donax* ecotypes, and the higher isoprene emitter exhibited greater drought tolerance and faster recovery (Ahrar et al., 2017). This implied that isoprene emission may play a positive role in plant adaptation to water-limited conditions.

2 Materials and methods

2.1 Growth condition & isoprene emission measurement

2.1.1 Seeds sterilization & growth chamber condition

AdoIspS over-expression lines used in this study were generated from *Arabidopsis thaliana* (ecotype Col-0). Seeds of Col-0 and transgenic lines were surface sterilized with 70% ethanol for 15 min, then with 5% (v/v) commercial sodium hydroxide for 10min, after washing twice with sterile deionized water, the seeds were sown on Murashige-Skoog (MS) agar medium containing 1% sucrose. The plates were kept at 4 °C for three days and moved to a growth chamber set at 23 °C and 40-50% relative humidity with fluorescent light intensity of 100-120 $\mu Em^{-2}s^{-1}$ under a long day condition (16hr light/8hr dark).

MS medium: 4.4 g/L Murashige and Skoog Salt Mixture, 10g/L sucrose, 8g/L phyto-agar, pH 5.7 (adjusted with 1M KOH).

2.1.2 Soil mixture & growth chamber condition

After germination on the medium for a few days, the seedlings were transferred and grown to maturity in soil mixture of Schildkrötensubstrat soil (Floragard products, Germany), Profi Substrat soil (Einheits Erde® Classic, Einheitserde-Werke, Germany) and perlite (3:3:1, v:v:v), and maintained at 23 °C and 40-60% relative humidity under fluorescent light (100-120 $\mu Em^{-2}s^{-1}$) with a long-day condition cycle (16h light/8h dark).

2.1.3 Hydroponic culture system

The protocol described by Tocquin *et al.* (2003) for plants growing in the

hydroponic solution system was used with slight changes. Seeds of the Col-0 and two *AdoIspS* transgenic lines were germinated and grown in the standard nutrient solution system containing 1.01 mM $Ca(NO_3)_2 \cdot 4H_2O$, 5.10 mM KNO_3, 0.13 mM $NH_4H_2PO_4$, 0.498 mM $MgSO_4 \cdot 7H_2O$, 8.70×10^{-3} mM NaOH, 2.03×10^{-3} mM $MnCl_2 \cdot 4H_2O$, 9.68×10^{-3} mM H_3BO_3, 2.10×10^{-4}mM $CuSO_4 \cdot 5H_2O$, 3.14×10^{-4} mM $ZnSO_4 \cdot 7H_2O$, 1.39×10^{-4} mM MoO_3, 8.59×10^{-5} mM $Co(NO_3)_2 \cdot 6H_2O$, 2.24×10^{-2} mM $C_{10}H_{12}N_2O_8FeNa$ and 2.93×10^{-2} mM NH_4NO_3. The plants were grown in a growth chamber under a controlled environmental condition as described previously in section 2.1.1.

2.1.4 Fitness parameters

The numbers of primary rosette-leaf (PRL) branches and the total number of branches were assessed by using eight-week-old plants (Aguilar-Martinez et al., 2007). Seeds were harvested by using Arasystems (Ara System 360 KIT; Beta Tech bvba, Gent, Belgium) to separate each individual. After 12-week of growth, when plants were mature and last siliques were ready to dehisce, seeds were collected from every single plant from two different temperature settings, normal growth condition (23 °C) and normal growth condition with a heat shock (42 °C) applied every day (12 o'clock) for two hours since plants were 7-day old.

For the measurement of flowering time, 72 plants (each genotype) of transgenic line 44 and wild type were grown in individual pots under the short-day condition (8h light/16h dark). Bolting time was counted as the number of days passed after germination when the flower primary shoots were 1 cm in height, and the flowering time was counted as the days when the first flower in the primary shoot was visible. The number of rosette leaves was counted correspondingly.

2.1.5 PTR-MS analysis from Arabidopsis leaves

A young and mature rosette leaf was cut from four-week old T1 transgenic plants and weighed before transferring into a 20 ml glass vials containing 300 µl of distilled water. The vials were kept open for 30 min to remove green leaf volatiles. After quickly closing metal lids sealed with silicone septa (Agilent, Cernusco sul Naviglio, Italy), the vials were incubated in a growth chamber for 3 hrs, at 30 °C with light intensity of 130±10 $\mu Em^{-2}s^{-1}$.

Samples were measured by PTR-ToF 8000 apparatus from Ionicon Analytik GmbH, Innsbruck (Austria). Each measurement lasted for 30 s and vials were automatically switched using an adapted GC auto sampler (MPS Multipurpose Sampler, GERSTEL). Vials were sampled in a randomized order. One Col-0 leaf was used in each auto sampler block as a reference. The data was extracted and converted to parts per billion (ppb) as previously described (Cappellin et al., 2011), and the concentration was converted to flux on a mass basis (nmol g^{-1}_{FW}) by using the corresponding individual leaf fresh weight (Li et al., 2017).

2.2 Molecular biology

2.2.1 Construction of over-expression plasmid

The full-length *AdoIspS* cDNA was amplified with primers *AdoIspS_For* and *AdoIspS_Rev* using Phusion High-Fidelity DNA Polymerase (NEB). The purified amplicon was cloned into pENTR/D-TOPO vector, which was further recombined in the destination vector pK7WG2 to generate the final constructs through LR reaction with LR clonase II (Invitrogen).

The primers used in section 2.2.1 are listed below:

AdoIspS_For	CACCATGGCAATGGCTACCTGTAGT
AdoIspS_Rev	GCACGATGTGTATACATACTCAC

2.2.2 Transformation of *Agrobacterium tumefaciens*

The positive *AdoIspS* construct was finally transformed into *Agrobacterium tumefaciens* strain GV3101-pMP90RK by electroporation, then, it was cultured with 1 mL of YEB with shaking for two hours at 28 °C. This liquid mixture was plated on YEB solid medium with 50 mg/L of Kanamycin and 100 mg/L Rifampicin antibiotics.

Colonies were picked from those plates and cultured in 50 ml fresh made selective YEB liquid medium with proper antibiotics (Kanamycin 50 mg/L, Rifampicin100 mg/L and 1M Mg_2SO_4 100µl). The culture was incubated at 28 °C and shaking at 270 rpm over night. This liquid culture was mixed into 250 ml fresh made selective YEB liquid medium with same antibiotics contents as described previously. This culture was incubated at 28 °C for another 18hr.

YEB medium: 1 g/L yeast extract, 5 g/L beef extract, 5 g/L peptone, 5 g/L

sucrose, 15 g/L agar, pH 7.2 (adjusted with NaOH).

2.2.3 Transformation and selection of *Arabidopsis thaliana*

The culture of *Agrobacterium* was mixed with 300 ml of 5% sucrose solution and 120 µl Silwet L-77. The mixture was placed on a magnetic mixer, and continuously stirred with a magnetic bar. The inflorescense of *A. thaliana* was dipped into *Agrobacterium* solution for about 15 seconds. After floral dipping, all the plants were kept in black and humid by covering black plastic bags for 22 hrs, and then plants were transferred into GMO green house.

Positive transgenic plants were selected by screening sterilized seeds on solid MS medium supplemented with 50 mg/L of kanamycin. The successful transgenic plants are green and robust, whereas non-transgenic plants were white and small.

2.2.4 Genotyping

Segregation analysis was carried out with segregating T2 plants of transgenic line *IspS*-44. T2 seeds stratified at 4 °C for three days were sown directly in the soil and cultured in the growth chamber under normal growth condition. Four-week-old plants were used for both PTR-MS isoprene emission measurement (described below) and genotyping with the CTAB method (Doyle and Doyle, 1987). DNA quality and presence of the *IspS* transgene were assayed respectively by PCR using primer pairs *AtActin2_For* and *AtActin2_Rev*, also *AdoIspS_Rev* and *pK7WG2_2S*. Thermal cycling was programmed as: 95 °C for 2 minutes, following with 35 cycles of 94 °C for 40 seconds, 60 °C for 30 seconds and 72 °C for 2 minutes (Li et al., 2017).

The primers used in section 2.2.4 are listed below:

pK7WG2_2S ACGCACAATCCCACTATCCTTCGCA

AtActin2_For	CTGAAAGGAAGTACAGTGTCTGGAT
AtActin2_Rev	AAACAAACAAATGGAGAAGCAAATA

2.2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from Col-0 and *AdoIspS* transgenic plants by using Trizol reagent (Invitrogen). Genomic DNAs of all RNA samples were eliminated by Sigma DNase I. After RNA quantification by using a spectrophotometer, 1 μg total RNA was used for individual cDNA synthesis with SuperScript^{TM} III First-Strand Synthesis Super-mix (Invitrogen) kit.

2.2.6 qPCR analysis

The PCR was programmed as: 50 °C for 2 minutes, 95 °C for 2 minutes, following with 40 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds. The qRT–PCR analysis was carried out with at least three technical replicas and three biological replicas. The relative transcript level of each gene was calculated with the $2^{-\Delta\Delta CT}$ method, the results were normalized to the expression of Actin Ⅱ.

The expression level of specific genes under dehydration treatment was assessed with 18-day-old plants. Plants were taken out of the hydroponic solution and directly exposed to air for two hours and four hours, then the aerial part of each plant was collected into liquid nitrogen. The expression level of specific genes under exogenous ABA treatment was assessed with two-week-old plants cultivated in a hydroponic system. Seedlings were transferred from the standard nutrition solution to an identical solution supplemented with 50 μM ABA. Roots and aerial parts were collected separately after one- and four hours treatments, respectively.

The primers used in section 2.2.6 are listed below:

COR15A	Forward: GATACATTGGGTAAAGAAGCTGAGA
	Reverse: ACATGAAGAGAGAGGATATGGATCA
NCED3	Forward: ACATGGAAATCGGAGTTACAGATAG
	Reverse: AGAAACAACAAACAAGAAACAGAGC
P5CS	Forward: GAAGGATTACTTACAACGAGATGGA
	Reverse: CTCTCCTCAAGTCTCAACCAAATAC
RAB18	Forward: GGAAGAAGGGAATAACACAAAAGAT
	Reverse: GCGTTACAAACCCTCATTATTTTTA
RD20	Forward: TGGTTTCCTATCTAAAGAAGCTGTG
	Reverse: ATACAAATCCCCAAACTGAATAACA
RD29A	Forward: CACAATCACTTGGCTCCACTGTTG
	Reverse: ACCTAGTAGCTGGTATGGAGGAACT
ABA2	Forward: AAGCATGAAACATGCAGCTCG
	Reverse: AAGAATGTGGACCAACGCCTC
RD29B	Forward: GTTGAAGAGTCTCCACAATCACTTG
	Reverse: ATACAAATCCCCAAACTGAATAACA

2.3 Plant stress treatments

2.3.1 Heat shock

2.3.1.1 Survival test

To test the thermal tolerance of *AdoIspS* transgenic plants at different developmental stages, heat survival test was done as follows. 7-day-old plants were incubated at 45℃ in a pre-heated chamber for one hour in the dark condition, and returned to 23℃ growth chamber for recovery. The survival rate of plants was recorded after one week. Plants with four green leaves were scored as alive. The wild type and *AdoIspS* transgenic plants were grown on the same plate. Six plates were tested in this experiment. Each plate contains about 14 plants for each genotype.

3-week-old plants of each genotype growing in pots were transferred to a 60℃ growth chamber under a dim light condition (5 $mmolm^{-2}$ s^{-1}). After 2.5 hours treatment, plants were moved back to the normal growth condition to recover. Percentages of surviving plants were calculated after 7-day recovery. Six replicas were tested and each replicate was performed with 20 plants.

2.3.1.2 Chlorophyll content

Leaf chlorophyll content was extracted with acetone. Plants of each genotype within the same petri dish were harvested and weighed the fresh weight separately in an eppendorf tube, then homogenized by using tissue lyser in the presence of 80% (v/v) ice cold acetone. After centrifugation for 13 min at 4600 rpm, the supernatant was collected and the pellet was macerated with acetone again and centrifuged for another 13 min as before. The combined supernatant was used to measure the absorbance at 663 nm for chlorophyll a and 645 nm for chlorophyll b

with a spectrophotometer according to the method described previously (Arnon, 1949). The equation for chlorophyll a concentration is Chl a=12.7 OD_{663}-2.69 OD_{645}; for chlorophyll b is Chl b=22.9 OD_{645}-4.68 OD_{663}; and the equation of total chlorophyll content is C= 20.2 OD_{645} + 8.02 OD_{663}. Each experiment was repeated six times.

2.3.2 Dehydration stress

2.3.2.1 Survival test

To test the survival ability of each genotype under a fast dehydration condition, Col-0 and *AdoIspS* transgenic plants were germinated and grown in the hydroponic culture system. After 18 days, whole plants were taken out from the hydroponic solution and hang on a shelf to expose both roots and aerial parts to air directly. After 24 hours plants were rehydrated by putting back into the hydroponic solution system. After 48 hours recovery, plants with rehydrated primary shoots were counted as the survived. This experiment was performed with five replicates having about 20 plants for each genotype per replica.

2.3.2.2 Water loss

In order to assess the drought tolerance of the leaf, the water loss rate was assayed as follows. For each genotype, 15 rosette leaves of equal size were detached from five different 3-week-old plants at the same developmental stage, and weighed immediately on a plastic weighting tray. Weighting trays with fresh leaves were placed on the shelf of a growth chamber (40% RH) and weighed at each indicated time points, with five replicates.

2.3.2.3 Lipid peroxidation assay

Leaf lipid peroxidation was quantified by measuring malondialdehyde (MDA) accumulation by using TBARS method (Heath and Packer, 1968) with a slight modification. Around 100mg of plant material frozen by liquid nitrogen was homogenized in 1ml of 0.1% (w/v) Trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 13000 *g* at 4 °C for 20 min; 0.5ml of the supernatant was transferred into 1.5ml Eppendorf tube containing fresh-made 20% TCA solution supplemented with 0.5% (w/v) thiobarbituric acid (TBA). The mixture was heated in boiling water bath for 30 min. Tubes were placed on ice immediately after heating process to stop the reaction. Samples were centrifuged again for 5 min at 10000 *g*. The absorbance of supernatant was measured with Ultrospec 3100 Pro UV/visible spectrophotometer (Amersham) and read at 532 nm, deducting the value recorded at 600nm to correct for non-specific turbidity. The extinction coefficient 155 $mM^{-1}cm^{-1}$ was used to calculate the content of MDA-TBA complex. We repeated the same experiment seven times.

