Agrochimica, Vol. LVIII – Special Issue (2014) 61-76

New evidence for the functional roles of volatile and non-volatile isoprenoids in stressed plants

C. BRUNETTI[1,*], F. FERRINI[1], A. FINI[1], M. TATTINI[2]
[1] Dipartimento delle Produzioni Agroalimentari e dell'Ambiente, Università di Firenze, Viale delle Idee 30, 50019 Sesto Fiorentino, Firenze (Italy)
[2] Istituto per la Protezione delle Piante, CNR, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Firenze (Italy)

Keywords: methylerythritol phosphate pathway, carotenoids, abscisic acid, isoprene, xanthophylls, reactive oxygen species.

SUMMARY. – Mediterranean plants are challenged against extreme, often unpredictable stress events which may pose severe constrains for the plant survival, as uncontrolled reactive oxygen species (ROS) generation ultimately lead to cell death. Secondary metabolites, which are highly responsive to environmental drivers, constitute a flexible system aimed at countering drastic perturbations in the cellular ROS homeostasis. Recent evidence supports the notion of isoprenoids (products of methylerythriol phosphate (MEP) pathway) as aimed at quenching ROS, not only devoted at avoiding ROS formation. Here we focus our attention on "*essential isoprenoids*" (i.e., carotenoids) and "*non-essential isoprenoids*" (e.g., isoprene), and how they constitute a well-coordinated system aimed at countering an excess of radiant energy to the chloroplast during stress progression. We also offer recent evidences of the functional roles of the end-product of MEP pathway, abscisic acid, as an essential regulator of the whole-plant metabolic machinery, and not merely of stomatal opening.

INTRODUCTION. – Isoprenoids constitute the oldest known class of plant metabolites with essential functions in the performance and regulation of basic physiological and biochemical processes in plants. Plants isoprenoids have essential roles in photosynthesis, respiration, membrane fluidity and regulation of growth and development (VRANOVÁ *et al.*, 2013). Isoprenoids are synthesized by plants through successive condensations of two activated forms of isoprene: the isopentenyl pyrophosphate (IPP) and the dimethylallyl diphosphate (DMAPP) (Fig. 1). These two isoprenoids precursors can be synthesized by two independent and non-homologous metabolic pathways: the cytosolic mevalonate (MVA) pathway and the plastidial MEP pathway (LOMBARD and MOREIRA, 2011). The MEP pathway, capable of generating IPP and DMAPP from D-glyceraldehyde-3-phosphate (G3P) and pyruvate, was discovered in the 1990s in bacteria and plants using ^{13}C-labelling experi-

* Corresponding author: cecilia.brunetti@unifi.it

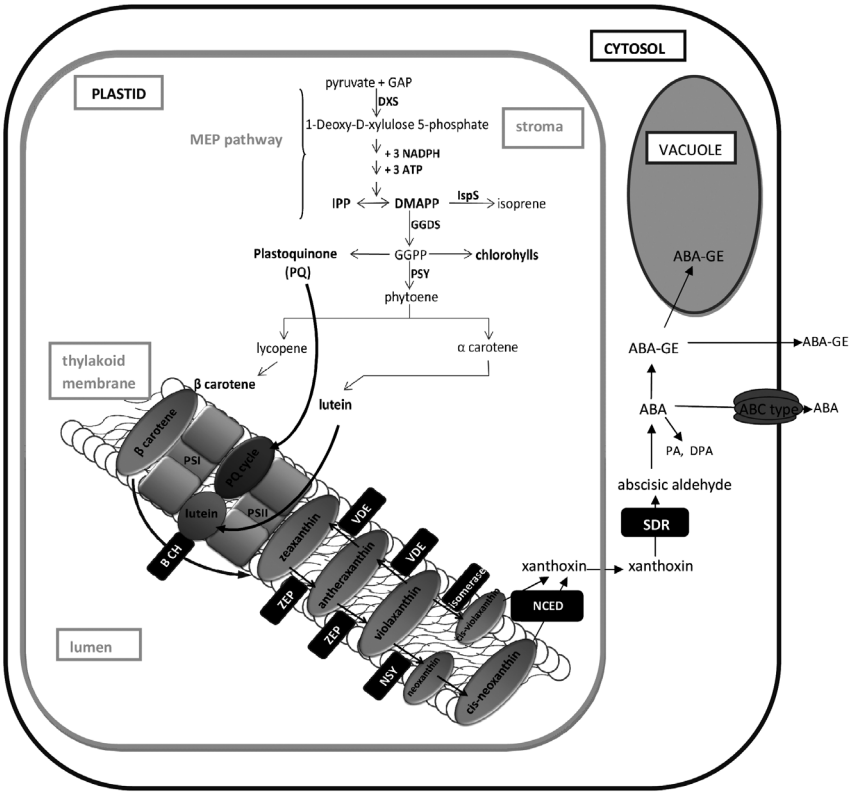

Fig. 1. – The carotenoid and ABA biosynthetic pathway in plants. In the plastid carotenoids are synthesized from the C5 precursor, IPP (isopentenyl pyrophosphate). Plastidic IPP is synthesized from glyceraldehyde-3-phosphate (GAP) and pyruvate via DXP (1-deoxy-D-xylulose-5-phosphate). Subsequently, the addition of three IPP molecules to DMAPP generates geranyl geranyl diphosphate (GGPP), a precursor for several groups of plastidial isoprenoids including carotenoids. The first step is the condensation of two GGPP molecules to produce phytoene. Subsequently, hydroxylation of the carotene rings generates xanthophylls such as lutein (from α-carotene) and zeaxanthin (from β-carotene). Zeaxanthin is epoxidated twice to make violaxanthin, which can be converted to 9-*cis*-violaxanthin and *trans*-neoxanthin. *Trans*-neoxanthin further gets converted to 9-*cis*-neoxanthin. 9-*Cis*-violaxanthin and 9-*cis*-neoxanthin are further oxidatively cleaved by NCED to generate xanthoxin. Xanthoxin, once formed, is then exported to the cytosol, where it is converted to ABA through a two-step reaction via ABA-aldehyde. ABA is inactivated through either oxidation (to phaseic (PA) and dihydrophaseic acid(DPA)) and conjugation (to ABA glucose ester (GE)). ABA-GE is involved in long distance transport of ABA and can be hydrolyzed in response to stress by β-glucosidases (AtBG1 and AtBG2), thus leading to an increase in the active ABA concentration. ABA can be transported from the cytoplasm to the apoplast through ATP-binding cassette (ABC) transporter.
DXS, DXP synthase; IspS, isoprene synthase; GGDS, GGPP synthase; PSY, phytoene synthase; BCH, β-carotene hydroxylase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxygenase; SDR, short-chain dehydrogenase/reductase.

