

Chapter 2

ABA Biosynthetic and Catabolic Pathways

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Abstract Absciscic acid (ABA) is a phytohormone that regulates physiological processes such as seed maturation, seed dormancy, and stress adaptation. These physiological responses are triggered by the fluctuation of endogenous ABA levels in accordance with changing surroundings or developmental stimuli. Endogenous ABA levels are largely controlled by the balance between biosynthesis and catabolism. ABA is also synthesized in various kinds of organisms other than plants. To manipulate ABA levels, we first need to understand the pathways for ABA biosynthesis and catabolism in each organism. The biosynthetic pathway has been extensively studied in plants and phytopathogenic fungi. The catabolic pathway has been mostly established in plants. Extensive investigations of mutants defective in ABA metabolism using biochemical, molecular genetic, and genomic approaches have helped to reveal the main framework of these pathways. This chapter reviews our current understanding of the pathways of ABA biosynthesis and catabolism. In addition, inhibitors of ABA biosynthesis and catabolism are introduced. These inhibitors can be used to manipulate endogenous ABA levels and are useful tools to investigate ABA action in plants.

Keywords ABA • Biosynthesis • Catabolism

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2.1 ABA Biosynthesis—Two Distinct Routes of ABA Biosynthesis

Absciscic acid (ABA) is a C₁₅ sesquiterpene containing fifteen carbon atoms in its structure that originates from isoprene known as isopentenyl pyrophosphate (IPP). Now, ABA is known to be synthesized via two distinct pathways (Nambara and Marion-Poll 2005; Oritani and Kiyota 2003; Schwartz and Zeevaart 2010). One is the direct pathway that occurs in phytopathogenic fungi. The other is the indirect pathway that operates in plants. IPP for the direct pathway is synthesized from the mevalonate (MVA) pathway that exists in prokaryotes and almost all eukaryotes (Newman and Chappell 1999). On the other hand, the indirect pathway uses the methylerythritol phosphate (MEP) pathway as a source of IPP. The MEP pathway seems to exist in cyanobacteria and all photosynthetic eukaryotes (Lichtenthaler 1999).

2.2 Direct Pathway in Fungi

In phytopathogenic fungi such as *Botrytis cinerea* and *Cercospora cruenta*, ABA is synthesized from MVA. When radiolabeled MVA or farnesyl diphosphate (FDP) was fed to ABA-producing fungi, the labels were effectively incorporated into ABA (Neill et al. 1982; Norman et al. 1983). In addition, when [1-¹³C] glucose was fed to ABA-producing fungi or plants, the positions of the labeled carbons in ABA were different between plants and fungi, because the labeled carbon was differently incorporated into IPP depending on its source, the MVA or MEP pathway (Hirai et al. 2000). These results strongly indicate that fungal ABA is synthesized from IPP produced in the MVA pathway (Hirai et al. 2000). Since all intermediates between FDP and ABA are sesquiterpenes, the ABA biosynthetic pathway in fungi has been referred to as the direct pathway (Nambara and Marion-Poll 2005; Oritani and Kiyota 2003; Zeevaart and Creelman 1988).

The direct pathway involves several modifications of FDP to generate ABA (Fig. 2.1). Isomers of ionylideneethanol and/or ionylideneacetic acid have been identified from several fungi and are supposed to be the endogenous precursors of ABA in fungi (Oritani and Kiyota 2003). As shown in Fig. 2.1, similar intermediates are utilized among diverse fungal genera, suggesting that the ABA biosynthetic pathway might be mostly similar but distinct among these fungi. Recently, allofarnesenes and ionylideneethanes were shown to be the endogenous precursors in *Botrytis cinerea* and *Cercospora cruenta* (Inomata et al. 2004a, b). The allofarnesenes were supposed to be converted from FDP to allofarnesenes via 4,5-didehydrofarnesyl diphosphate or 6*E*,10*E*-2,6,10-trimethyl-2,6,10-dodecatriene (Inomata et al. 2004a, b). Then, cyclization and isomerization of allofarnesenes give ionylideneethanes (Fig. 2.1) (Inomata et al. 2004a, b). In addition, possible carotenoid precursors having a γ -ring to produce ionylideneacetaldehyde were not detected in *C. cruenta* (Inomata et al. 2004b). These results