2.3.3 Osmotic stress

2.3.3.1 Root length

Root growth under osmotic stress treatment was assessed by using polyethylene glycol (PEG) 6000. In the hydroponic culture system, wild type and *AdoIspS* transgenic plants were germinated and grown in standard nutrition solution for four days, then transferred to the same nutrition solution containing various concentrations of PEG 6000. Plants were photographed and measured after 7 days. Root length was captured with a stereomicroscope (MZ75, Leica Microsystem) equipped with a color-CDD camera (Leica DFC 420C); meanwhile, the fresh weight of each plant was measured and recorded correspondingly. Root length of

each plant was later measured by using the Leica Application Suite 2.8.1 software. At least 15 plants of each genotype were measured per treatment; each treatment was repeated five times.

2.3.4 Exogenous ABA treatment

2.3.4.1 Germination rate

For the germination test, after seed sterilization, 72 seeds of each genotype were sown on petri dishes which contained ½ MS medium supplemented with 0.3 μM or 0.5 μM ABA, meanwhile, equal amount of 99% ethanol was added into control plates. Germination rate, which was based on radicle protrusion and green cotyledon number were recorded after 3-, 5- and 7-day growth in the growth chamber. Each experiment was performed three times.

2.3.4.2 Seedling growth

To measure the sensitivity of root growth to ABA during the post-germination stage, seeds from Col-0 and *AdoIspS* transgenic lines were germinated on ½ MS agar medium for four days, and followed by transferring into square petri dishes containing fresh medium supplemented with 10 μM and 20 μM ABA or equal amount of 99% ethanol (mock) and grown for additional seven days vertically, the root growth length was captured with a stereomicroscope as described previously; the fresh weight of each genotype was measured and recorded correspondingly. Images were later analyzed with the same software as mentioned above. This experiment was performed six times.

2.3.4.3 Stomatal aperture assay

Stomatal closing assays were assessed as previously described (Ren et al., 2010)

with slight modifications. Rosette leaves of 4-week-old plants were detached and incubated in a stomatal opening solution containing 10 mM MES, 50 μM $CaCl_2$, 10 mM KCl (pH 6.15), and exposed under fluorescent light (150 $\mu Em^{-2}s^{-1}$) for three hours. Then the buffer was replaced with fresh-made opening solution containing 10μM ABA for stomatal closing. After 3-hour treatment with ABA, about 40 stomata of each genotype were observed randomly under a Differential Interference Contrast (DIC) microscope (Leica DM 2500) with Leica Application Suite LAS V3.7 software. Image J 1.50i software (http://imagej.nih.gov/ij) was used to measure the length and width of individual stoma. Each experiment was repeated five times.

3 Results and Discussion

3.1 Transgenic plants over-expressing the *AdoIspS* gene

3.1.1 Identification of *AdoIspS* transgenic lines

By using sequence homology between several terpene synthase genes which had been identified in the transcriptome of *A. donax* (Sablok et al., 2014) and *IspS* from poplar, the best hit was selected to do a further analysis.

An overexpression construct containing the full-length cDNA of putative *AdoIspS* gene was transformed into Col-0 wild-type Arabidopsis plants under the control of CaMV 35S promoter. In a group of 120 independent T1 transgenic lines overexpressing *AdoIspS*, the majority of them have been detected to emit isoprene (Fig. 3.1).

Isoprene emission ability of transgenic Arabidopsis was further verified by semi-quantitative RT-PCR, confirmed that isoprene emission was due to the transformation of *AdoIspS* gene into Arabidopsis (Li et al., 2017).

To conclusively demonstrate that isoprene emission from Arabidopsis was due to the transformation of the *AdoIspS* gene, isoprene emission measurement was conducted concurrently with PCR amplification with primers specific to the *AdoIspS* transgene in the T2 segregating population of IspS-44, a highly emitting single copy line (Fig. 3.2).

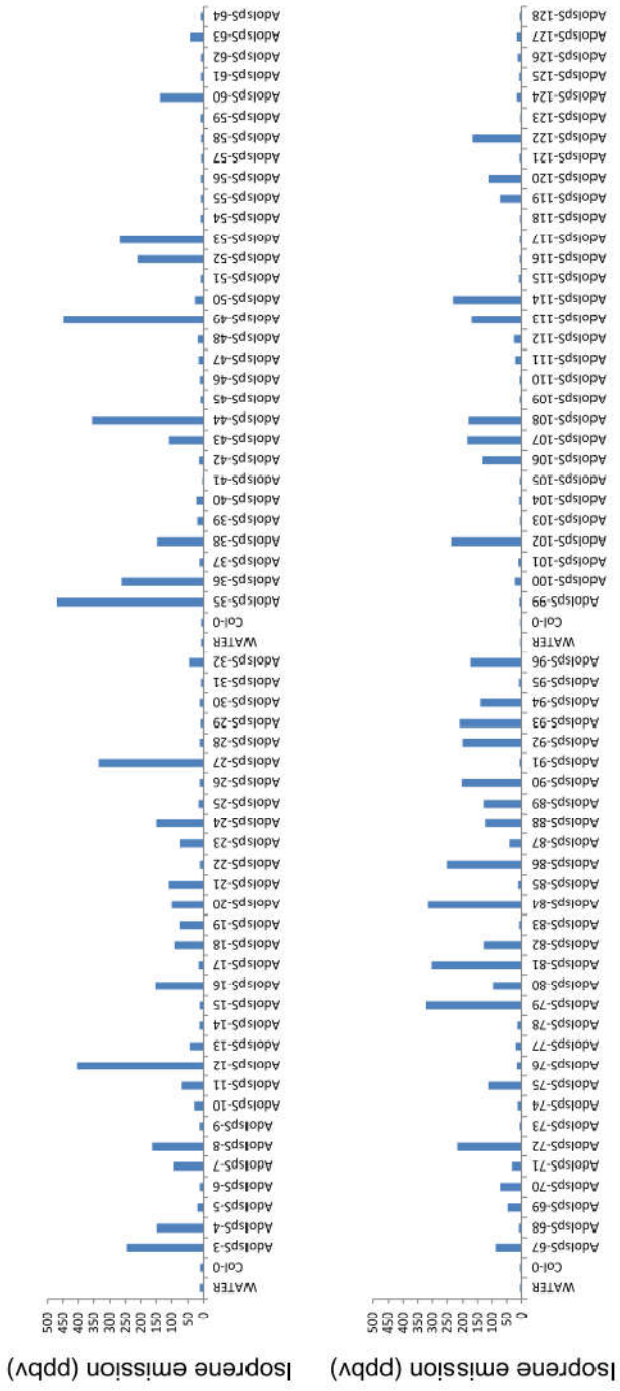


Fig. 3.1 Isoprene emission screening of T1 transgenic plants overexpressing *AdoIspS* (p35S:*AdoIspS*-WT). Isoprene emission from a single 4-week-old rosette leaf of 120 independent transgenic lines was measured by PTR-ToF-MS.

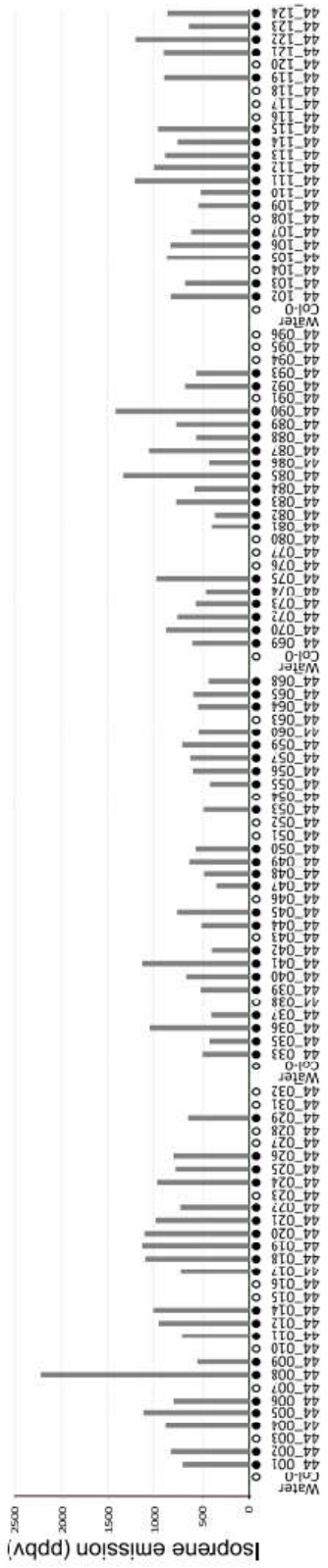


Fig. 3.2 Segregation analysis of Arabidopsis T2 population (line IspS-44). A total of 108 individuals were genotyped for presence/absence of the *AdoIspS* transgene (filled/empty circles, respectively). Isoprene emission was measured for the same plants (gray bars), using empty vials (water) or WT Arabidopsis plants (Col-0) as controls. ppbv: parts per billion volume.

The combined results showed that isoprene emission above background (Col-0) level was detected only in individuals positive for the presence of the *AdoIspS* transgene (n=78) and not in the negative ones (n=30) (Fig. 3.2). In the analyzed samples, the null hypothesis of isoprene emission being randomly associated with transgene presence was rejected at $P<0.001$ (two tailed Fisher's exact test; P=2.27E-27). These results demonstrate that the *AdoIspS* gene from *A. donax* is the first isoprene synthase gene identified so far among monocot species.

Isoprene synthase genes have been characterized from several plant species of dicots, particularly from the rosids (Sharkey, 2013). The *AdoIspS* gene is the first *IspS* identified and characterized from a monocot species (Li et al., 2017). From a functional point of view, some features of *AdoIspS* shared similarity with *IspS*s from dicots. 1) They are necessary and sufficient to make the non-emitter Arabidopsis to synthesize and emit isoprene; 2) Only chloroplast-located isoprene synthases catalyze the formation of isoprene; 3) Like poplar and kudzu *IspS*, *AdoIspS* is a highly specific isoprene synthase; 4) The genomic structure is highly conserved; 5) The expression is mainly responsive to heat stress (Li et al., 2017).

Based on the levels of isoprene emission, two homozygous lines emitting high amounts of isoprene were selected from approximately 60 single-copy transgenic lines obtained from the original screening, which named as: IspS-44 and IspS-79. All downstream work was performed with these lines (T3 generation).

3.1.2 Growth advantage of *AdoIspS* transgenic plants

It has been suggested that exogenous isoprene fumigation could trigger an earlier flowering with several plant species (Terry et al., 1995), while limited studies have been performed in detail to elucidate the differences between isoprene emitters and non-emitters on morphological characteristics and fitness.

Compared to Col-0, the seed production was significantly higher in *IspS* transgenic plants (line 44) under normal growth condition. Moreover, the seeds yield was also moderately higher in *IspS* transgenic line 79. (Fig. 3.3)

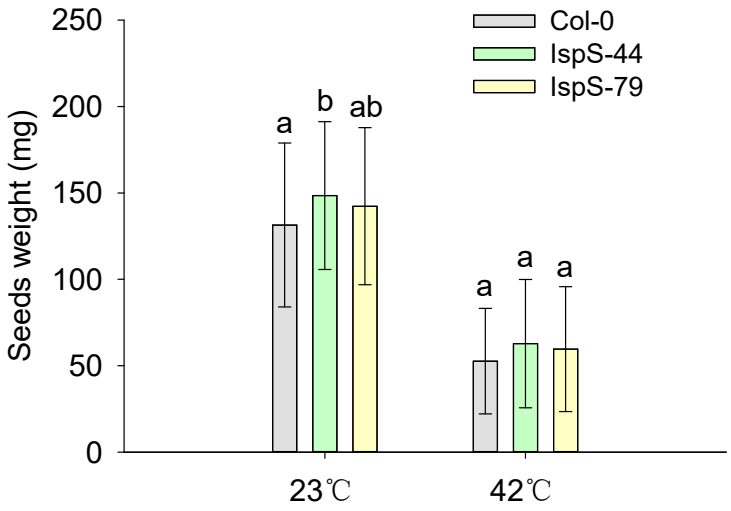
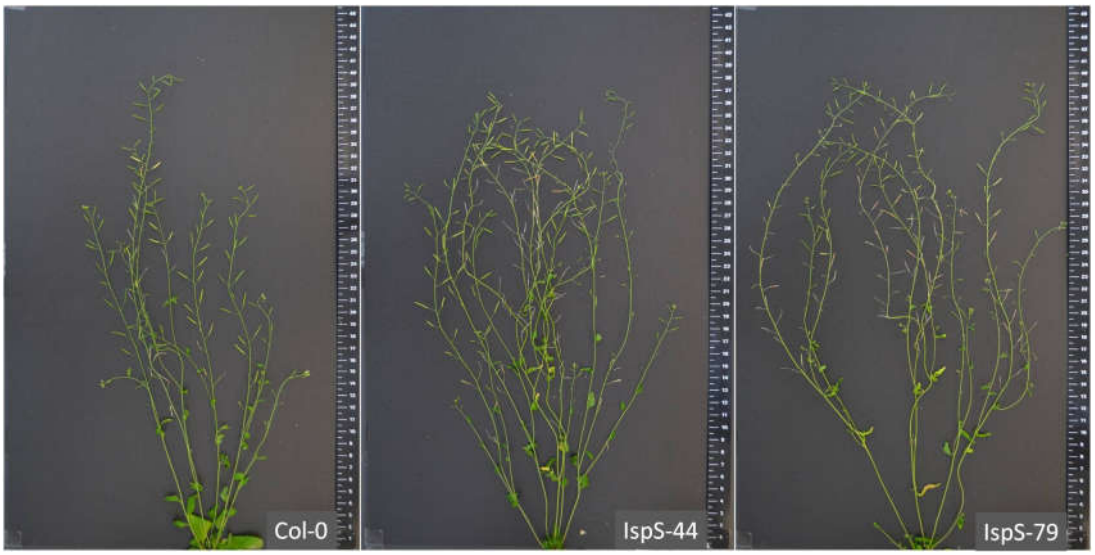


Fig. 3.3 Seed production in Col-0 and *AdoIspS* transgenic plants under normal growth condition and heat shock condition, data shown indicated seed weight per plant.



Pic. 3.1 Phenotypic differences between Col-0 and *AdoIspS* transgenic plants.