ments (ROHMER *et al.*, 1996; LICHTENTHALER *et al.*, 1997). Despite their prokaryotic origin during endosymbiosis, all the genes coding for the enzymes for IPP synthesis through the MEP pathway are encoded in the nucleus, and this pathway constitute a robust trait maintained during evolution from algae to higher plants (LANGE *et al.*, 2000). By compartmentalizing the two pathways, plants may have gained a selective advantage optimizing isoprenoids biosynthesis according to fixed carbon and ATP availability and thus overcoming their sessile lifestyle constraints (VRANOVÁ *et al.*, 2013). Indeed, despite an exchange of isoprenoids precursors takes place among different subcellular locations (LICHTENTHALER, 2007), the loss of one of the two pathways cannot be compensated by the remaining pathway (PULIDO *et al.*, 2012). In a broader sense, the MEP and MVA pathways are not evolutionary remnants, but could enable to channel rapid production of specific isoprenoids for different functions (e.g. for growth or defence), ensuring that plants survive under different stress conditions (KLIEBENSTEIN, 2004).

As sessile organisms in a dynamic environment, plants are inevitably subjected to a diverse array of abiotic stresses. For example, plants suffer from excess light on both daily and seasonal basis (LI *et al.*, 2009), and hence exposed to massive generation of ROS in the photosynthetic apparatus (FOYER and SHIGEOKA, 2011). Environment-induced alterations in ROS homeostasis may be severe in plants inhabiting Mediterranean regions. Mediterranean plants are challenged against extreme, often unpredictable stress events (broadly referred as to global climate change effects) which may strongly depress the usage of visible light in photosynthetic processes (CHAVES *et al.*, 2009). Under these circumstances, the impact of high sunlight irradiance when coupled with hot air temperature and water scarcity, poses severe constrains for the plant survival, as uncontrolled ROS generation ultimately lead to cell death. Actually, plants have evolved mechanisms to use ROS for efficient signalling (to activate a network of responses leading to acclimation to a changing environment), and hence mechanisms have to operate in plants to keep ROS under tight control, not to merely remove them (MITTLER, 2004). Secondary metabolites, which are essential in plant-environment interactions, and highly responsive to environmental drivers, constitute a flexible system aimed at countering drastic perturbations in the cellular ROS homeostasis. Recent evidence supports the notion of isoprenoids as aimed at quenching ROS, not only devoted at avoiding ROS formation.

The MEP pathway is tightly involved in the control of accumulating ROS levels during abiotic stress, and constitutes a highly flexible mechanism, which integrates information from the environment and cellular metabolism. Here we focus our attention on some "products" of MEP pathway: volatile isoprenoids, particularly isoprene, and non-volatile isoprenoids such as carotenoids, xanthophylls and the phytohormone abscisic acid (ABA). In this brief review article, the functional roles of isoprene and carotenoids in plant responses to severe abiotic stress conditions will be examined. It appears evident that "*essential isoprenoids*" (i.e., carotenoids) and "*non-essential isoprenoids*" (e.g., isoprene) cooperate not only during leaf development (RASULOV *et al*., 2013) but also during stress progression. We also offer recent evidences of the functional roles of another essential isoprenoid, ABA, as regulator of the whole-plant metabolic machinery, and not merely of stomatal opening.

ISOPRENE. – Isoprene (2-methyl-1,3-butadiene) emitting species occur in almost all major groups of land plants including dicots, monocots, gymnosperms, pteridophytes, and mosses. Isoprene is biosynthesized in the chloroplast by isoprene synthase (IspS) (Fig. 1) and its emission is probably a genetic ancestral character which evolved during the colonization of land by plants. Since isoprene helps plants withstand rapid temperature fluctuations, the thermal stress experienced by early land plants could have conferred a selective pressure favouring the evolution of isoprene emission. SHARKEY and SINGAAS (1995) indeed reported that isoprene emission is much more common in mosses and ferns than in later divergent land plants. In contrast, isoprene biosynthesis is absent in liverworts and hornworts, which are restricted to moist and shady environments, and unlikely benefit from isoprene emission. Therefore, isoprene synthase may have evolved multiple times, possibly from a reservoir of monoterpene synthase genes, and this may explain the distribution of isoprene emission among plants (SHARKEY, 2005). Isoprene is neither stored nor metabolized in leaves, so emission reflects biosynthesis (DELWICHE and SHARKEY, 1993; SCHNITZLER *et al.*, 2004). Isoprene emission represents a considerable investment for the plant in terms of carbon and energy consumption (SHARKEY and YEH, 2001). Since constitutive emission in strong isoprene emitters is in the range 1-100 nmol m^{-2} sec^{-1}, emitting plants lose as much as 1-2% of fresh assimilated carbon to isoprene biosynthesis (VICKERS *et al.*, 2009). Even when the fresh carbon budget becomes limited, e.g., during severe

drought stress, isoprene emission is slightly reduced (SHARKEY and LORETO, 1993; BRÜGEMANN and SCHNITZLER, 2002). Alternative carbon sources are used for isoprene biosynthesis when assimilation rates are severely constrained (WOLFERTZ *et al*., 2004; BRILLI *et al.*, 2007).

It is now clear that terrestrial plants have developed a very sophisticated secondary metabolism and invest carbon and energy to produce compounds that play more than one functional role. Actually, the biosynthesis of most secondary metabolites is a safety way to dissipate an excess of photoassimilates and energy (HERNANDEZ and VAN BREUSEGEM, 2010). This role as "safety valve" also applies to isoprene, but it is not the main function. Indeed, 'several' functions for isoprene biosynthesis and emission in maintaining plant fitness during stress events (by increasing broad-spectrum tolerance to the production of oxidative compounds) have been recently suggested. A large number of experiments support the hypothesis of isoprene both conferring thermotolerance to thylakoid membranes and serving antioxidant functions in response to abiotic stresses (LORETO and SCHNITZLER, 2010).