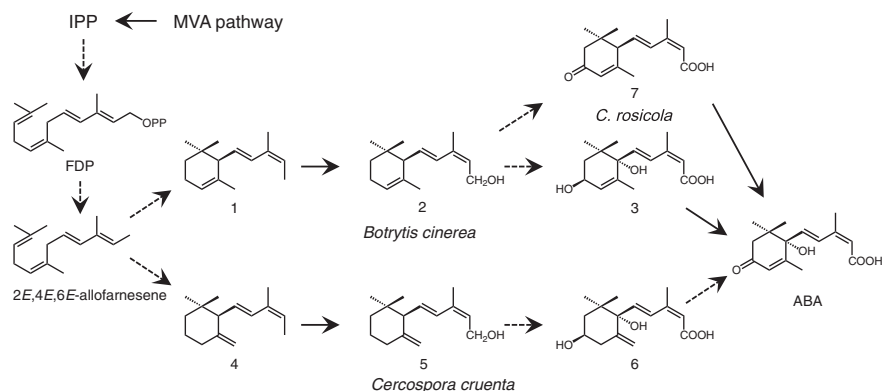


Fig. 2.1 Proposed biosynthetic pathways of ABA in fungi. The direct pathway starts with FDP produced from IPP via the MVA pathway. *Solid arrows* indicate one-step modification of an intermediate and *dashed arrows* represent multistep modifications of an intermediate. Abbreviations: *IPP* isopentenyl pyrophosphate, *FDP* Farnesyl diphosphate, *1* 2Z,4E- α -ionylideneethane, *2* 2Z,4E- α -ionylideneethanol, *3* 1',4'-*t*-diol-ABA, *4* 2Z,4E- γ -ionylideneethane, *5* 2Z,4E- γ -ionylideneethanol, *6* 2Z,4E-1',4'-Dihydroxy- γ -ionylideneacetic acid, *7* 1'-deoxy ABA

indicate that ABA is synthesized by the direct pathway via the isomerization and cyclization of allofarnesene and several oxidation steps of ionylideneethanes in these fungi (Fig. 2.1). After the synthesis of ionylideneethanes, ionylideneethanol is supposed to be generated by their oxidation (Inomata et al. 2004a, b). In *C. rosicola*, α -ionylideneethanol and α -ionylideneacetic acid were converted to ABA and 1'-deoxy ABA (Fig. 2.1) (Neill and Horgan 1983). 1'-deoxy ABA is thought to be the precursor of ABA in this fungus, because it is also oxidized to ABA (Fig. 2.1) (Neill et al. 1982). On the one hand, in *B. cinerea* and *C. pinidensiflorae*, 1',4'-trans-diol ABA is likely the predominant precursor whose endogenous levels are correlated with ABA synthesis (Fig. 2.1) (Hirai et al. 1986; Okamoto et al. 1988). On the other hand, 1',4'-trans-dihydro- γ -ionylideneacetic acid is thought to be the intermediate of ABA biosynthesis in *C. cruenta* (Fig. 2.1) (Oritani et al. 1985). During the conversion from ionylideneethanes to ABA, atmospheric oxygen is incorporated into ABA at C-1, -1, C-1', and C-4' in *C. cruenta* and *B. cinerea* (Inomata et al. 2004a, b). P450 was supposed to be involved in these oxidation steps since P450 inhibitors could effectively block ABA production (Norman et al. 1986). Based on this notion, Siewers et al. (2004) tried to knock out two genes in *Botrytis cinerea*, *BcCPR1* and *BcABA1*, encoding P450 oxidoreductase and P450, respectively. The ABA level in the *Bccpr1* mutant was reduced compared with the wild type. ABA production was completely abolished in the *Bcabal* mutant (Siewers et al. 2004). The same group also found three additional genes located around the *BcABA1* gene on the genome (Siewers et al. 2006). These genes were named *BcABA2*, *BcABA3*, and *BcABA4* and encoded P450, an unknown protein and a short-chain dehydrogenase/reductase, respectively (Siewers et al. 2006). ABA was undetectable in the *Bcaba3* mutant but not in

Bcaba2 and *Bcaba4*. The authors speculated that the *Bcaba2* and *Bcaba4* mutants likely accumulated ABA precursors that could not be distinguished from ABA by their immunological ABA detection system (Siewers et al. 2006). Biochemical analysis of these proteins or feeding experiments combining several known precursors with these mutants will be powerful tools to investigate the direct pathway in detail.