To find out the cause of this growth advantage of *AdoIspS* transgenic plants, further investigation on the morphological features of the plant aerial part was conducted. The size and weight of seeds from three genotypes were evaluated and exhibited no difference, and the assessment of seed set per silique showed a comparable result. However, compared to Col-0, both *AdoIspS* transgenic lines produced more primary rosette-leaf (PRL) branches, which consequently increased total silique numbers of each *IspS* transgenic plant (Fig. 3.4 and Pic. 3.1). Total seeds yield of *AdoIspS* transgenic line 44 and line 79 under normal growth condition increased 13% and 8.4% over Col-0, respectively. Although under the heat shock condition, seed productions of both *AdoIspS* transgenic plants were not significantly greater than those of Col-0 anymore, line 44 and line 79 still produced 19.3% and 13.2% more seeds than Col-0.

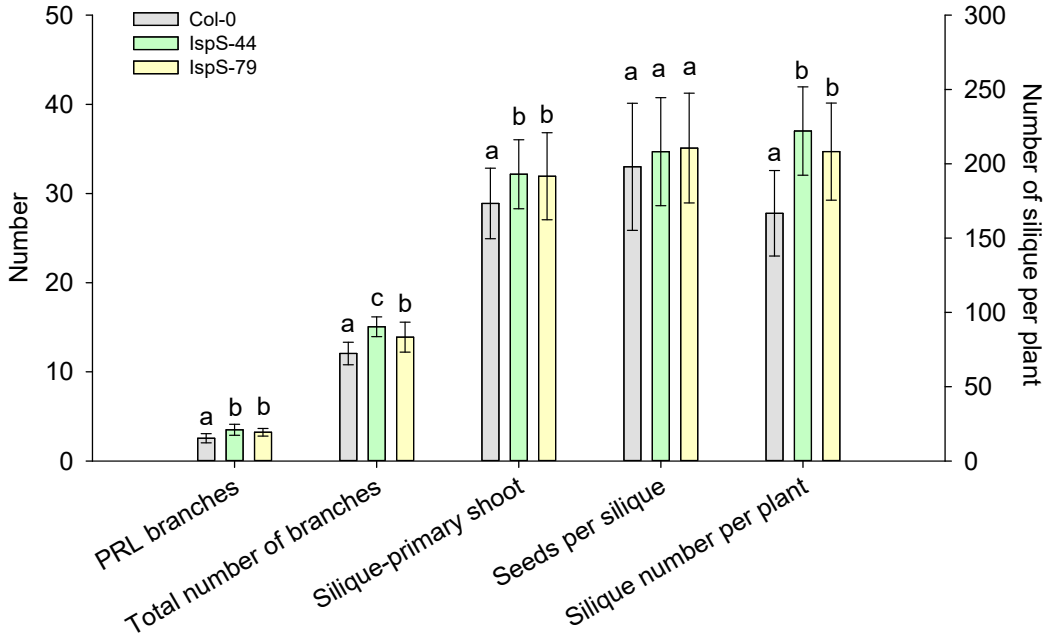


Fig. 3.4 Analysis of phenotypic differences between Col-0 and *AdoIspS* transgenic plants, comparison of branch development, silique number and the number of seed per silique.

Pic. 3.2 Photograph of flowering phenotype.

AdoIspS transgenic line 44 plants displayed early flowering under short day condition accompanied with a reduction of total leaf number. This result confirmed the previous finding that isoprene promotes flowering (Terry et al., 1995). (Fig. 3.5 and Pic. 3.2)

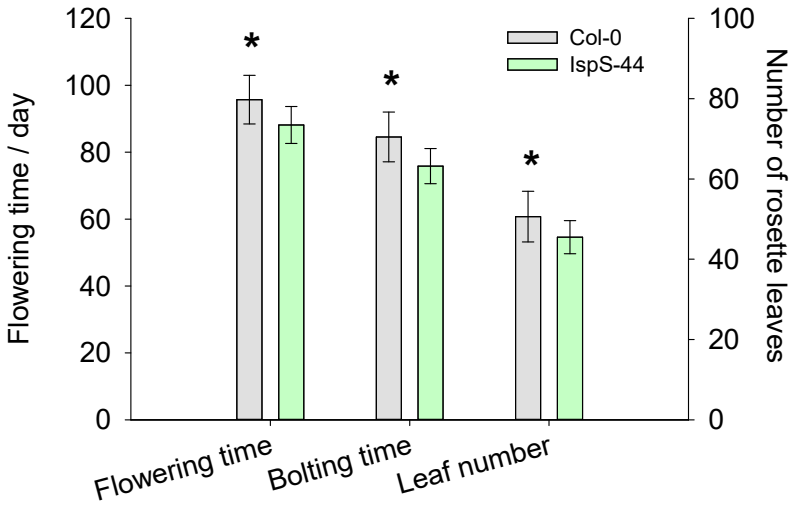


Fig. 3.5 Average bolting time, flowering time and rosette leaf numbers of Col-0 and *AdoIspS* transgenic line 44 at the time of flowering under short-day conditions.

Discussion

Considering the results of the morphological investigation, it could be concluded that the fitness of Arabidopsis plants has been improved due to the over-expression of the *AdoIspS* gene, indicating a general role of isoprene emission in promoting plant growth.

Over-expression of poplar *IspS* in Arabidopsis resulted in transiently enhanced growth rates compared to the wild type under normal growth condition and moderate thermal stress (Loivamäki et al., 2007). Besides higher growth rates, higher dimethylallyl diphosphate levels and enzyme activity were also detected in *IspS* transgenic plants during their vegetative growth phase (Loivamäki et al., 2007). Isoprene fumigation was shown to promote plant growth with barley, oil-seed rape and Arabidopsis (Terry et al., 1995). On the other hand, previous studies reported no difference in biomass between transgenic isoprene-emitting and non-isoprene emitting tobacco (Vickers et al., 2009b; Ryan et al., 2014).

In the present study, we noticed an increased seed production in *AdoIspS* over-expression lines. Further study discovered that more branches were generated in *AdoIspS* transgenic plants in 8-week growth period, leading to more siliques and seed production per transgenic plant. There were no differences in leaf biomass in 5-week-old plants and seed amount per silique between Col-0 and *AdoIspS* transgenic plants. It seems possible that *IspS* promoted plant developmental rate rather than biomass accumulation. However, *IspS* transgenic plants lost this growth advantage under a long-term spell of heat stress.

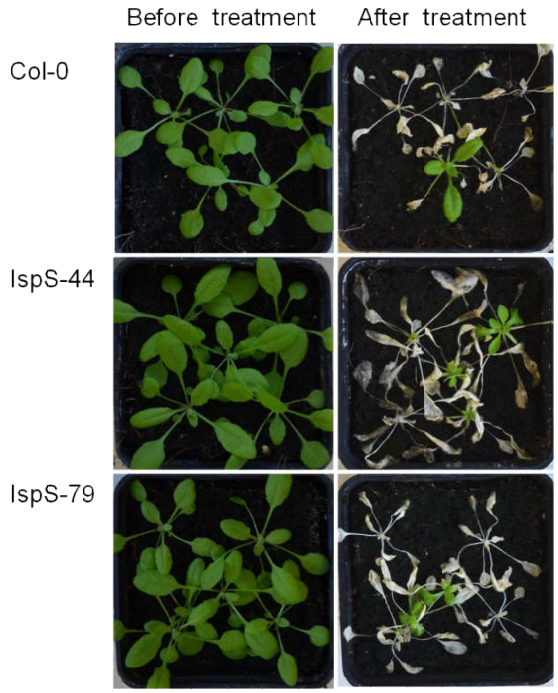
3.2 Physiological characterization of *AdoIspS* transgenic plants in relation to abiotic stresses

The continuous investigation of isoprene emission from plants has lasted over 60 years (Sharkey and Monson, 2017). Since the first finding of the correlation between isoprene and temperature has been established (Sharkey and Singsaas, 1995), scientists have done plenty of researches to test whether isoprene emitters have a better thermal tolerance than non-emitters. They found that plant thermotolerance increases in natural isoprene-emitting leaves or non-emitting leaves which were supplied with isoprene gas stream (Sharkey et al., 2001; Velikova and Loreto, 2005). They also found that thermotolerance decreased when isoprene emission ability was down regulated in a natural emitter (Behnke et al., 2007) and increased when isoprene emission ability was generated from a non-emitter by over-expressing *IspS* with *Agrobacterium*-mediated transformation (Sasaki et al., 2007). Besides thermotolerance, *IspS* has been suggested to provide a better drought tolerance in plants, too (Tattini et al., 2014; Ryan et al., 2014).

Phenotypic comparisons among two *AdoIspS* transgenic plants highly emitting isoprene and non-emitter Col-0 wild type which were treated with exogenous abscisic acid (ABA), osmotic stress, dehydration and heat shock were performed.

3.2.1 Enhanced tolerance of *AdoIspS* transgenic plants to heat shock

Previous study demonstrated that transgenic plants over-expressing *IspS* gene from poplar in Arabidopsis could increase thermotolerance (Sasaki et al., 2007). In order to evaluate the functional relevance of the newly identified *AdoIspS* from *A. donax*, a monocot, on thermotolerance, the resistance to heat shock among Col-0 and *AdoIspS* transgenic plants was examined at two different developmental stages. 3-week old plants were treated at 60 °C for 2.5 hours under a dim light condition. After 7-day recovery, the percentage of surviving plants from Col-0 was lower than those from both *AdoIspS* transgenic plants and the difference was statistically significant. (Fig. 3.6 and Pic. 3.3)



Pic. 3.3 Phenotypic comparisons among Col-0 and two *AdoIspS* transgenic lines using 3-weekold plants before treatment and 7-day recovery after treatment at 60 °C.

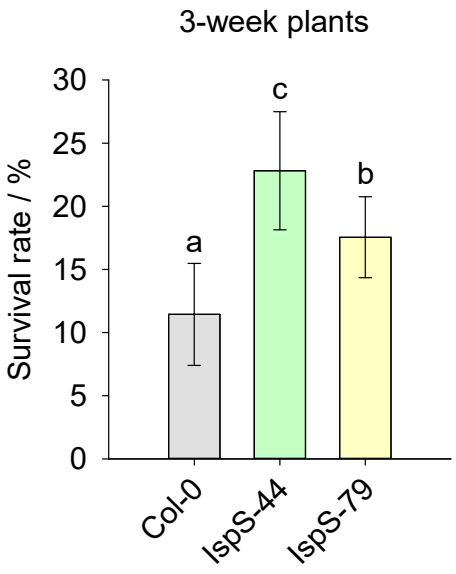


Fig. 3.6 Survival rate of Col-0 and two *AdoIspS* transgenic plants using 3-week old plants treated at 60 °C.

Furthermore, the survival analysis on thermotolerance was also conducted on 7-day-old seedlings which were treated at 45 °C for 1 hr. After the recovery phase, more *AdoIspS* transgenic seedlings than Col-0 seedlings survived. The fresh weights of both *AdoIspS* transgenic plants was also higher than Col-0. Compared to Col-0, recovered transgenic seedlings showed fewer leaf bleaching, and chlorophyll analysis indicated that the content was significantly higher in *AdoIspS* transgenic plants (Fig. 3.7 and Pic. 3.4).

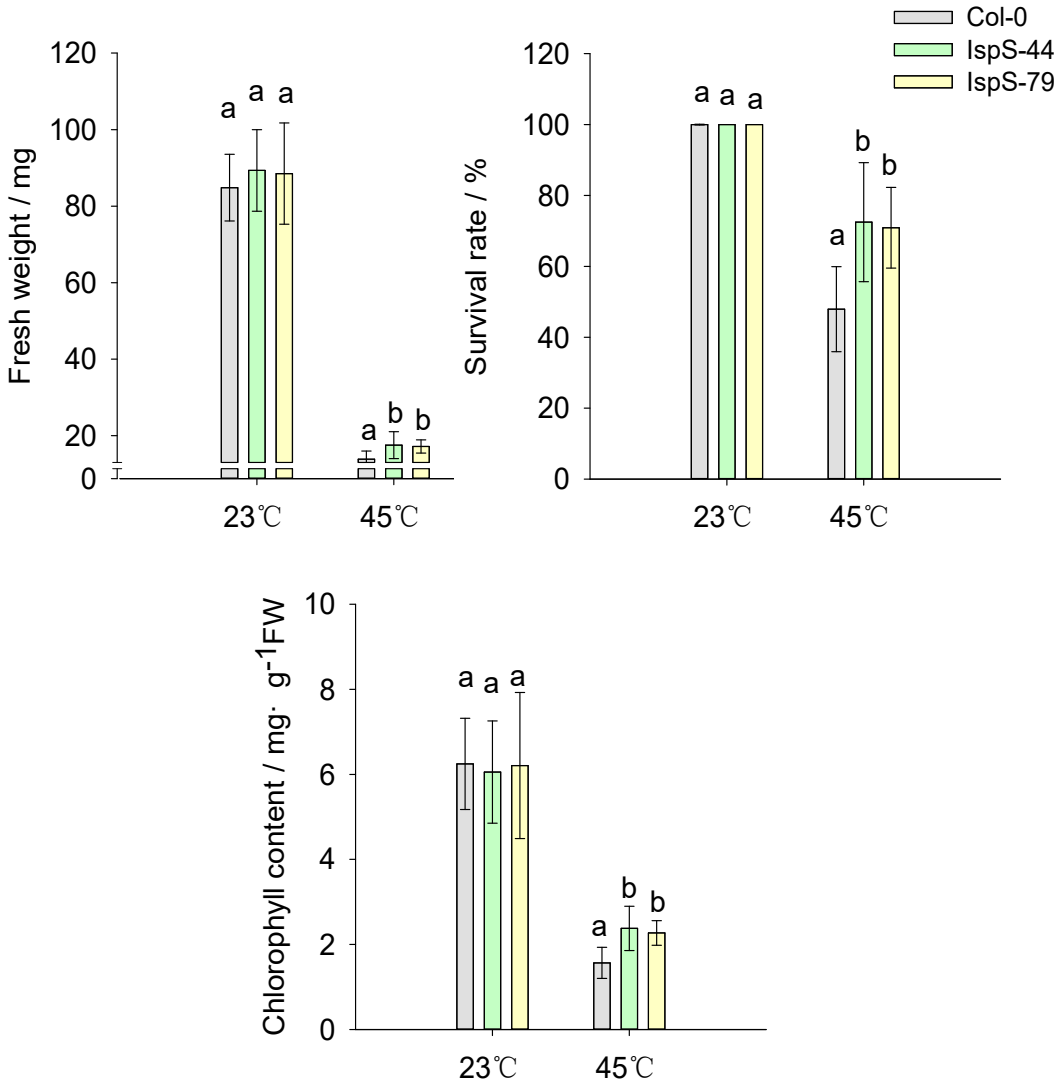
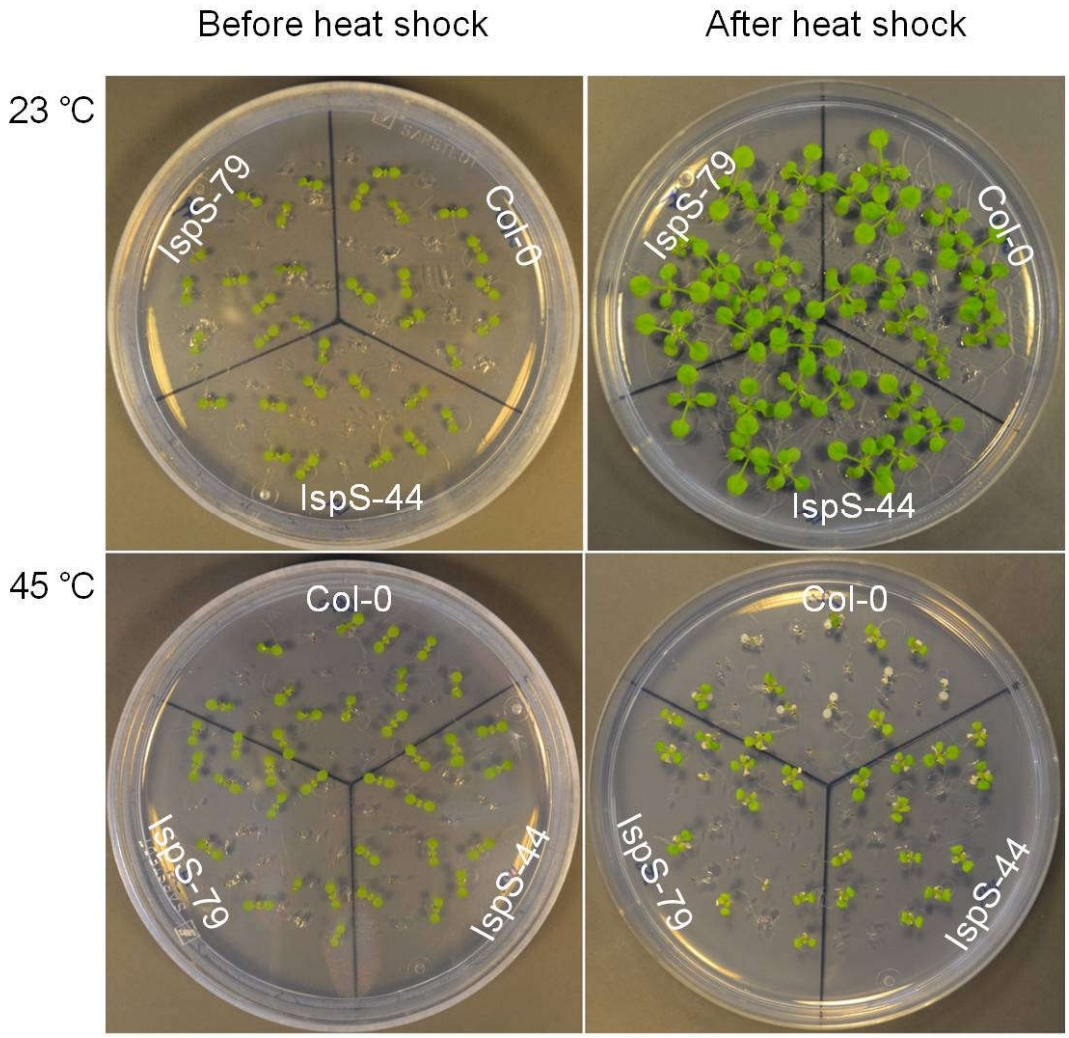