Isoprene emission is likely the most rapidly induced thermo-tolerance mechanism (SINGAAS *et al.*, 1997; SHARKEY *et al.*, 2008). BEHNKE *et al.* (2007) showed that transgenic non-isoprene-emitting poplars exposed to short stress treatments, at 38-40°C, displayed reduced photosynthesis rates and photosynthetic electron transport, and increased nonphotochemical quenching as compared with wild-type poplars. This is because isoprene biosynthesis takes place rapidly in response to high T, whereas thermo-tolerance mechanisms sustained through de-epoxidation of violaxanthin-cycle pigments take much longer time (HAVAUX, 1993). Isoprene is highly lipophilic so that it may accumulate inside chloroplast membranes (COPOLOVICI and NÏINEMETS, 2005) changing the features of secondary electron acceptor of PSII (plastoquinone, QB), thus enabling plants to perform efficient primary photochemistry of PSII at higher temperatures (VELIKOVA *et al.*, 2011). Membrane stabilisation at high T is to be considered an antioxidant function *sensu lato* (HALLIWELL, 2009), as prevents peroxidation of membrane lipids. Furthermore, isoprene is a quencher of singlet oxygen ($^1O_2$) (AFFEK and YAKIR, 2002), nitric oxide (NO) and other ROS that are formed under abiotic stress conditions (VELIKOVA *et al.*, 2005; LORETO *et al.*, 2001). It was shown that in elevated ozone isoprene both reduced/delayed ozone-induced lipid peroxidation (LORETO and VELIKOVA, 2001) through thylakoid membrane stabilization, and, in addition directly reacted with

ozone, particularly if the reaction occurs in a humid environment such as in the mesophyll layer (Loreto *et al.*, 2001).

It has been suggested that isoprene emission evolved when plants conquered the land and started experiencing thermal extremes of terrestrial environments. Indeed, isoprene is still a trait more widespread in hygrophytes than in xerophytes. For example, in Mediterranean regions, characterized by aridity coupled with high temperatures and high sun light irradiance, isoprene emission is more common in the hygrophytes rather than in xeric species (Loreto *et al*., 2013). Isoprene emission by plants living in Mediterranean water-buffered environments, could be an initial mechanism which protects the chloroplast against sharp temperature increases (heat flecks) (Sharkey et al., 2008) due to sudden heat waves.

Carotenoids. – Carotenoids comprise a large family of polyisoprenoid pigments synthesized by all photosynthetic organisms (Cazzonelli, 2011; Rodríguez-Conceptión, 2010). The conjugated double bonds of their C_{40} backbone act as a chromophore and are responsible for the yellow colour found in many fruits and vegetables. Carotenoid biosynthesis (details given in Fig. 1) requires an available source of isoprenoids substrates derived from the MEP pathway: the first committed step in the carotenoids pathway is indeed the conversion of geranylgeranyl diphosphate to phytoene (the first carotenoid) driven by phytoene synthase (PSY). PSY is the most important regulatory enzyme of the MEP pathway, and PSY genes are mostly responsive to abiotic stresses (Cazzonelli, 2011). Then several enzymes and two photoisomerisation processes lead to the production of all-trans-lycopene, which undergoes modifications to produce two different branches, α- and β-carotene respectively. These carotenoids serve as substrates for the synthesis of xanthophylls including lutein, violaxanthin cycle pigments and neoxanthin (Cazzonelli and Pogson, 2010). Carotenoids play diverse biological functions in plants: (1) accessory pigments in photosynthesis, namely energy transfer to chlorophyll (Demming-Adams and Adams, 1996); (2) phyto-hormone precursors (Schwartz *et al*., 2003); (3) modulators of membrane protein function (Ruban and Johnson, 2010) and (4) first line of defence against $^1O_2$ generation through well-known physical-quenching (Havaux and Niyogi, 1999; Triantaphylidès and Havaux, 2009) and recently discovered chemical-quenching mechanisms (Ramel *et al.*, 2012).

Here we focus our discussion on the role of carotenoids, particularly β-carotene and zeaxanthin, as quenchers of $^1O_2$ and membrane stabilizers in the chloroplast, two essential functions during stressful abiotic conditions. The localization of each carotenoids species within the different components of the photosynthetic machinery is highly conserved and it is the basis for their specific functions. β-carotene is bound to reaction centre subunits of both photosystems (PSI and PSII), while the major trimeric light harvesting complex of photosystem II (LHCII) of higher plants binds two luteins (L1 and L2), one violaxanthin/zeaxanthin (V1) and one neoxanthin (N1) (Kühlbrandt *et al.*, 1994; Amunts *et al.*, 2010; Umena *et al.*, 2011).

β-carotene in the core complex quenches $^3Chl^*$ and $^1O_2$ primarily by a physical reaction, in which excess excited state energy from $^1O_2$ is absorbed by β-carotene and then dissipated as heat by the polyene chain (Triantaphylidès and Havaux, 2009). Notably, a concomitant chemical-quenching mechanism involving β-carotene oxidation has been proven not only *in vitro* (Stratton *et al.*, 1993), but also in plant leaves (Ramel *et al.*, 2012a, b) (Fig. 2B). Indeed, Ramel *et al*. (2012a, b) reported the accumulation of a large number of aldehydic oxidation products and of different volatile derivatives originated from β-carotene in *Arabidopsis* plants under photooxidative conditions. The volatile products derived from β-carotene oxidation (mainly β-cyclocitral) are bioactive molecules. They have been proposed as acting as messengers in the $^1O_2$-signalling pathway (retrograde signalling, from chloroplast to nucleus), and hence involved in the reprogramming of gene expression, which leads to stress acclimation (Ramel *et al*., 2012b). Furthermore, β-carotene plays a key role in freezing tolerance (Huner *et al.*, 1984). Indeed, β-carotene, due to the absence of hydroxyl groups on the carbon chain, is homogenously distributed in membranes but without well-defined orientation. Therefore, β-carotene maintains thylakoid membranes in a fluid state during low-temperature stress (Havaux, 1996) (Fig. 2A). In addition to participate in the maintenance of thylakoid membrane fluidity during cold hardening of plants, β-carotene has a crucial role in photoprotection of PSI in low temperature conditions (Cazzaniga *et al.*, 2012).