2.3 Indirect Pathway in Higher Plants

ABA was discovered in the 1960s (Addicott et al. 1968; Ohkuma et al. 1965; Schwartz and Zeevaart 2010) and subsequently xanthoxin (Xan) was isolated as a plant growth inhibitor like ABA (Taylor and Burden 1970b; Taylor and Smith 1967). Xan had structural similarity to part of an epoxycarotenoid (Fig. 2.2). Indeed, the cleavage of all-*trans*- or 9-*cis*-epoxycarotenoids by light or lipoxygenase successfully generated 2-*trans*,4-*trans*- or 2-*cis*,4-*trans*-Xan (Firn and Friend 1972; Taylor and Burden 1970a). A conversion experiment from Xan to ABA using cell-free extracts from various plant species indicated that 2-*cis*,4-*trans*-Xan was the possible precursor of ABA that originates from 9-*cis*-epoxycarotenoids (Sindhu and Walton 1987). In addition, most carotenoid deficient mutants were also ABA deficient (Moore and Smith 1985; Neill et al. 1986). These facts suggested that epoxycarotenoids could be the precursors of ABA in higher plants. On the other hand, terpenoids such as carotenoids, plastoquinone, sterol, and phytol are synthesized from IPP produced in the MEP pathway in plastids (Lichtenthaler 1999). The *Arabidopsis chloroplasts altered 1 (cla1)* mutant has a defect in the synthesis of 1-deoxy-D-xylulose-5-phosphate in the MEP pathway and presents carotenoid deficiency resulting in decreased levels of ABA (Estevez et al. 2001). These facts support that C15 ABA is synthesized via C40 epoxycarotenoids that are composed of IPP from the MEP pathway in plants. Therefore, this ABA biosynthetic pathway is referred to as the indirect pathway since C40 epoxycarotenoids are the intermediates of ABA in contrast to the direct pathway in fungi (Figs. 2.1 and 2.2). The indirect pathway has been revealed by biochemical and molecular genetic studies of ABA-deficient mutants as described below. Mutants impaired in carotenoid biosynthesis or molybdenum cofactor (MoCo) synthesis present ABA deficiency as part of a pleiotropic phenotype. We will not deal with these biosynthetic pathways or their corresponding mutants in this chapter since they are not specifically involved in ABA biosynthesis. The epoxidation steps of zeaxanthin are set as the starting point of the indirect pathway.

Zeaxanthin epoxidase (ZEP) converts zeaxanthin into violaxanthin via antheraxanthin by a two-step epoxidation (Fig. 2.2). Zeaxanthin can be also produced from violaxanthin by violaxanthin de-epoxidase (VDE) (Fig. 2.2). This cyclic reaction is called the xanthophyll cycle and is involved in nonphotochemical quenching for photoprotection (Li et al. 2009). The *ZEP* gene was first identified in the study of a tobacco ABA-deficient mutant, *Npaba2*, in *Nicotiana*