Fig. 3.7 Thermal tolerance of Col-0 and *AdoIspS* transgenic seedlings. 7-day-old seedlings were exposed to 60 °C heat shock. After 7-day recovery, the chlorophyll content, fresh weight and survival rate in each genotype were measured, correspondingly.



Pic. 3.4 Seven-day-old seedlings were exposed to 45 °C heat shock. After 7 days recovery, the representative images were taken.

Discussion

It is believed that isoprene-emitting plants have a better tolerance to heat, because the photosynthesis in isoprene-emitting leaf recovers rapidly after the thermal stress (Velikova & Loreto 2005). In the previous study, our group found that *AdoIspS* expression in *A. donax* was mainly responsive to heat stress. The largest transcriptional response of *AdoIspS* was caused by heat shock, at all time points tested (Li et al. 2017). Besides, it has been reported that transgenic plants over-expressing the *IspS* gene from poplar in Arabidopsis showed a striking

thermotolerance with a much higher survival rate than wild type plants, due to the decreased leaf temperature on the transgenic leaf surface which concomitant with increased isoprene emission (Sasaki et al. 2007).

In the present work, we find that after recovering from the heat shock, *AdoIspS* transgenic plants exhibited a higher survival rate, chlorophyll content and fresh weight at different developmental stages. These results indicate that, like the *IspS* gene from poplar, the over-expression of *AdoIspS* gene from *A. donax* could also enhance plant tolerance to heat shock, and confirmed that isoprene emission has an important role in protecting plants against heat.

3.2.2 Enhanced tolerance of *AdoIspS* transgenic plants to exogenous ABA treatment

Seeds of Col-0 and two *AdoIspS* transgenic lines were germinated on half-strength MS medium with different concentrations of ABA. The seed germination rates from both transgenic lines were significantly higher than that of Col-0 in MS medium supplemented either with 0.3 uM or 0.5μM ABA in the first three day growth and there were no differences observed from the fifth day on. The rate of opened green cotyledon seedlings was significantly higher in both *AdoIspS* transgenic lines than that in Col-0 from the third day after sowing on medium containing ABA, and the same growth patterns were maintained till the end of this test. (Fig. 3.8 and Pic. 3.5)

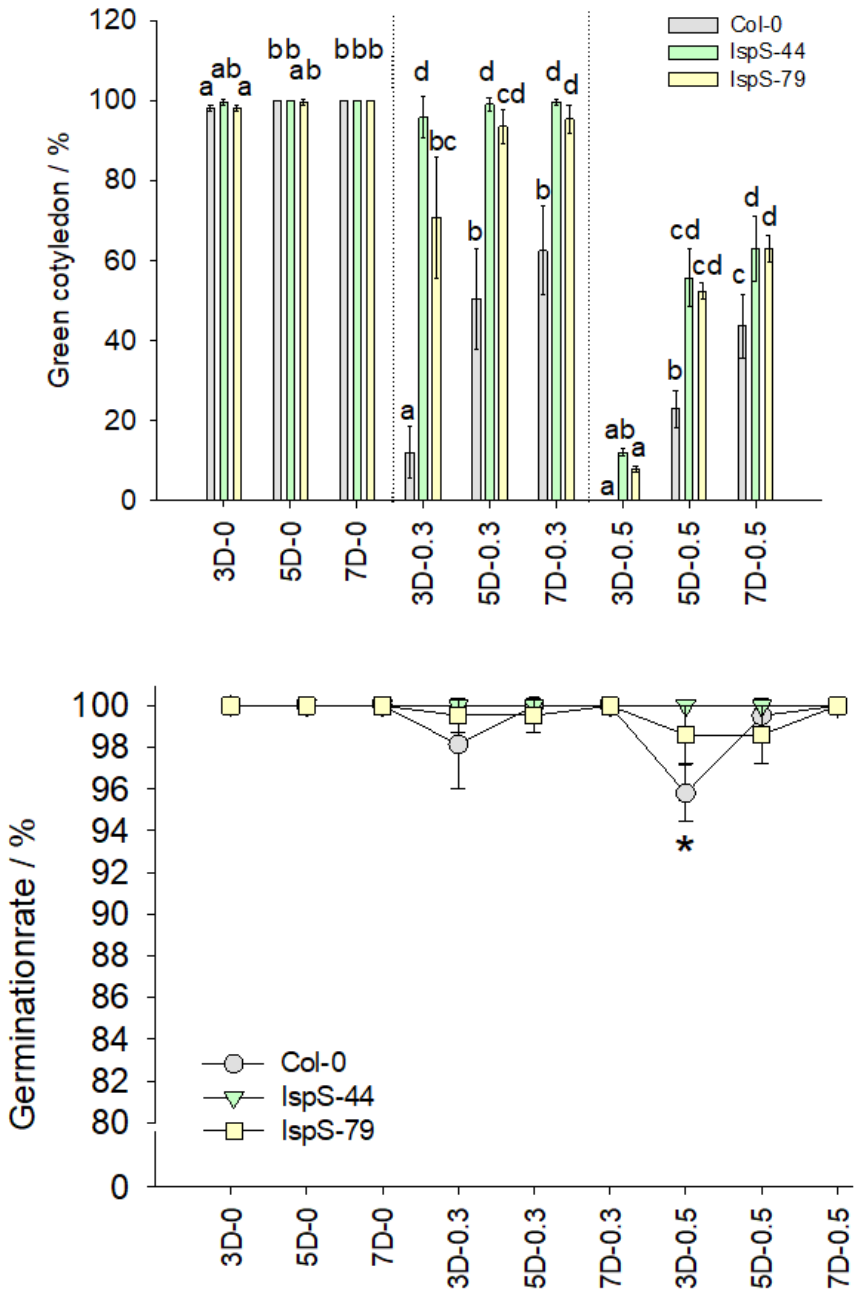
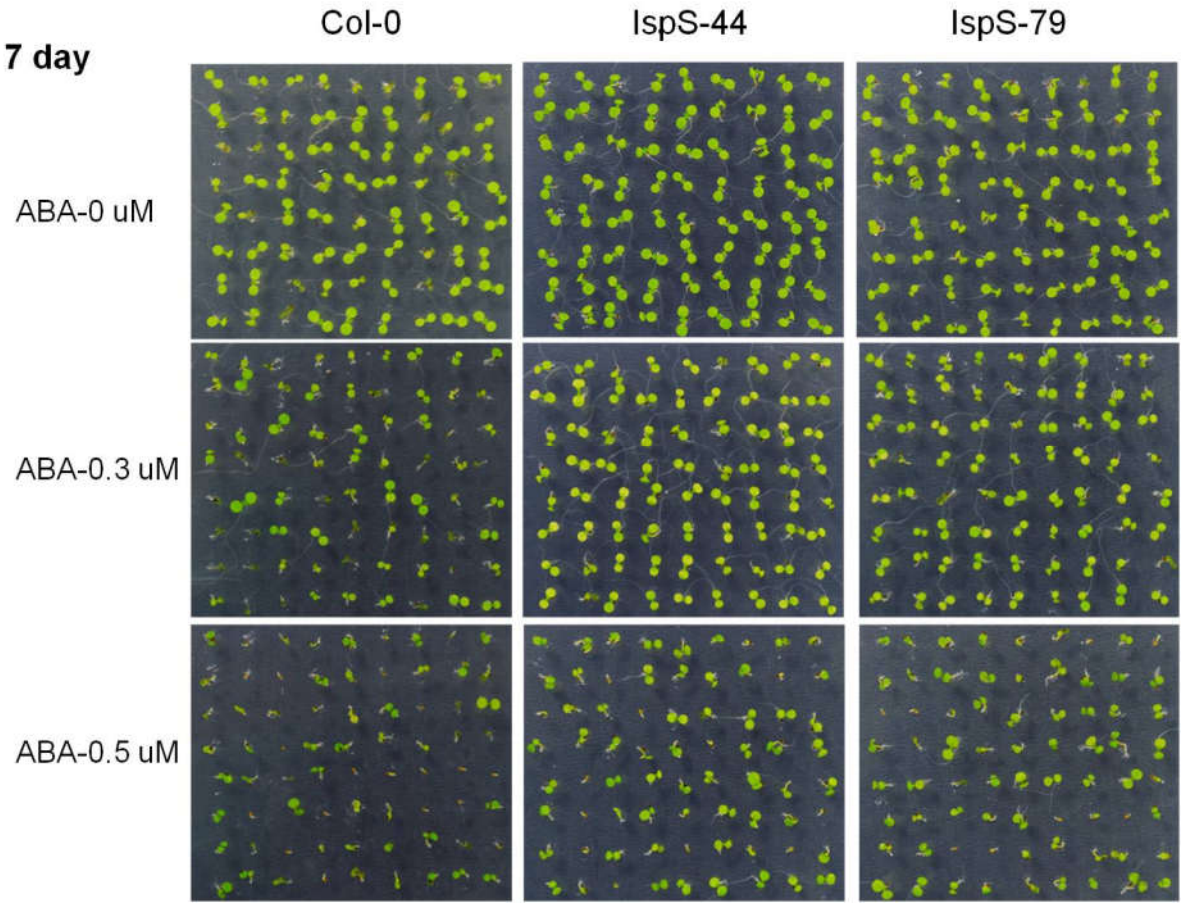


Fig. 3.8 Germination rates of Col-0 and *AdoIspS* transgenic plants treated with ABA.



Pic. 3.5 Germination rates of Col-0 and *AdoIspS* transgenic plants exposed to ABA.