In addition to β-carotene, thylakoid membrane contains other carotenoids, involved in specialized tasks depending on their structure and their localization (Havaux, 1996). Lutein has specific property of quenching harmful $^3Chl^*$, thus preventing ROS formation (Dall'osto

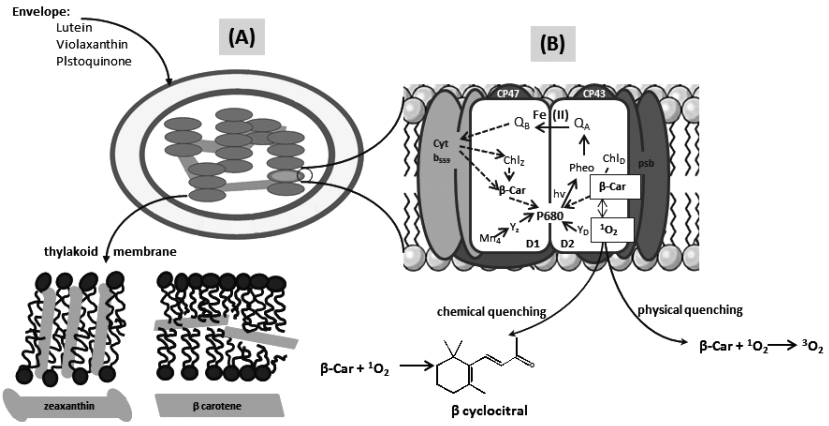

Fig. 2. – (A) Structural model of β-carotene and zeaxanthin in chloroplast membranes: the two carotenoids are oriented differently in thylakoid membranes and thereby zeaxanthin increases membrane rigidity while β-carotene increases membrane fluidity. (B) β-carotene plays different roles in active PSII complexes: an accessory pigment of the photosystem reaction centers and core antenna, an effective quencher of $^1O_2$, acting as a physical quencher as well as a chemical quencher leading to the production of volatile compounds (i.e. β-cyclocitral).

et al., 2006), neoxanthin has protective function of PSII against superoxide anion (O_2^-) diffusion from stroma membranes to grana partitions (Dall'osto *et al.*, 2007), while the VAZ cycle (violaxanthin-antheraxanthin-zeaxanthin cycle) can be seen as an elegant mechanism that rapidly converts within the LHCs, an efficient accessory pigment in weak light (violaxanthin) to an efficient photoprotector in strong light (Z) (Havaux and Niyogi, 1999).

Zeaxanthin has long been recognized as serving functions of primary significance in plants exposed to a wide range of abiotic stresses, including, but not limiting to high solar irradiance. Zeaxanthin plays an antioxidant function on thylakoids lipids through two different both mechanisms and pools of convertible violaxanthin. The fraction of zeaxanthin bound to the antenna components enhances NPQ (non-photochemical quenching) by promoting both a rapidly "flexible" dissipation (qE, rapidly reversible NPQ) and a "sustained" dissipation in long-term (under severe stress) quenching of excited chlorophylls (^{1}Chl*) (Demming-Adams and Adams III, 1996). On the other side, the non-protein-bound pool of zeaxanthin is free in the lipid phase of thylakoids, where it increases membrane rigidity through stabilization of phospholipids layers (Havaux, 1996) (Fig. 2A). Indeed, plant thylakoids have a highly conserved lipid composition

characterized by a conspicuous amount of galactolipids: the outer leaflet of thylakoid is enriched in monogalactosyldiacylglicerol (MGDG, about 60%) while the inner leaflet is enriched in digalactosyldiacylglycerol (DGDG, about 80%) (RAWYLER *et al.*, 1987). This great proportion of highly unsaturated fatty acids confer an extreme fluidity to the thylakoid membrane system, which is essential for the photosynthetic process but, at the same time, constitutes a weak point when active forms of oxygen are inevitably produced during the photosynthetic process. Zeaxanthin, an amphiphilic molecule with two hydroxyl groups on the rings, can increase membranes viscosity. The regular unsaturation of C-C bonds enables zeaxanthin to act as "pillar" in the lipid bilayer, with the long axis of the carotenoid almost perpendicular to the membrane surface, and the two polar heads "fastened" to the head-group regions on both sides of the membrane (RUBAN and JOHNSON, 2010; HAVAUX, 1996) (Fig. 2A). Besides its membrane-stabilizing properties, zeaxanthin also functions as a chemical trap for hydroxyl radicals and $^1O_2$, a distinct mechanism from NPQ that can occur at the protein/lipid interface. Indeed, the *in vivo* antioxidant capacity of zeaxanthin has been shown to be higher than that of all other xanthophylls (HAVAUX *et al*., 2007). HAVAUX (2007) showed that zeaxanthin-deficient mutants of *Arabidopsis* were much less tolerant to oxidative stress than zeaxanthin-overexpressing mutant. Under severe stress conditions, e.g., when plants are concomitantly challenged against water deficit and high solar irradiance, the ratio of VAZ to chlorophyll largely exceeds 100 mmol mol^{-1}. Zeaxanthin is therefore unlikely bound to light-harvesting chlorophyll-protein complexes, and hence its contribution to NPQ is negligible (BECKETT *et al.*, 2012). Zeaxanthin may be indeed synthesized from β-carotene (DU *et al*., 2010) and resides in other parts of thylakoid, increasing rigidity of thylakoid membranes and enhancing their resistance to peroxidative damage under severe drought stress (HAVAUX, 1996; BECKETT *et al.*, 2012). Therefore, the functional roles of carotenoids in plants challenged against multiple stress agents, as typically occurs in the Mediterranean region, have to be re-considered. Each carotenoid may serve more than one function depending on the stress severity. For example, under natural conditions, the predawn to midday enhancement in zeaxanthin concentration is unlikely correlates with predawn to midday enhancement in NPQ, i.e. zeaxanthin-dependent NPQ. Instead, zeaxanthin behaves as a chloroplast antioxidant at midday, when high solar irradiance becomes severe excess light stress for plants and severe excess energy for the chloroplast.

Carotenoids share the same biochemical precursors as volatile "non-essential" isoprenoids, e.g., isoprene. During leaf development, the chloroplastic DMADP pools are preferentially consumed to synthesize "essential" isoprenoids, i.e. carotenoids, while isoprene biosynthesis occurs when leaves have built up a mature photosynthetic system. Indeed, isoprene can play a role in maintaining the MEP pathway active in adult leaves, thereby allowing for rapid synthesis of "essential" isoprenoids when they are needed during a stress event (RASULOV *et al.*, 2013), and lastly in preventing the overflow of chloroplastic DMADP (ROSENTIEL *et al.*, 2002).

This trade-off between 'essential' and 'non-essential' isoprenoid synthesis has been observed not only during leaf development but also during severe drought stress. Since isoprene biosynthesis is steeply depressed under severe stress conditions, and very recent evidence show that some carotenoids, especially zeaxanthin, may complement the action of isoprene (BECKETT *et al.*, 2012) through a well-coordinated mechanism aimed at preserving thylakoid membranes from more severe oxidative damage/disruption (HAVAUX *et al.*, 2007).

ABSCISIC ACID (ABA). – ABA is a ubiquitous phytormone, which regulates plant growth and development under recurring environmental stress conditions such as drought, salinity, and cold (MELCHER *et al.*, 2010). ABA is required to fine-tune growth and development under non-stress conditions, including stomatal aperture and hydraulic conductivity, as well as seed dormancy and fruit abscission (RAGHAVENDRA *et al.*, 2010). Nonetheless, ABA has been termed as "stress hormone" because ABA is an endogenous messenger in plant abiotic stress responses. The onset of ABA signalling begins with ABA biosynthesis which occurs in the cytosol from xanthoxin, a 15 skeleton carbon molecule, which is oxidized to xanthoic acid and undergoes further oxidation and rearrangement to ABA (Fig. 1) (MILBORROW, 2001). ABA can be considered as the end-product of MEP-carotenoids pathway, because xanthoxin is produced in the plastid through the oxidative cleavage of a 9-*cis*-epoxycarotenoid precursor (9-*cis*-violaxanthin or 9'-*cis*-neoxanthin) (TAYLOR *et al.*, 2000). This is the first committed step in the ABA biosynthesis pathway and the rate-limiting step in the *de-novo* ABA synthesis. However, LEE *et al.* (2006) showed that bioactive ABA can be produced also from its conjugated form with glucose (ABA-GE) (Fig. 1), which has long been reported as an inactive pool of ABA (DIETZ *et*

al., 2000). Glucosyltransferases can glucosylate ABA, and ABA-GE accumulates in vacuoles. In response to dehydration stress as well as to day/night conditions, ABA-specific β-glucosidase (AtBG1), localized in the endoplasmic reticulum, could produce free ABA from ABA-GE (Lee *et al.*, 2006).

It is well-known that enhanced biosynthesis of ABA occurs in response to hyperosmotic stresses caused by high salinity and drought, which cause the intracellular redistribution and accumulation of ABA in guard cells. Increased ABA concentration leads to regulation of ion channels, resulting in the efflux of ions, loss of turgor of the guard cells and closure of stomata (Wang and Song, 2008). Although most studies on ABA functions have been conducted using angiosperms, ABA-induced increase in stress tolerance has been reported both in vascular plants and in non-vascular bryophytes. Detection of ABA in mosses, liverworts, and hornworts suggests that acquisition of this mechanism for stress tolerance in vegetative tissues was one of the critical evolutionary events for adaptation of plants to the land (Takezawa *et al.*, 2011). Recent evidence suggests that active stomatal responsiveness to environmental and endogenous cues originated after the evolution of the ferns ~ 360 mya (McAdam and Brodribb, 2012). Indeed, as reported in McAdam and Brodribb (2013), in ferns and lycophytes the augmentation of ABA levels occurs during drought but it is not associated with the closing of stomata. In these plants, the passive stomatal control still provides an efficient way of regulating leaf hydration in response to changes in water availability. These findings are now supported by findings of Pantin *et al.* (2013), who proposed that ABA promotes stomatal closure in a dual way, through the well-known effect on guard cell metabolism as well as through an 'indirect' hydraulic effect, inactivating bundle sheath aquaporins and decreasing water permeability within the leaf vascular tissues. While in angiosperms ABA acts as key modulator of stomatal sensitivity to ambient CO_2 in angiosperm, drought-induced accumulation of foliar ABA does not affect stomata sensitivity to CO_2 in conifers (McAdam *et al.*, 2011). Authors conclude that there is phylogenetic variation in ABA-dependent stomatal sensitivity related to drought and ambient CO_2. Overall, this supports the hypothesis that regulation of stomatal opening unlikely was the primary function served by ABA during the establishment of early plants on the land.

The plethora of ABA responses is initiated by sensing ABA by soluble cytosolic ABA receptor proteins (PYR/PYL proteins) (Kline *et al.*,

2010). The ABA binding to PYR/PYL protein, through the inhibition of the PP2C (Protein Phosphtase 2C), enables the activation of the SnRK2 protein kinases, which are free to phosphorylate downstream components, including NADPH oxidases and transcription factors (Hauser *et al.*, 2011). The activation of NADPH oxidase (NOX) activity leads to H_2O_2 and NO production and ROS mediated signalling. Indeed, changes in ROS homeostasis are pivotal signalling events, which can allow flexible responses to environmental stress events (Tossi *et al.*, 2012), in addition to their role in stomatal closure (Bright *et al*., 2006). Moreover, ABA signalling cascade in response to environmental stresses includes the expression of ABA-responsive genes, by phosphorylating transcription factors (TFs), particularly bZIP TFs belonging to the AREB/ABFs family and MYB TFs (Fujita *et al.*, 2011). In particular, one of the four AREB/ABF proteins, ABF2, is a positive component of glucose signal transduction. Indeed, one of the early effects of drought-induced ABA accumulation includes an increased starch degradation and increased hexoses levels. Furthermore, by increasing the transcripts of several *myb* genes (Denekamp and Smeekens, 2003), ABA may up-regulate the activity of chalcone synthase (CHS) and chalcone isomerase (CHI) (Tossi *et al*., 2009; Perrone *et al.*, 2012) increasing the biosynthesis of phenylpropanoids, particularly flavonols and anthocyanins. Flavonoids represent a more long term adaptive antioxidant response (Agati *et al*., 2013) to abiotic stresses, and together with vacuolar sugars could play a crucial role in oxidative stress defence in the vacuole (Keunen *et al.*, 2013).