In order to find out whether the insensitivity to exogenous ABA treatment in germination stage of transgenic plants is also maintained at post-germination stage, seeds of Col-0 and two *AdoIspS* transgenic lines were germinated on normal half MS medium for four days, then transferred to the same medium containing 10 μM, 20 μM ABA or a mock treatment to grow for another seven days. Compared to Col-0, root growths of both transgenic lines were consistently increased and the lengths of primary roots in transgenic plants were significantly longer than that of Col-0. (Fig. 3.9)

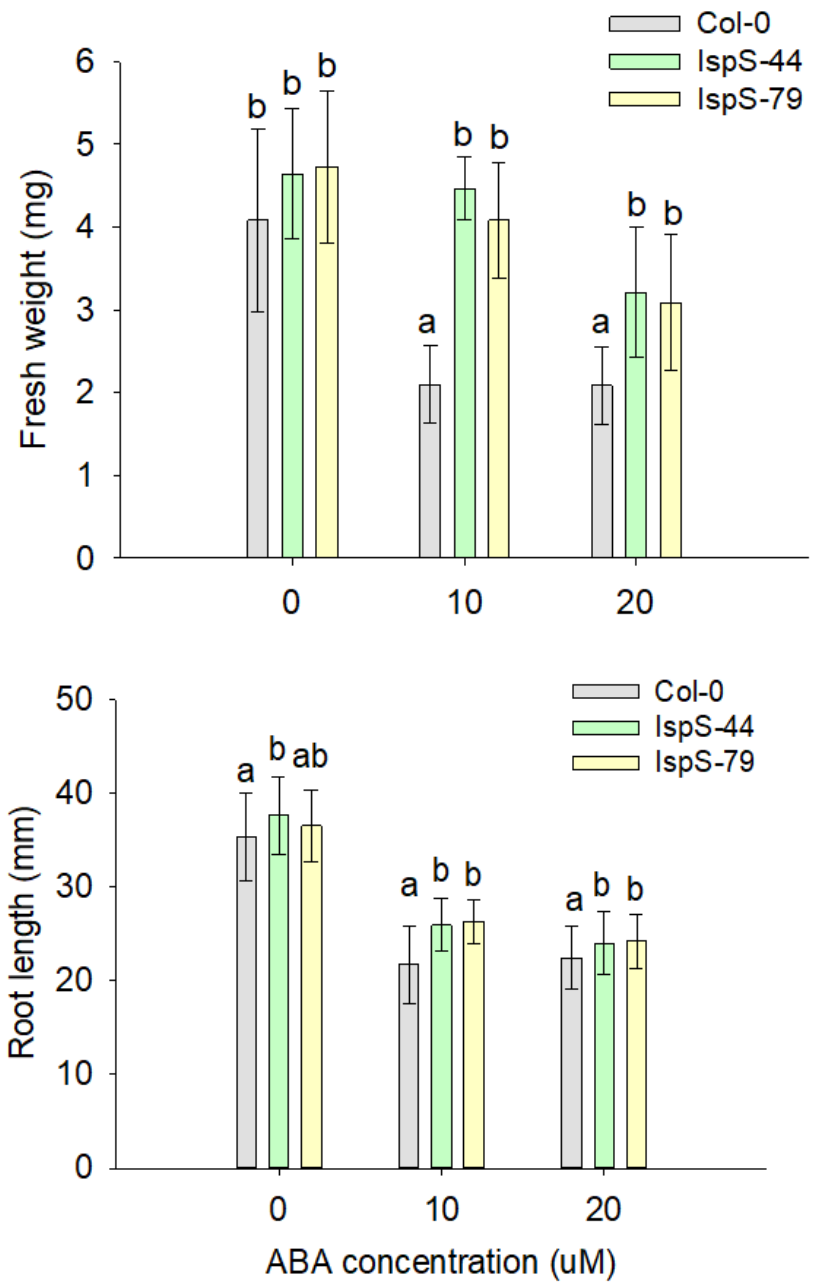
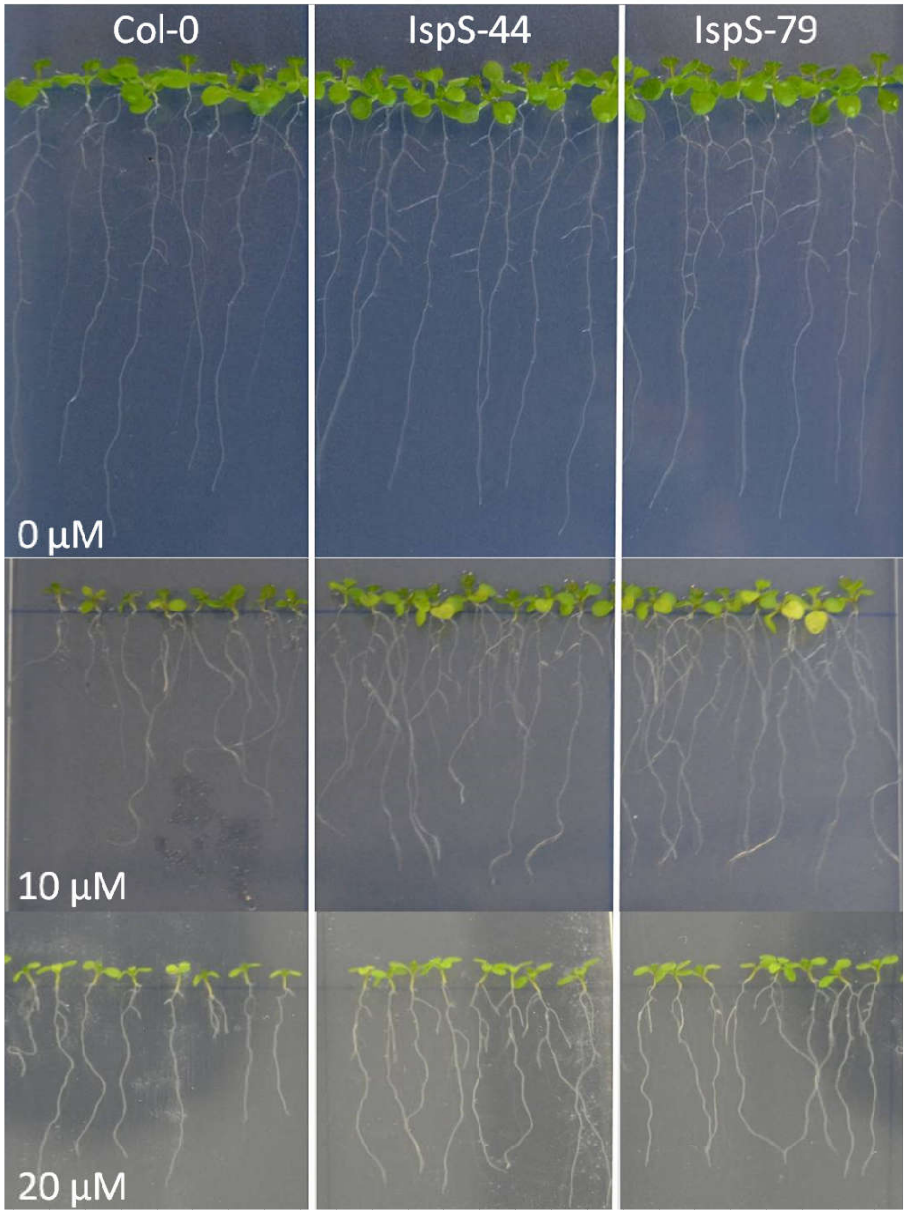


Fig. 3.9 Root growth of Col-0 and *AdoIspS* transgenic plants treated with exogenous ABA after germination. Four-day-old seedlings grown on 1/2MS were transferred to media containing different concentration of ABA. After 7 days, the root length and fresh weight in each genotype were measured.



Pic. 3.6 Root growth of Col-0 and *AdoIspS* transgenic plants treated with ABA after germination.

Fresh weight of treated plants was measured correspondingly with root length. The result indicated that the fresh weights of both transgenic lines were significantly higher than that of Col-0 after treatment. This result implies that the presence of *AdoIspS* gene not only protected the growth of root, but also the growth of plants aerial part was protected from the treatment of exogenous ABA. (Pic. 3.6)

Therefore, *AdoIspS* transgenic plants not only show enhanced plant growth under normal culture condition (as described in section 3.1.2), but also show growth advantage under exogenous ABA treatment.

According to these results, the presence of *AdoIspS* reduces plant sensitivity to exogenous ABA treatment in both leaf and root organs. *AdoIspS* gene in *A. donax* is mainly expressed in leaf, not in root (Li et al., 2017). It is possible that transgenic leaves over-expressing *AdoIspS* are less sensitive to ABA-induced signals from root, thus promoted a higher biomass accumulation which in turn led to a better growth in root.

To determine how ABA biosynthesis and signaling pathway were affected in *AdoIspS* over-expression plants, four genes were analyzed by qRT-PCR, including *RAB18* and *RD29B*, which are related to ABA signaling; *NCED3* and *ABA2*, which are related to ABA biosynthesis. ABA-responsive marker genes *RAB18* and *RD29B* from leaf and root tissue of each genotype were highly induced after exogenous ABA treatment.

The expression levels of *RAB18* and *RD29B* were higher in *AdoIspS* transgenic plants than in Col-0, for both leaf and root. The expression of *NCED3* was also elevated after the treatment, and the expression level was significantly higher in Col-0 leaf tissue than that in two *AdoIspS* transgenic lines, while there was no difference observed in the root part of these three genotypes. Interestingly, the expression level of *ABA2* was slightly reduced in leaf after the treatment, and it was lower in *AdoIspS* transgenic plants than that in Col-0. On the contrary, the expression of *ABA2* in root was slightly increased after the treatment, but there was no significant difference between *AdoIspS* transgenic plants and Col-0 (Fig.3.10 and Fig. 3.11).

These results indicate that in leaf tissue, *AdoIspS* transgenic plants exhibit up-regulation of ABA-responsive gene expression, and down-regulation of ABA biosynthetic gene expression under exogenous ABA treatment. But this pattern is not consistent in root tissue.

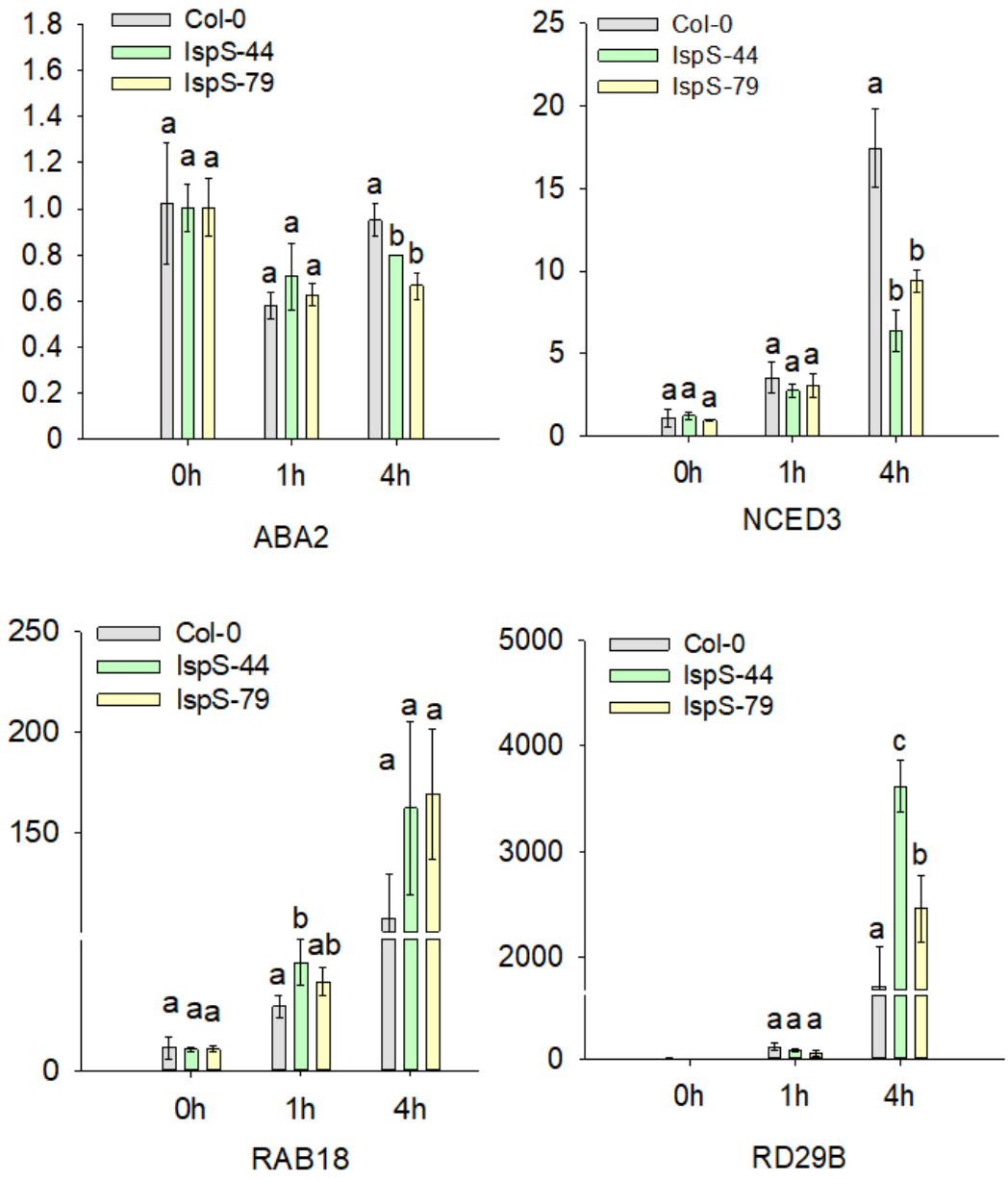


Fig. 3.10 qRT–PCR analysis of ABA signaling genes and biosynthesis-related genes in the leaf tissue of *AdoIspS* transgenic plants and Col-0 after ABA treatment.

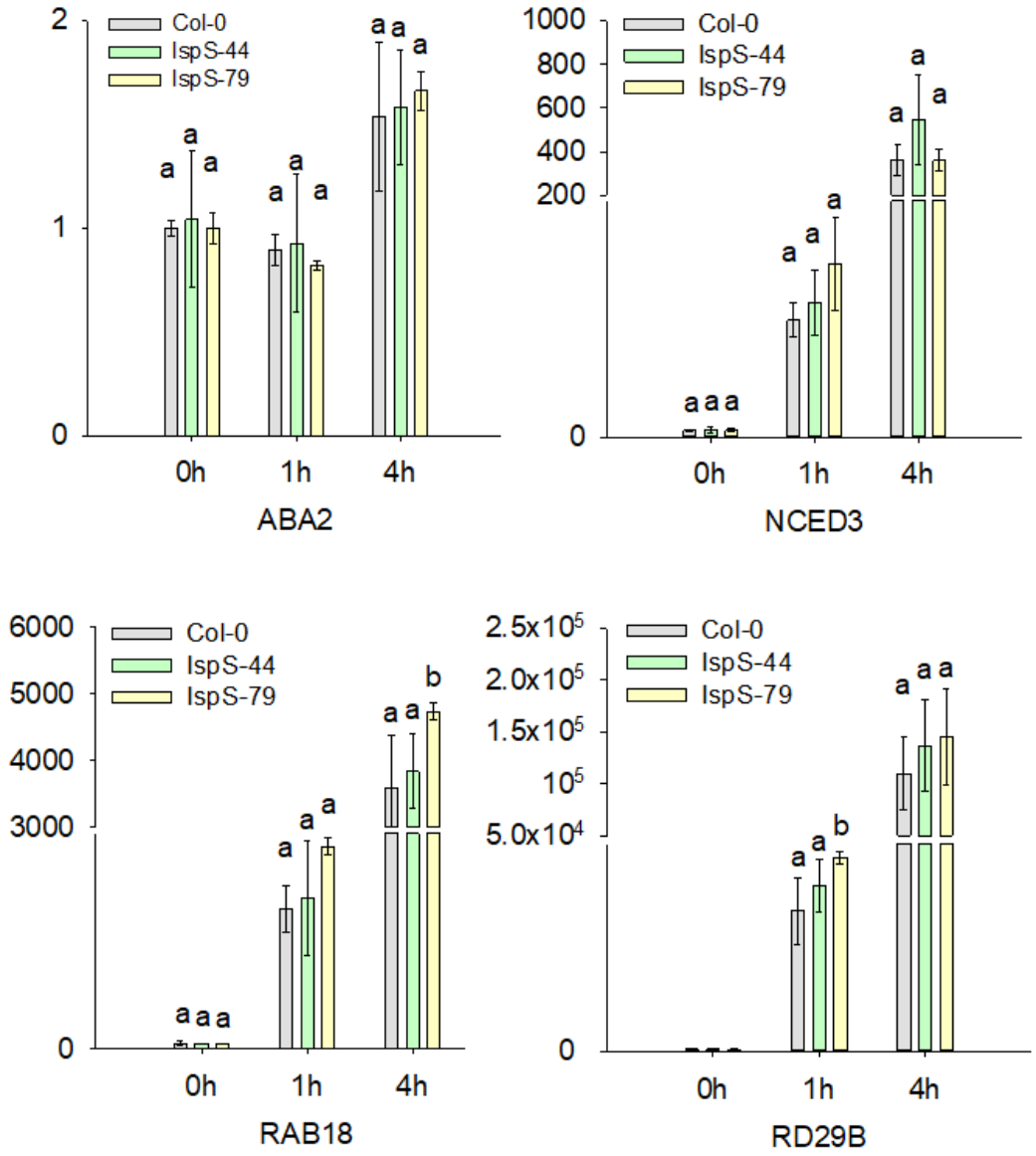


Fig. 3.11 qRT–PCR analysis of ABA signaling and biosynthesis-related genes in roots of *AdoIspS* transgenic plants and Col-0 after ABA treatment.