Conclusions. – The emerging picture for the functional roles of isoprenoids in plants suffering from severe stress conditions reveals not only the multiplicity of their functions but also that these secondary metabolites constitute a well-coordinated system aimed at countering an excess of radiant energy to the chloroplast. Under conditions that severely depress photosynthesis, e.g., at severe leaf dehydration, the decline in isoprene biosynthesis is paralleled by an increased biosynthesis of zeaxanthin. Zeaxanthin biosynthesis originates not only through de-epoxidation of violaxanthin but additionally from hydroxylation of β-carotene. Chemical features allow zeaxanthin to increase membrane rigidity, thus protecting thylakoid membranes from oxidative damage. Severe drought usually occurs in concomitance with high both solar irradiance and air temperature in the field, thus greatly enhancing the ratio of violaxanthin-cycle pigments to chlorophyll. Therefore, zea-

xanthin likely serve a minor role in nonphotochemical quenching and a major role as a chloroplast antioxidant, replacing the function served by isoprene.

Abscisic acid, the biosynthesis of which steeply increases in response to drought stress as well as from predawn to midday does not serve a direct antioxidant function. Rather, ABA generates H_2O_2 and nitric oxide, which are also essential components in ABA-mediate stomatal opening. However, ABA greatly affects primary and secondary metabolism, enhancing the biosynthesis of hexoses and phenylpropanoids. Phenylpropanoids, which are located in various cells and cellular compartments, are efficient scavenger of ROS. The significance of their ROS-scavenger activities is relevant in plants challenged against severe stress conditions, when the activity of primary antioxidant defenses, i.e. antioxidant enzymes, declines steeply. It may be not a mere coincidence that very same conditions enhance the expression of genes of flavonoid biosynthesis, while down-regulate photosynthetic gene expression and decrease the activities of antioxidant enzymes.

ACKNOWLEDGEMENTS. – Work in the author's laboratory was supported by MIUR, Rome, project PRIN (2010-2011) TreeCity.

REFERENCES

AFFEK H.P. and YAKIR D.: Protection by isoprene against singlet oxygen in leaves. *Plant Physiol. 129*, 269-277 (2002).

AGATI G., BRUNETTI C., DI FERDINANDO M., FERRINI F., POLLASTRI S. and TATTINI M.: Functional roles of flavonoids in photoprotection: New evidence, lessons from the past. *Plant Physiol. Biochem. 72*, 35-45 (2013).

AMUNTS A., TOPORIK H., BOROVIKOVA A. and NELSON N.: Structure determination and improved model of plant photosystem I. *J. Biol. Chem. 285*, 3478-3486 (2010).

BECKETT M., LORETO F., VELIKOVA V., BRUNETTI C., DI FERDINANDO M., TATTINI M. et al.: Photosynthetic limitations and volatile and non-volatile isoprenoids in the poikilochlorophyllous resurrection plant *Xerophyta humilis* during dehydration and rehydration. *Plant Cell Environ. 35*, 2061-2074 (2012).

BEHNKE K., EHLTING B., TEUBER M., BAUERFEIND M., LOUIS S., HÄNSCH R. et al.: Transgenic, non-isoprene emitting poplars don't like it hot. *Plant J. 51*, 485-499 (2007).

BRIGHT J., DESIKAN R., HANCOCK J.T., WEIR I.S. and NEILL S.J.: ABA-induced NP generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. *Plant J. 45*, 113-122 (2006).

BRILLI F., BARTA C., FORTUNATI A., LERDAU M., LORETO F. and CENTRITTO M.: Response of isoprene emission and carbon metabolism to drought in white poplar (*Populus alba*) saplings. *New Phytol. 175*, 244-254 (2007).

BRÜGGEMANN N. and SCHNITZLER J.P.: Comparison of isoprene emission, intercellularisoprene concentration and photosynthetic performance in water-limited oak (*Quercus pubescens* Willd. and *Quercus robur* L.) saplings. *Plant Biol. 4*, 456-463 (2002).

CAZZANIGA S., LI Z., NIYOGI K.K., BASSI R. and DALL'OSTO L.: The Arabidopsis *szl1* mutant reveals a critical role of β-carotene in photosystem I photoprotection. *Plant Physiol. 159*, 1745-1758 (2012).

CAZZONELLI C.I. and POGSON B.J.: Source to sink: regulation of carotenoid biosynthesis in plants. *Trends Plant Sci. 15*, 266-274 (2010).

CAZZONELLI C.I.: Carotenoids in nature: insights from plants and beyond. *Funct. Plant Biol. 38*, 833-847 (2011).

CHAVES M.M., FLEXAS J. and PINHEIRO C.: Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann. Bot. 103*, 551-560 (2009).

Copolovici L.O. and Niinemets U.: Temperature dependencies of Henry's law constants and octanol/water partition coefficients for key plant volatile monoterpenoids. *Chemosphere 61*, 1390-1400 (2005).
Dall'osto L., Lico C., Alric J., Giuliano G., Havaux M. and Bassi R.: Lutein is needed for efficient chlorophyll triplet quenching in the major LHCII antenna complex of higher plants and effective photoprotection *in vivo* under strong light. *BMC Plant Biol. 6*, 32 (2006).
Dall'osto L., Cazzaniga S., North H., Marion-Poll A. and Bassi R.: The *Arabidopsis aba4-1* mutant reveals a specific function for neoxanthin in protection against photooxidative stress. *Plant Cell 19*, 1048-1064 (2007).
Danekamp M. and Smeekens S.C.: Integration of wounding and osmotic stress signals determines the expression of the AtMYB102 transcription factor gene. *Plant Physiol. 132*, 1415-1423 (2003).
Delwiche C.F. and Sharkey T.D.: Rapid appearance of 13C in biogenic isoprene when $^{13}CO_2$ is fed to intact leaves. *Plant Cell Environ. 16*, 587-591 (1993).
Demming-Adams B. and Adams III W.W.: The role of xanthophyll cycle carotenoids in the photoprotection of photosynthesis. *Trends Plant Sci 1*, 21-26 (1996).
Dietz J-K., Sauter A., Wichert K., Messdaghi D. and Hartung W.: Extracellular β-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. *J. Exp. Bot. 51*, 937-944 (2000).
Du H., Wang N., Cui F., Li X., Xiao J. and Xiong L.: Characterization of the β-carotene hydroxylase gene DSM2 conferring drought and oxidative stress resistance by increasing xanthophylls and abscisic acid synthesis in rice. *Plant Physiol. 154*, 1304-1318 (2010).
Foyer C.H. and Shigeoka S.: Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiol. 155*, 93-100 (2011).
Fujita Y., Fujita M., Shinozaki K. and Yamaguchi-Shinozaki K.: ABA-mediated transcriptional regulation in response to osmotic stress in plants. *J Plant Res 124*, 509-525 (2011).
Halliwell B.: The wanderings of a free radical. *Free Radical Biol. Med. 46*, 531-542 (2009).
Hauser F., Waadt R. and Schroeder J.I.: Evolution of abscisic acid synthesis and signaling mechanisms. *Curr. Biol. 21*, 346-355 (2011).
Havaux M.: Carotenoids as membrane stabilizers in chloroplasts. *Trends Plant Sci. 3*, 147-151 (1996).
Havaux M. and Niyogi K.K.: The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. *Proc. Natl. Acad. Sci. USA 96*, 8762-8767 (1999).
Havaux M., Dall'osto L. and Bassi R.: Zeaxanthin has enhanced antioxidant capacity with respect to all other xanthophylls in *Arabidopsis* leaves and functions independent of binding to PSII antennae. *Plant Physiol. 145*, 1506-1520 (2007).
Hernandez I. and Van Breusegem F.: Opinion on the possible role of flavonoids as energy escape valves: novel tools for nature's Swiss army knife? *Plant Sci 179*, 297-301 (2010).
Huner N.P.A., Elfman B., Krol M. and Mcintosh A.: Growth and development at cold-hardening temperatures. Chloroplast ultrastructure, pigment content, and composition. *Can. J. Bot. 62*, 53-60 (1984).
Keunen E., Peshev D., Vangronsveld J., Van Den Ende W. and Cuypers A.: Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. *Plant Cell Environ. 36*, 1242-1255 (2013).
Kliebenstein D.J.: Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. *Plant Cell Environ. 27*, 675-684 (2004).
Kline K.J., Sussman M.R. and Jones A.M.: Abscisic acid receptors. *Plant Physiol. 154*, 479-482 (2010).
Kühlbrandt W.: Structure and function of the plant light-harvesting complex, LHC-II. *Curr. Opin. Struc. Biol. 4*, 519-528 (1994).
Lange B.M., Rujan T., Martin W. and Croteau R.: Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *Proc. Natl. Acad. Sci. USA 97*, 13172-13177 (2000).
Lee K., Piao H.L., Kim H-Y., Choi S.M., Jiang F., Hartung W. et al.: Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Plant Cell. 126*, 1109-1120 (2006).
Li Z., Wakao S., Fischer B.B. and Niyogi K.K.: Sensing and responding to excess light. *Ann. Rev. Plant Biol. 60*, 239-260 (2009).
Lichtenthaler H.K., Rohmer M. and Schwender J.: Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. *Physiol. Plant. 101*, 643-652 (1997).
Lichtenthaler H.K.: Biosynthesis, accumulation and emission of carotenoids, α-tocopherol, plastoquinone, and isoprene in leaves under high photosynthetic irradiance. *Photosynth. Res. 92*, 163-179 (2007).
Lombard J. and Moreira D.: Origins and early evolution of the mevalonate pathway of isoprenoid biosynthesis in the three domains of life. *Mol. Biol. Evol. 28*, 87-99 (2011).
Loreto F. and Velikova V.: Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiol. 127*, 1781-1787 (2001).
Loreto F., Mannozzi M., Maris C., Nascetti P., Ferranti F. and Pasqualini S.: Ozone quenching properties of isoprene and its antioxidant role in leaves. *Plant Physiol. 126*, 993-1000 (2001).
Loreto F. and Schnitzler J.P.: Abiotic stresses induced BVOCs. *Trends Plant Sci. 15*, 154-166 (2010).

Loreto F., Bagnoli F., Calfapietra C., Cafasso D., De Lillis M., Filibeck G. et al.: Isoprenoid emission in hygrophyte and xerophyte European woody flora: ecological and evolutionary implications. *Global Ecol. Biogeogr*. DOI: 10.1111/geb.12124 (2013).

McAdams S.A.M. Brodribb T.J., Ross J.J. and Jordan G.J.: Augmentation of abscisic acid (ABA) levels by drought does not induce short-term stomatal sensitivity to CO_2 in two divergent conifer species. *J. Exp. Bot. 62*, 195-203 (2011).

McAdam S.A.M. and Brodribb T.J.: Fern and lycophyte guard cells do not respond to endogenous abscisic acid. *Plant Cell 24*, 1510-1521 (2012).

McAdam S.A.M. and Brodribb T.J.: Ancestral stomatal control results in a canalization of fern and lycophyte adaptation to drought. *New Phytol. 198*, 429-441 (2013).

Melcher K., Zhou X.E. and Xu H.E.: Thirsty plants and beyond: structural mechanisms of abscisic acid perception and signaling. *Curr. Opin. Struc. Biol. 20*, 722-729 (2010).

Millborrow B.V.: The pathway of biosynthesis of abscisic acid in vascular plants: a review of the present state of knowledge of ABA biosynthesis. *J. Exp. Bot*. 52, 1145-1164 (2001).

Mittler R., Vanderauwera S., Gollery M. and Van Breusegem F.: Reactive oxygen gene network of plants. *Trends Plant Sci. 9*, 490-498 (2004).

Pantin F., Monnet F., Jannaud D., Costa J.M., Renaud J., Muller B. et al.: The dual effect of abscisic acid on stomata. *New Phytol. 197*, 65-72 (2013).

Perrone I., Pagliarani C., Lovisolo C., Chitarra W., Roman F. and Schubert A.: Recovery from water stress affects grape leaf petiole transcriptome. *Planta 235*, 1383-1396 (2012).

Pulido P., Perello C. and Rodriguez-Concepcion M.: New insights into plant isoprenoid metabolism. *Mol. Plant 5*, 964-967 (2012).