Discussion

ABA is a plant hormone, participating in drought perception and signal transduction pathways (Jensen et al., 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). Drought-induced ABA biosynthesis initiates ABA relocation and accumulation in guard cells, causing a loss of guard cell turgor pressure which results in stomatal closure (Desikan et al., 2004). In various plant species, ABA-induced stomatal conductance reduction requires ROS production as a pivotal signal (Pei et al., 2000; Desikan et al., 2004; Yan et al., 2007). The decrease of ROS accumulation impairs ABA-induced stomatal closure (Pei et al., 2000; Mittler and Blumwald, 2015) and other ABA-induced antioxidant activities (Furlan et al., 2016). As mentioned earlier, ROS production can be induced in varied forms of stressful conditions, which include the exogenous ABA treatment (Furlan et al., 2016; Kwak et al., 2003). The application of exogenous ABA can decrease plant transpiration rate (Lake and Woodward, 2008), inhibit seed germination (Rock, 2000) and plant growth under non-stressful conditions (Xiong and Zhu, 2003). Since drought tolerance mechanisms include ABA-dependent and ABA-independent pathways, most drought-inducible genes in ABA-dependent pathway are also induced by exogenous ABA treatment (Shinozaki and Yamaguchi-Shinozaki, 1997; Lee et al., 2015). In this study, we explored responses to exogenous ABA treatment for the first time in *IspS* transgenic plants.

Previous studies compared the foliar ABA accumulation in transgenic isoprene-emitting and non-emitting plants under drought stress, concluding that the effect of ABA in isoprene emission plants under drought stress is not consistent with current understanding of this hormone's mechanism of action (i.e. lower stomatal conductance accompanied by higher ABA levels) (Tattini et al., 2014). For instance, Ryan *et al.*, (2014) discovered a stronger reduction of

stomatal aperture in isoprene-emitting transgenic tobacco than non-emitting tobacco under water deficiency, but found no difference in foliar ABA concentration between isoprene-emitting and non-emitting leaves. Tattini *et al.*, (2014) suggested that the presence of *IspS* switched tobacco plants from isohydric behavior to anisohydric behaviour. Under water deficiency, isohydric behaviour plants synthesize high amounts of ABA and prefer to maintain leaf water potential caused by ABA effect on stomatal conductance, while anisohydric behaviour plants are in favour of the maintenance of CO_2 assimilation and accumulate much less ABA (Pantin et al., 2013). Anisohydric behaviour results in faster recovery and better performance under short term and moderate drought conditions (Sade et al., 2012; Pou et al., 2012). ABA is a phytohormone, involved in abiotic stress response and stress-resistant gene expression (Fujita et al., 2006; Leung and Giraudat, 1998). Drought-regulated gene expression can also be affected by exogenous ABA application (Zhu, 2002). Exogenous ABA application inhibits shoot and root growth, and causes leaf yellowing in well-watered plants (Chen et al., 2005; Mutui et al., 2005). Using exogenous ABA to chemically simulate "drought" stress under non-stressful conditions can provide insights into the influence of ABA alone (Franks and Farquhar, 2001), though at the whole-plant level, it cannot be expected to mimic every physiological response to drought (González et al., 2001).

Here, for the first time, we performed the investigation of *AdoIspS* transgenic plants in response to exogenous ABA. Compared to wild type plants, *AdoIspS* transgenic plants reduced ABA sensitivity to transgenic plants at germination stage, and accumulated higher fresh weight and root length at seedling stage after exogenous ABA application. These results indicated that *IspS* plants had higher carbon assimilation during the period of treatment, and supported the idea that *IspS* enhanced plant anisohydric behaviour tendency under drought stress (Tattini et al., 2014). In addition, the following qPCR results showed that the expression

levels of tested genes in root tissue were similar while in leaf tissue they were not. The expression levels of ABA-induced genes were higher in *IspS* transgenic leaves but the expression levels of ABA synthesis genes were lower. Since isoprene emission mainly occurs in leaf tissue (Cinege et al., 2009), the differential gene expression should be due to the capability of emitting isoprene. One possible explanation for this result is that ABA concentration is lower in *AdoIspS* transgenic plants compared to wild type due to isoprene emission in leaf and isoprene synthesis in root, therefore, the inhibition from exogenous ABA treatment would be less and promote transgenic plants growing better. This is however at odds with previous studies reporting no differences in ABA content between *IspS* over-expressing and control plants (Tattini et al., 2014). Alternatively, isoprene emission may down-regulate ABA biosynthesis by ROS quenching. Cellular ROS has been largely documented to elevate ABA synthesis in leaf tissue, as reviewed in (Mittler and Blumwald, 2015). This may be another reason why *IspS* transgenic plants showed growth advantage under exogenous ABA treatment.

3.2.3 Enhanced tolerance of *AdoIspS* transgenic plants to dehydration stress

In order to examine how *AdoIspS* transgenic plants respond to osmotic stress, 4-day-old seedlings from Col-0 and two *AdoIspS* transgenic lines were grown in hydroponic nutrition solution supplemented with different concentrations of PEG 6000. After 7-day treatment, a big reduction of plant growth was observed, and transgenic plants showed enhanced tolerance to osmotic stress at the seedling stage. Root growth of *AdoIspS* transgenic plants was significantly longer than that of Col-0 in 4% (w/v) PEG solution. However, the fresh weight was not significantly different between Col-0 and *AdoIspS* transgenic plants at every concentration of PEG solution. These results suggested that over-expressing *AdoIspS* may increase plant tolerance to osmotic stress (Fig. 3.12 and Pic.3.7).

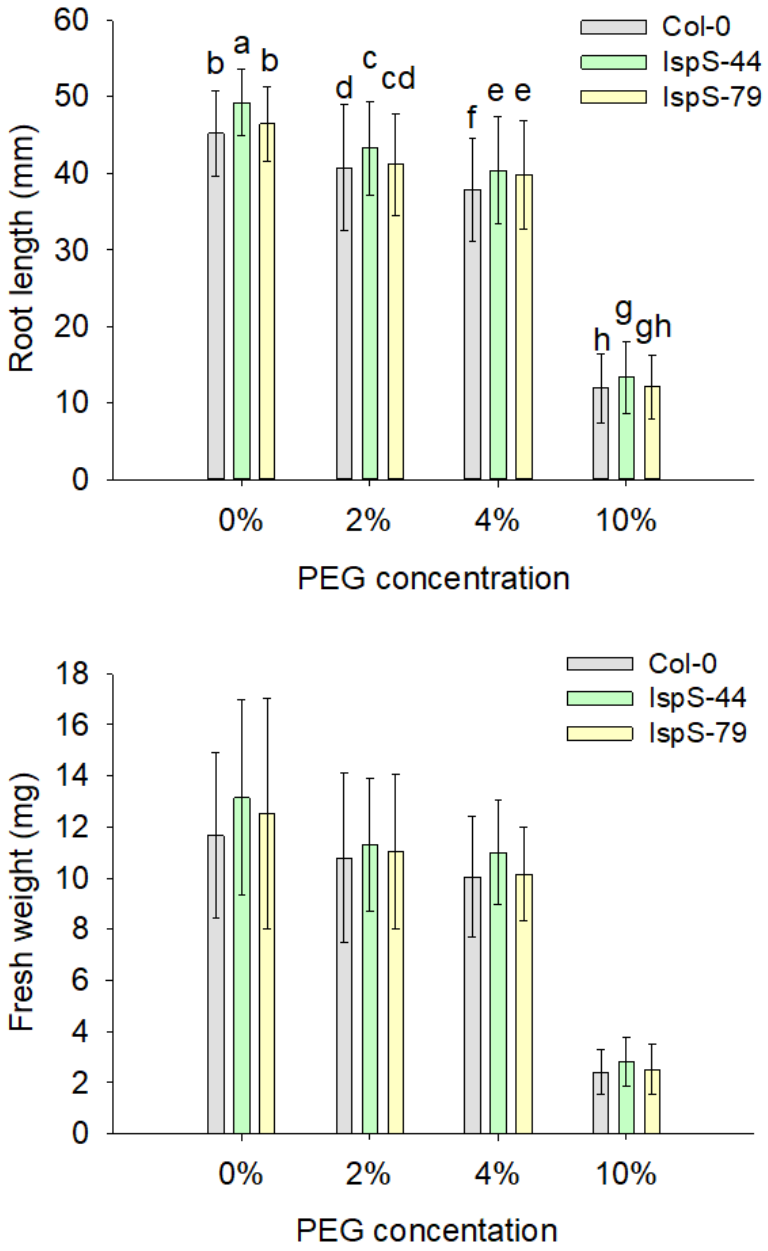
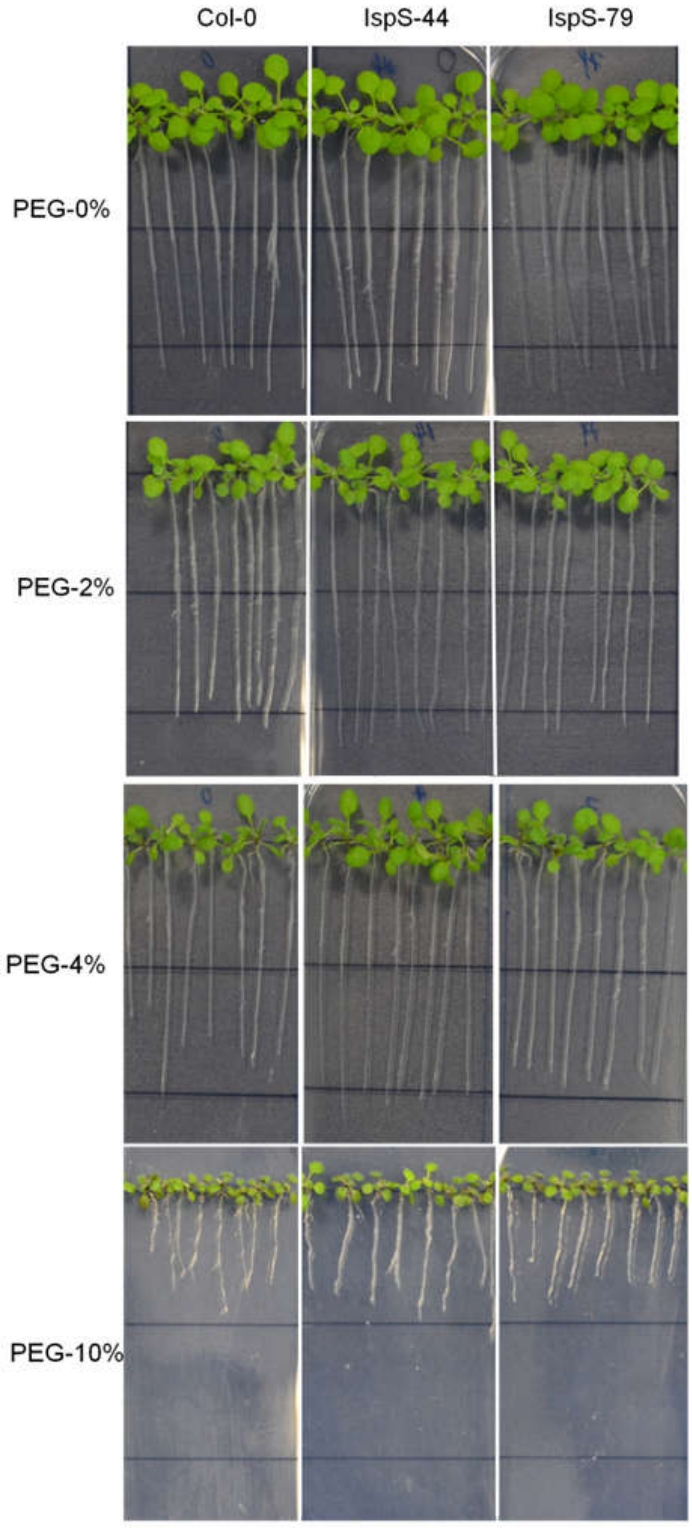


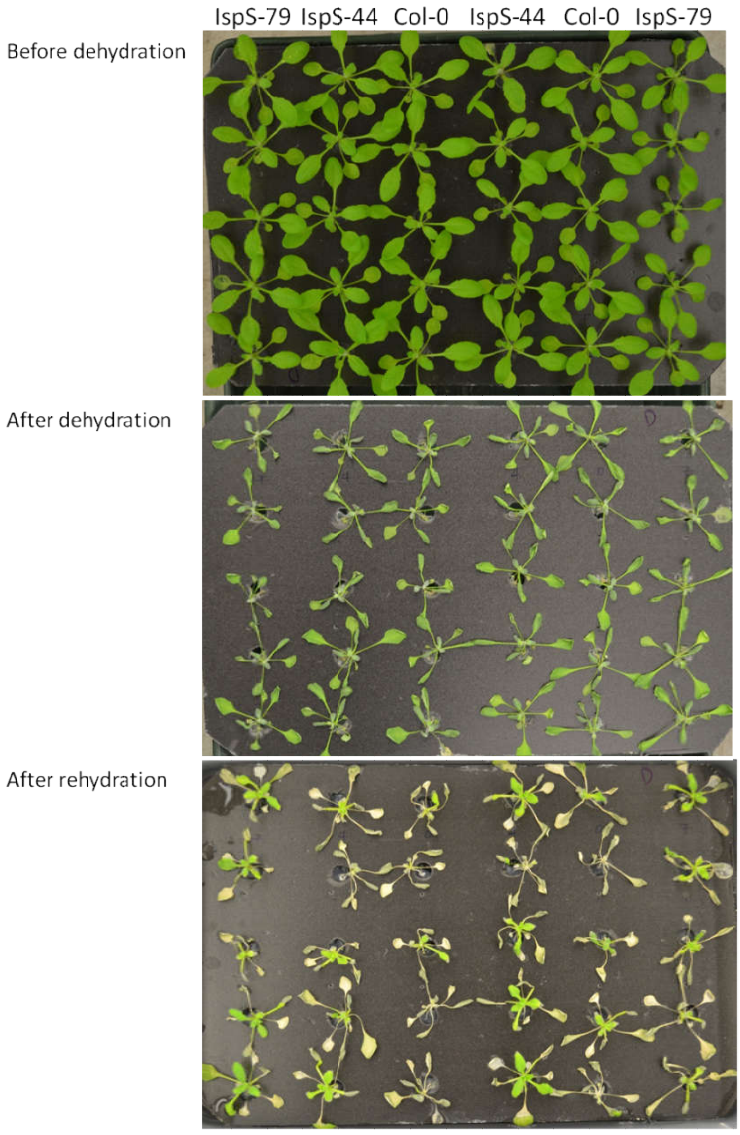
Fig. 3.12 Root growth of Col-0 and *AdoIspS* transgenic plants treated with various concentration of PEG.



Pic. 3.7 Root growth of Col-0 and *AdoIspS* transgenic plants treated with different concentration of PEG 6000.

Since *AdoIspS* transgenic plants exhibited an increased tolerance to exogenous ABA treatment and PEG-induced osmotic stress in seedling stages, it is reasonable to check whether *AdoIspS* transgenic plants could exhibit an enhanced tolerance to dehydration stress as well at later developmental stage.

The survival rates were recorded after 2-day recovery after dehydration treatment. Compared to Col-0, more plants of two *AdoIspS* transgenic lines appeared alive. This result demonstrates that *AdoIspS* transgenic plants have an increased drought resistance compared to Col-0. (Pic.3.8 and Fig.3.13)



Pic. 3.8 Col-0 and *AdoIspS* transgenic plants before and after 24hr dehydration treatment.

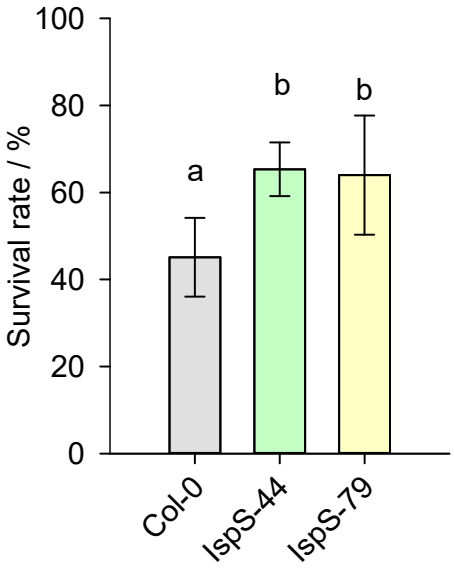


Fig. 3.13 The percentage of plants that survived after 24hr dehydration stress.

The ability to emit isoprene increases plant dehydration tolerance, which may lead to an enhanced tolerance to drought stress.

To further investigate the tolerance of water deficiency on cellular level, transpirational water loss rate, stomatal aperture and lipid peroxidation were examined. ABA and drought related genes expression was also analyzed here to examine the increased dehydration tolerance of *AdoIspS* transgenic plants at the molecular level.

Transpirational water loss rate was measured with two independent tests. Detached rosette leaves were weighed every hour to determine the rates of transpirational water loss in Col-0 and *AdoIspS* transgenic lines. Water loss rate of leaf tissue was lower in *AdoIspS* transgenic plants in the first hour. This suggested that the enhanced tolerance of *AdoIspS* transgenic plants to dehydration treatment

was possibly due to the decrease of leaf transpiration rate under water loss conditions. (Fig. 3.14)

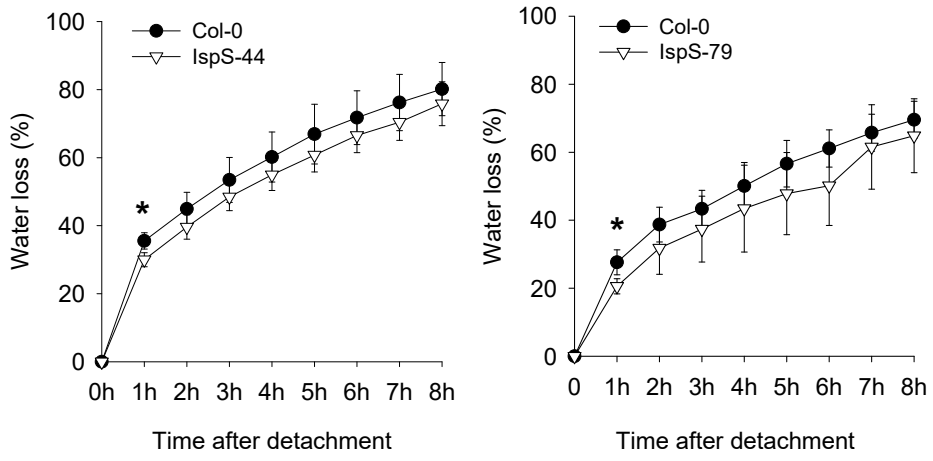


Fig. 3.14 Water loss from the leaves at various times after leaf detachment.

In order to find out the cause of the lower transpiration rate of *AdoIspS* transgenic plants under dehydration conditions, stomatal aperture was measured after leaves of Col-0 and *AdoIspS* transgenic plants were treated with ABA. In the absence of ABA, no obvious difference in stomatal aperture was detected between Col-0 and *AdoIspS* transgenic plants. However, after incubating leaves in the presence of ABA, transgenic plants exhibited a significantly lower width: length ratio than that of Col-0. *AdoIspS* transgenic plants showed enhanced ABA-induced stomatal closure compared to Col-0 and this could be the cause of lower transpiration rate of detached leaves from *AdoIspS* transgenic plants. (Fig. 3.15 and Pic.3.9)

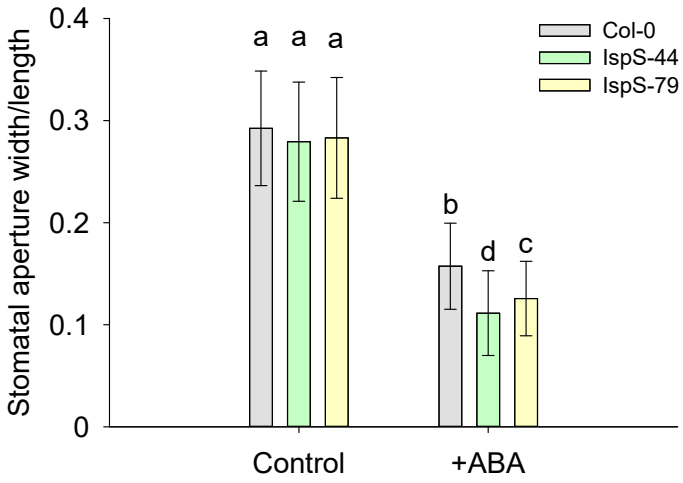
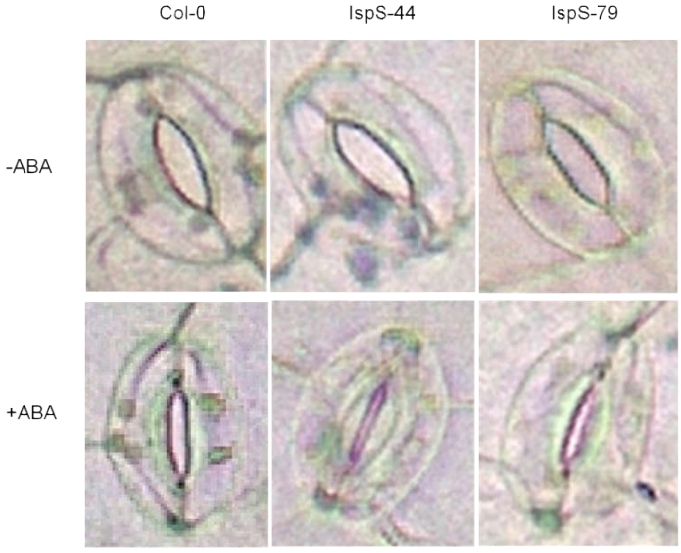


Fig. 3.15 Stomatal apertures in Col-0 and *AdoIspS* transgenic plants treated with ABA.



Pic. 3.9 Stomatal apertures in Col-0 and *AdoIspS* transgenic plants treated with ABA.

After dehydration stress, plants lipid peroxidation has been analyzed by assessing the accumulation of malondialdehyde (MDA). MDA contents of both Col-0 and *AdoIspS* transgenic plants were significantly accumulated after dehydration treatment, and the content was lower in *AdoIspS* transgenic lines. Line 44 showed a significantly lower content of MDA accumulation compared to Col-0 after 4 hours treatment. Previous studies reported that MDA accumulation was induced by oxidative stress and negatively correlated with dehydration tolerance, thus MDA content is an indicator of lipid peroxidation (Saha et al., 2016; Vickers et al.,

2009a). This result corroborated the former conclusion that *IspS* transgenic plants have an increased dehydration resistance, which may be due to the reduced oxidative damage affected by isoprene emission. (Fig. 3.16)

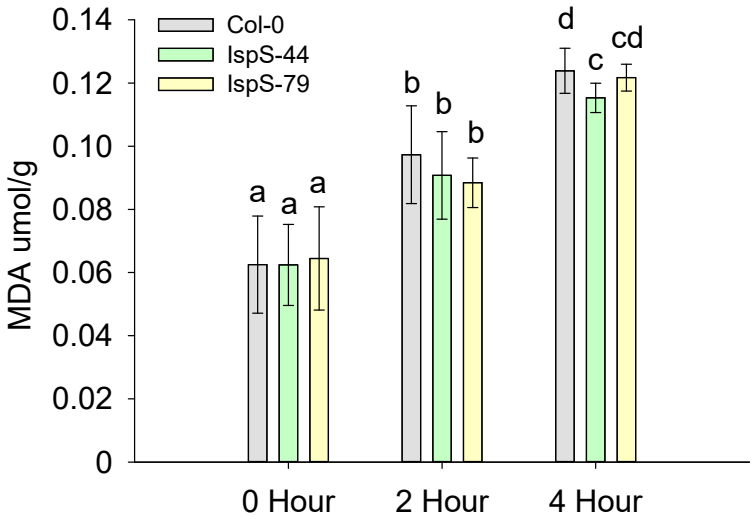


Fig. 3.16 MDA content in Col-0 and *AdoIspS* transgenic plants after exposed to dehydration stress.

Next we analyzed the expression level of four dehydration stress-inducible genes (*COR15A*, *P5CS1*, *RD20* and *RD29A*) to check their relevance with increased dehydration resistance in *AdoIspS* transgenic plants. Dehydration treatment caused a large increase in four stress-inducible gene expression levels, while no difference in *RD20* or *RD29A* expression levels was detected between each genotype in response to dehydration stress. The expression levels of *COR15A* and *P5CS1* were more elevated in Col-0. The expression of *COR15A* is induced by ROS to decrease membrane damage caused by stressful conditions, thus maintains the integrity of membranes (Lee et al., 2002). One aspect of dehydration tolerance in plants is the control of ROS or limitation of the damage caused by ROS (Verslues et al., 2006). The expression level of *COR15A* was more elevated in Col-0, indicating that the increased ROS production caused more membrane damage in Col-0 than in *AdoIspS* transgenic plants under dehydration stress. (Fig. 3.17)

P5CS1 is a rate-limiting enzyme which is responsible for synthesizing proline under drought conditions (Kavi Kishor and Sreenivasulu, 2014; Liu et al., 2012b). The accumulation of compatible solutes, such as proline, is triggered by low water potential in plants (Verslues et al., 2006). Under the same dehydration condition, the genes responsible for proline synthesis were drastically increased in Col-0. The expression level of *P5CS1* in *IspS* transgenic plants after 4 hours dehydration is similar to that in Col-0 after 2 hours dehydration, suggesting that *IspS* transgenic plants accumulated much less proline during dehydration stress. (Fig. 3.17)

Taken together, these results indicate that higher membrane integrity and lower induction of proline biosynthetic genes were present in *IspS* transgenic plants during dehydration stress. Thus, we could further suggest that after the same period of transient dehydration stress, isoprene-emitting plants could maintain a better balance between water uptake and loss than non-emitters, which could lead to a better plant performance under stressful conditions (Verslues et al., 2006). (Fig. 3.17)