Raghavendra A.S., Gonugunta V.K., Christmann A. and Grill E.: ABA perception and signalling. *Trends Plant Sci. 15*, 395-401 (2010).

Ramel F., Birtic S., Cuiné S., Triantaphylidès C., Ravanat J.L. and Havaux M.: Chemical quenching of singlet oxygen by carotenoids in plants. *Plant Physiol. 158*, 1267-1278 (2012a).

Ramel F., Birtic S., Ginies C., Soubigou-Taconnat L., Triantaphylidès C. and Havaux M.: Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. *Proc. Natl. Acad. Sci. USA 109*, 5535-5540 (2012b).

Rasulov B., Bichele I., Laisk A. and Niinemets Ü.: Competition between isoprene emission and pigment synthesis during leaf development in aspen. *Plant Cell Environ.* DOI: 10.1111/pce.12190 (2013).

Rawyler A., Unitt M., Giroud C., Davies H., Mayor J-P., Harwood J.L. et al.: The transmembrane distribution of galactolipids in chloroplast thylakoids is universal in a wide variety of temperate climate plants. *Photosynth. Res. 11*, 3-13 (1987).

Rodríguez Concepción M.: Supply of precursors for carotenoids biosynthesis in plants. *Arch. Biochem. Biophys. 504*, 118-122 (2010).

Rohmer M., Seemann M., Horbach S., Bringer-Meyer S. and Sahm H.: Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. *J. Am. Chem. Soc. 118*, 2564-2566 (1996).

Rosenstiel T.N., Fisher A.J., Fall R. and Monson R.K.: Differential accumulation of dimethylallyl diphosphate in leaves and needles of isoprene and methylbutenol-emitting and non-emitting species. *Plant Physiol. 129*, 1276-1284 (2002).

Ruban A.V. and Johnson M.P.: Xanthophylls as modulators of membrane protein function. *Arch. Biochem. Biophys. 504*, 78-85 (2010).

Schnitzler J.P., Graus M., Kreuzwieser J., Heizmann U., Rennenberg H. and Wisthaler A.: Contribution of different carbon sources to isoprene biosynthesis in poplar leaves. *Plant Physiol. 135*, 152-160 (2004).

Schwartz S.H., Tan B.C., McCarty D.R., Welch W. and Zeevaart J.A.D.: Substrate specificity and kinetics for VP14, a carotenoid cleavage dioxygenase in the ABA biosynthetic pathway. *BBA 1619*, 9-14 (2003).

Sharkey T.D. and Loreto F.: Water stress, temperature, and light effects on the capacity for isoprene emission and photosynthesis of kudzu leaves. *Oecologia 95*, 328-333 (1993).

Sharkey T.D. and Singsaas E.L.: Why plants emit isoprene. *Nature 374*, 769-769 (1995).

Sharkey T.D. and Yeh S.: Isoprene emission from plants. *Annu. Rev. Plant Physiol. 52*, 407-436 (2001).

Sharkey T.D., Yeh S., Wiberley A.E., Fabel T.G., Gong D. and Fernandez D.E.: Evolution of the isoprene biosynthetic pathway in kudzu. *Plant Physiol. 137*, 700-712 (2005).

Sharkey T.M., Wiberley A.E. and Donohue A.R.: Isoprene emission from plants: why and how. *Ann. Bot. 101*, 5-18 (2008).

Singaas E.L., Lerdau M., Winter K. and Sharkey T.D.: Isoprene increases thermotolerance of isoprene-emitting species. *Plant Physiol. 115*, 1413-1420 (1997).

Stratton S.P., Schaefer W.H. and Liebler D.C.: Isolation and identification of singlet oxygen oxidation products of β-carotene. *Chem. Res. Toxicol. 6*, 542-547 (1993).

Takezawa D., Koomatsu K. and Sakata Y.: ABA in bryophytes: how a universal growth regulator in life became a plant hormone? *J. Plant Res. 124*, 437-453 (2011).

TAYLOR I.B., BURBIDGE A. and THOMPSON A.J.: Control of abscisic acid synthesis. *J. Exp. Bot. 51*, 1563-1574 (2000).

TOSSI V., LAMATTINA L. and CASSIA R.: An increase in the concentration of abscisic acid is critical for nitric oxide-mediated plant adaptive responses to UV-B irradiation. *New Phytol. 181*, 871-879 (2009).

TOSSI V., CASSIA R., BRUZZONE S., ZOCCHI E. and LAMATTINA L.: ABA says no to UV-B: a universal response? *Trends Plant Sci. 17*, 510-517 (2012).

TRIANTAPHYLIDÈS C. and HAVAUX M.: Singlet oxygen in plants: production, detoxification and signaling. *Trends Plant Sci. 14*, 219-227 (2009).

UMENA Y., KAWAKAMI K., SHEN J.-R. and KAMIYA N.: Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. Nature 473, 55-60 (2011).

VELIKOVA V., TSONEV T., PINELLI P., ALESSIO G.A. and LORETO F.: Localized O_3-fumigation for field-studies of the impact of different ozone levels on photosynthesis, respiration, electron transport rate and isoprene emission in Mediterranean oak species. *Tree Physiol. 157*, 905-916 (2005).

VELIKOVA V., VARKONYI Z., SZABÒ M., MASLENKOVA L., NOGUES I., KOVACS L. et al.: Increased thermostability of thylakoid membranes in isoprene-emitting leaves probed with three biophysical techniques. *Plant Pysiol. 157*, 905-916 (2011).

VICKERS C.E., GERSHENZON J., LERDAU M.T. and LORETO F.: A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nat. Chem. Biol. 5*, 283-291 (2009).

VRANOVÁ E., COMAN D. and GRUISSEM W.: Network analysis of the MVA and MEP pathways for isoprenoid synthesis. *Annu. Rev. Plant Biol. 64*, 665-700 (2013).

WANG P. and SONG C.-P.: Guard-cell signalling for hydrogen peroxide and abscisic acid. *New Phytol. 178*, 703-718 (2008).

WOLFERTZ M., SHARKEY T.D., BOLAND W., KÜHNEMANN F., YEH S. and WEISE S.E.: Biochemical regulation of isoprene emission. *Plant Cell Environ. 26*, 1357-1364 (2003).