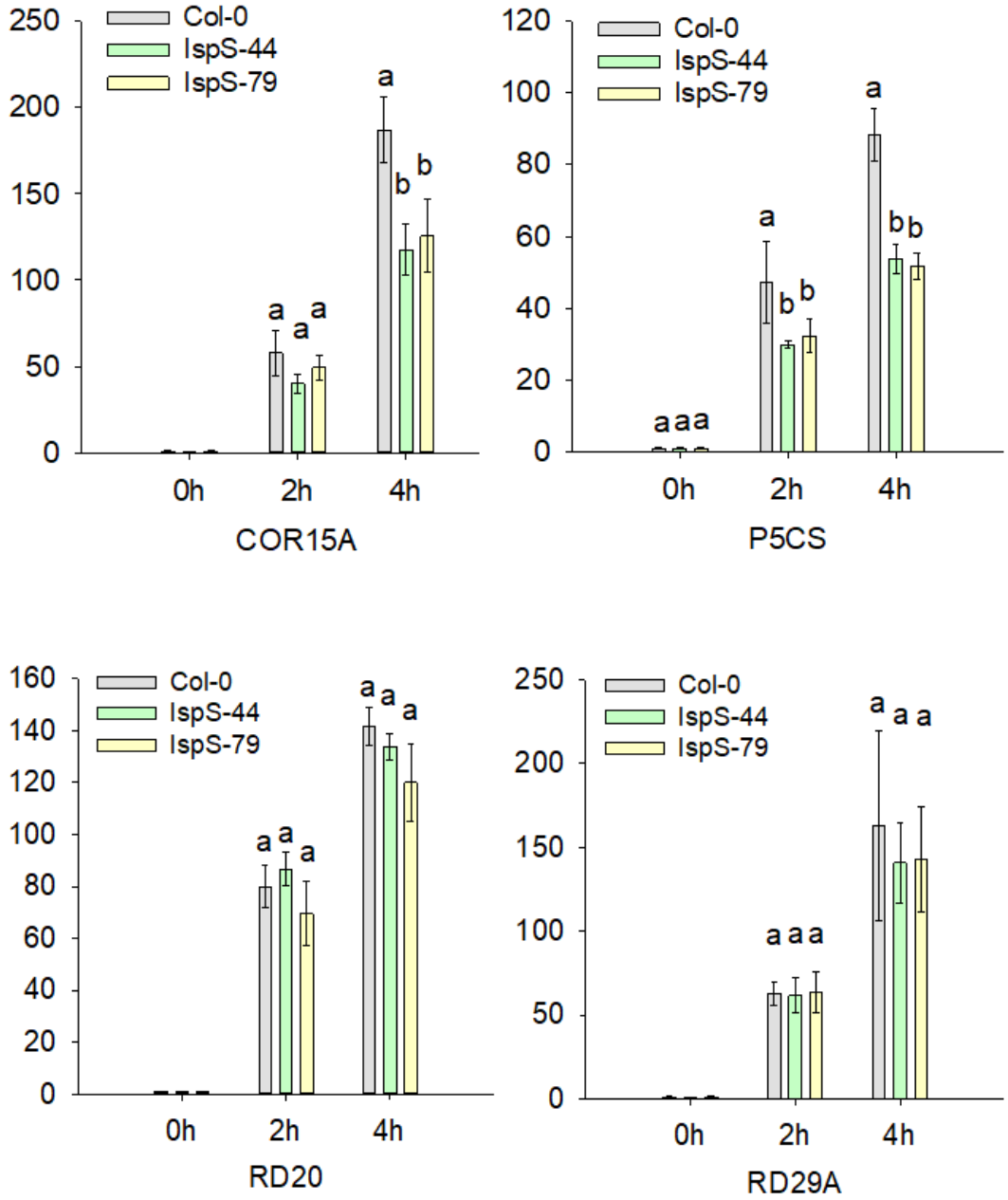


Fig. 3.17 qRT–PCR analysis of drought-inducible genes in Col-0 and *AdoIspS* transgenic plants in response to dehydration treatment.

Discussion

Besides thermotolerance, isoprene has been suggested to provide a better drought tolerance in plants, too. Some researches in *IspS* transgenic tobacco suggested a protective role of isoprene emission for plant photosynthesis under short term (less than a week) drought stress (Ryan et al., 2014; Tattini et al., 2014). However, Sasaki *et al.*, (2007) found that there was no difference of survival ability between wild type and *IspS* transgenic Arabidopsis after 3-week drought treatment.

In this study we asked whether there is any increased tolerance of *IspS* transgenic Arabidopsis under short term drought stress. The answer is yes.

According to our investigation, *AdoIspS* lines showed a lower water loss rate in detached rosette leaves. This result was confirmed by ABA-induced stomatal closure test, which indicates that the presence of *IspS* could increase stomatal closure under a similar ABA concentration or dehydration condition. This finding supports the result of Ryan *et al.* (2014), in which isoprene-emitting plants showed a lower leaf conductance when leaf ABA content was similar to wild type plants. As a consequence of fast stomatal closure in response to water deficiency, isoprene-emitting plants could maintain a higher water potential in leaf under stressful conditions and prolong the first responsive phase of plants to dehydration stress (Verslues et al., 2006). Therefore, *AdoIspS* transgenic lines showed a higher survival rate after 24 hours dehydration stress, which was applied directly exposing the whole plant to air. The reason why we used hydroponic solution to culture plants instead of soil is that the dehydration process takes much longer time in soil, and we confirmed that there is no difference in survival rate between wild type plants and *AdoIspS* transgenic plants after 3 weeks drought stress in soil. This result is similar to the one previously reported in Sasaki *et al.*, (2007), supporting the idea that isoprene-emitting plants have no enhanced tolerance to long-term stress compared to non-emitters (Vickers et al., 2009a). The first

response in plants to dehydration stress is to avoid low water potential by maintaining a balance between water loss and uptake; if the plant fails to maintain the balance, low water potential will trigger the accumulation of compatible solutes, such as proline (Verslues et al., 2006). Compatible solutes may protect proteins and cell structure, which is one aspect of dehydration tolerance. Another aspect is the control of the ROS level, or the limitation of oxidative damage in cells (Verslues et al., 2006). MDA is an indicator of the degree of lipid peroxidation and membrane damage in the leaf tissue (Vickers et al., 2009a), and the finding that there was less MDA accumulation in *AdoIspS* transgenic plants during the dehydration treatment indicates that there was less oxidative damage in *AdoIspS* plant cells. This result was corroborated by the qPCR result, which showed that the expression levels of *COR15A* in *AdoIspS* lines were significantly lower than that in wild type plants. The function of *COR15A* is to stabilize membrane structure by reducing the formation of lamella-to-hexagonal II phase (Steponkus et al., 1998). The expression of *COR15A* is induced by ROS accumulation. ROS are toxic molecules, which can damage cellular membranes and trigger cell death (Baier et al., 2005; Alvarez et al., 1998). Lee *et al.* (2002) suggested that constitutive ROS generation in *fro1* mutant may desensitize plant cells, thus causing a defect in cold-responsive gene expression. They reported that unlike *RD29A*, the expression of *COR15A* cannot be induced by cold stress in *fro1* mutant, which means the expression of *COR15A* in the *fro1* mutant was affected more dramatically by constitutive ROS accumulation than that of *RD29A* (Lee et al., 2002). Thus, unlike *COR15A*, which is predominantly induced by ROS signal, *RD29A* was subjected to a more complex regulation (Lee et al., 2002). Interestingly, although the expression level of *COR15A* in *AdoIspS* lines was significantly lower, the expression level of *RD29A* was similar to that in wild type plants.

Isoprene emission has been repeatedly documented as a ROS scavenger (Affek and Yakir, 2002; Vickers et al., 2009a), and capable of quenching and removing ROS (Vickers et al., 2009b). Drought stress affects plant photosynthesis, increases ROS accumulation in cell, thus enhances oxidative stress in plants (Tattini et al., 2015). It has been proposed that isoprene emission could increase plant's drought tolerance by reducing ROS damage in cell (Ryan et al., 2014). Thus, it is reasonable to assume that the presence of *IspS* reduced the accumulation of ROS in cells, and indirectly preserved the integrity of the membrane during the dehydration stress.

Besides *COR15A*, the expression level of *P5CS1* in *AdoIspS* lines was also significantly lower than that in wild type plants. *P5CS1* is a rate-limiting enzyme which is responsible for synthesizing proline under stress conditions (Kavi Kishor and Sreenivasulu, 2014; Liu et al., 2012b). Many plant species accumulate proline as a compatible solute to offset cellular imbalances caused by various stresses, including drought (Liang et al., 2013). Stress-induced proline synthesis can be triggered by ABA (Hare and Cress, 1997; Szabados and Savouré, 2010). *RD20* is predominantly induced by ABA (Aubert et al., 2010), so, considering the similarity of the expression of *RD20* between *AdoIspS* transgenic and wild type plants, the effect caused by dehydration-induced ABA should also be similar in *AdoIspS* transgenic and wild type plants. Thus, the difference of the expression levels of *P5CS1* should not be attributed to ABA. Up to now, the roles of proline in drought tolerance still remain controversial in plant stress biology (Hare and Cress, 1997; Nanjo et al., 1999; Bhaskara et al., 2015). Some studies suggested proline as a ROS scavenger, which could be induced by and react with stress-related ROS (Ben Rejeb et al., 2014; Szabados and Savouré, 2010). Meanwhile, proline catabolism has also been proved to increase the production of endogenous ROS (Miller et al., 2009; Liu et al., 2012b; Zhang and Becker, 2015). Compatible solutes accumulation is triggered by decreased water potential, and

can be energy intensive for the plant, may provide only small effect on water uptake under severe stress conditions (Verslues et al., 2006). Kesari *et al.* (2012) discovered that proline accumulation was not associated with an arid environment, but associated with humidity and mild climate. They proposed that, in addition to proline accumulation, plants which acclimatized to a dryer and more changeable weather have already acquired other drought-stress adaptive mechanisms (Kesari et al., 2012). Under the same dehydration condition, the rate-limiting gene for proline biosynthesis was drastically increased in Col-0. Therefore, it is reasonable to assume that isoprene emission could act as an additional adaptive mechanism to improve plants acclimatization to the drought environment and variable temperature, maybe associated with its ability as a ROS scavenger. Intriguingly, a similar drought-adaptive strategy was also found in *Arundo donax* plants, where *AdoIspS* gene was isolated from. Pompeiano *et al.* (2016) reported an unusually slow proline accumulation in *A. donax* in response to drought. Furthermore, a recent study characterized transcriptional responses of *A. donax* to water stress and identified gene *HAI1*, which could control and prevent excessive proline accumulation (Fu et al., 2016). However, dehydration-induced proline accumulation in another Poaceae species, *Saccharum* spp., was strongly correlated with days after drought treatment (Molinari et al., 2007). Unlike *A. donax*, *Saccharum* spp. is not an isoprene emitter (Hardacre et al., 2013). Even though these two plant species belong to the same family and share a similar morphology, these evidences indicate that they likely developed two separate acclimation mechanisms in osmotolerance.

Taken together, these results indicate that the presence of isoprene emission ability is highly relevant to the reduced production of proline under dehydration conditions. As suggested previously, plants acclimatized to dryer growth conditions have already acquired other drought-stress adaptive mechanisms other than proline (Kesari et al., 2012). Therefore, maybe it is not sufficient to conclude

that isoprene-emitting capability is the cause of this adaptive advantage in plants to drought stress, referred to the retarded proline accumulation. But the ability to emit isoprene is certainly involved in the evolutionary process of this acclimation mechanism.

It has been reported that under drought stress, isoprene-emitting tobacco accumulated less biomass than non-emitting tobacco (Ryan et al., 2014). Interestingly, water deficiency has been proposed as the biggest environmental stress factor on the growth of *A. donax* (Pompeiano et al., 2016, 2013). Therefore, it is reasonable to speculate that the reduced biomass accumulation in these plants is attributed to isoprene emission. This may be one possible reason why isoprene-emitting plants showed no growth or tolerance advantage to long-term stress, not only to drought stress. After all, the production of isoprene represents a substantial investment for the plant in terms of carbon and energy (Vickers et al., 2009a). The proposed protective mechanisms of isoprene emission enhances plant tolerance to abiotic stresses, such as membrane stabilization, ROS scavenging and signal responses change, probably not sufficient to provide better protections for plants under long-term stressful conditions. However, thanks to the growth advantages under normal conditions and better tolerance to mild/transient stresses, isoprene emission could certainly be a selective advantage for emitting plants to cope better with more changeable environmental conditions in the future.

4 Conclusion and outlook

In conclusion, the results obtained so far strongly support that *IspS* gene plays important roles in regulating plant growth and tolerance to several abiotic stresses such as heat, drought and osmotic stresses.

The response to heat stress has been reported previously by using *IspS* gene from other plant species; however, several novel findings of this study further our understanding of the physiological role played by isoprene emission in the tolerance to dehydration and osmotic stresses, indicating that its mechanism of action could possibly involve the modulation of sensitivity/dependence to ABA. Therefore, the cross talk between ABA and isoprene emission under dehydration stress should be further studied in the future. Under dehydration stress, only *AdoIspS* transgenic line 44 showed statistical significance on reduced MDA content to Col-0. In order to elucidate the protective role of isoprene against oxidative stress induced by drought or dehydration, ROS accumulation induced by dehydration stress should be further assessed by measuring intracellular ROS levels or by checking the expression levels of ROS marker genes, e.g. *DEFL*.

The increase in plant fitness provided by isoprene emission could contribute to the maintenance of the trait by conferring an additional selective advantage to the individuals/species that acquired it also in the absence of environmental stresses.

Taken together, the results of this study indicate that the benefits acquired by plants emitting isoprene outweigh the energy costs associate to its biosynthesis, especially under abiotic stress conditions, which proves the importance of isoprene emission capacity for natural emitters.

Studies carried out in natural isoprene-emitters have made a major contribution to the current understanding of isoprene emission involvement in physiological and

biochemical basis of abiotic stress tolerance in plants. The results presented here proved that molecular genetic approaches could be a new direction to gain more understanding of biological functions of the *IspS* gene.

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6 Acknowledgements

I express my deepest thanks to my supervisor Dr. Claudio Varotto who inspired me, and offered indispensable support and patient guidance to my studies.
I would like to express my sincere gratitude to Dr. Mingai Li, who invited me in this group and led me to start my research in the field of molecular biology. Without her help these studies were unlikely to be conducted.
I want to thank my co-supervisor Prof. Trainotti for his help and assistance in the University of Padova. He was always there to help, even if the time was busy for him.
I would also like to thank my evaluators, Prof. Fineschi and Prof. Morosinotto, for their helpful criticism and suggestions on these studies.

I express my thanks to China Scholarship Council for giving me this opportunity to study in Italy and supported me financially for 4 years, and to Fondazione Edmund Mach (FEM), especially the Ecogenomics group, for providing the place and equipment for this study (This work was supported by the Autonomous Province of Trento (Italy) through core funding of the Ecogenomics group).

Thanks to all the colleagues in Ecogenomics group, especially Bo, Michele and Enrico for helping me in the beginning of my time in the laboratory. Thanks also to Sylvie and Jeroen, in particular to Dr. Elisabetta Perini and my classmate Silvia, who offered a lot of help and warm care in private life.
I express my special thanks to Wentao and Junning who continuously offered me help since I was a master student, also to Yimo and Chuangbin, for helping and visiting my mother when I was abroad. Thanks to Zhirong, Zengfan and Anming for their special visits to me during these four years.

I express my heartfelt thanks to Dr. Qiushi Yin. It's rough to study abroad, but you made my life so much easier. Thanks for your devotion, which can be proved by the tremendous contribution you've paid to Trenitalia.
I express my deepest love and gratitude to my parents. You are my strongest support forever.