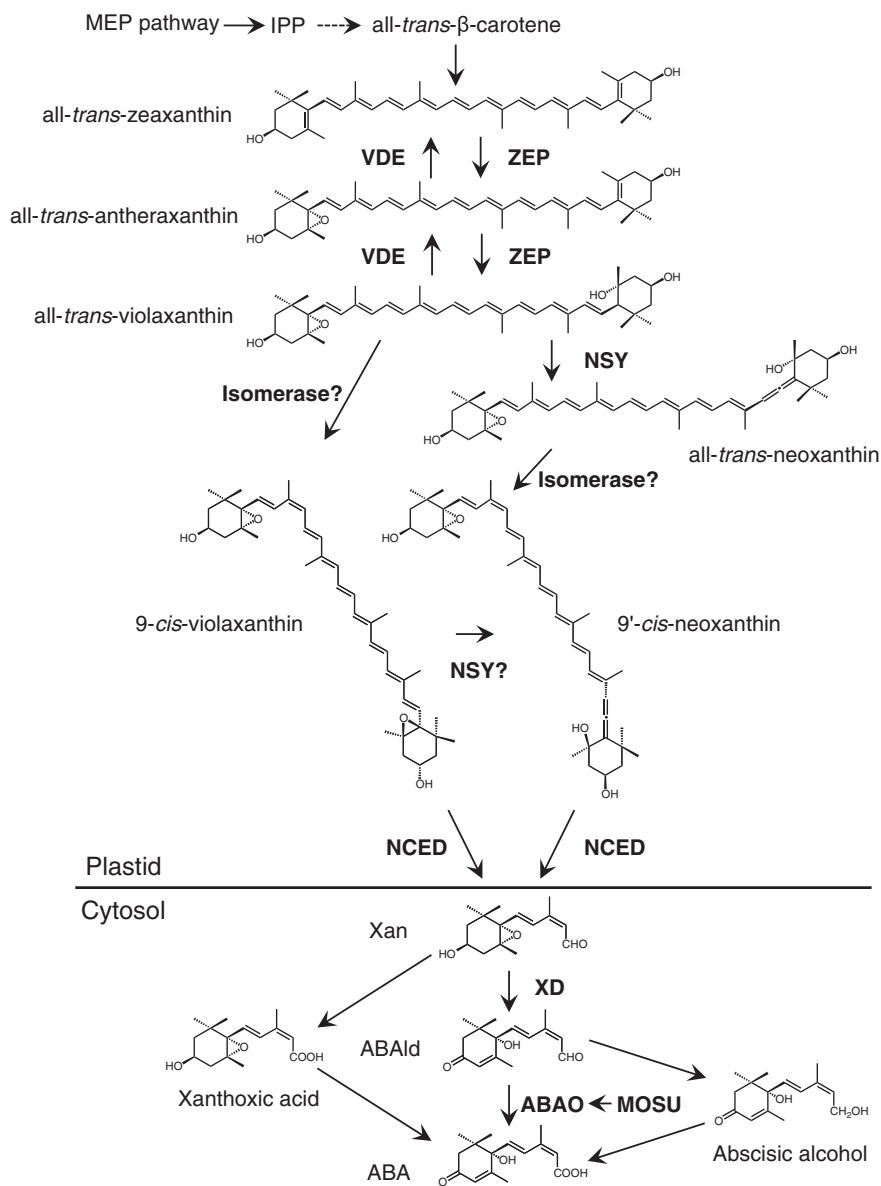


Fig. 2.2 ABA biosynthetic pathway in higher plants. The ABA precursor, a C40 carotenoid, is synthesized from IPP originating from the MEP pathway. *Solid arrows* indicate one-step modification of an intermediate and *dashed arrows* represent multistep modifications of an intermediate. Enzyme names are given in **bold**. Abbreviations: *IPP* isopentenyl pyrophosphate, *ZEP* zeaxanthin epoxidase, *VDE* violaxanthin de-epoxidase, *NSY* neoxanthin synthase, *NCED* 9-cis-epoxycarotenoid dioxygenase, *XD* xanthoxin dehydrogenase, *ABAO* abscisic aldehyde oxidase, *MOSU* MoCo sulfuryase, *Xan* xanthoxin, *ABAld* abscisic aldehyde

plumbaginifolia (Marin et al. 1996). The *ZEP* gene encodes a monooxygenase with a FAD binding domain. Recombinant *ZEP* protein required additives existing in the stroma fraction for its conversion activity from zeaxanthin to violaxanthin (Marin et al. 1996). Then, it was revealed that the activity of *ZEP* depended on a reduced ferredoxin (Bouvier et al. 1996). Tobacco *Npaba2*, *Arabidopsis Ataba1*, rice *Osaba1*, and tomato *hp3* mutants impaired in *ZEP* genes show wilted and non-dormant seed phenotypes, and accumulate high levels of zeaxanthin but not epoxycarotenoids such as violaxanthin and neoxanthin (Table 2.1) (Agrawal et al. 2001; Duckham et al. 1991; Galpaz et al. 2008; Marin et al. 1996; Rock and Zeevaert 1991). *VDE* may not be responsible for ABA biosynthesis, as the *Arabidopsis npq1* mutant defective in the *VDE* gene accumulated significant amounts of epoxycarotenoids such as violaxanthin and neoxanthin that are precursors of ABA (Niyogi et al. 1998).

After the epoxidation step, all-*trans*-violaxanthin is converted to the 9-*cis* isomer prior to oxidative cleavage of the epoxycarotenoids to form xanthoxin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Fig. 2.2). There are two possible substrates for NCED in plants, the 9-*cis* isomers of violaxanthin and neoxanthin (Fig. 2.2). In *Arabidopsis* leaves, all-*trans*-violaxanthin and 9'-*cis*-neoxanthin were found to be the two major epoxycarotenoids (40.2 and 51.8 %, respectively) of the total epoxyxanthophyll content. All-*trans*-neoxanthin and 9-*cis*-violaxanthin represented only 5.4 and 2.6 %, respectively (North et al. 2007). In tomato leaves, a similar composition of epoxyxanthophylls was observed (Parry and Horgan 1991). Therefore, 9'-*cis*-neoxanthin was supposed to be the major substrate of NCED in leaves. Two enzymatic steps are thought to be involved in the synthesis of 9'-*cis*-neoxanthin from all-*trans*-violaxanthin, involving neoxanthin synthase (NSY) and an unknown isomerase (Fig. 2.2). Three different types of NSY have been reported to date (Table 2.1). The first NSYs were biochemically isolated from tomato and potato as homologs of capsanthin capsorubin synthase or lycopene β -cyclase (Al-Babili et al. 2000; Bouvier et al. 2000). The tomato NSY enzyme was then found to be identical to the *B* gene encoding a fruit-specific lycopene β -cyclase isoform (Ronen et al. 2000), suggesting that NSY might be capable of converting both lycopene to β -carotene and violaxanthin to neoxanthin in tomato. However, neoxanthin was still produced in the *old-gold* mutant, a loss of function allele of the *B* gene (Hirschberg 2001; Ronen et al. 2000). Other genes responsible for neoxanthin synthesis were identified in the *Arabidopsis aba4* and tomato *neoxanthin-deficient 1 (nxd1)* mutants lacking both isomers of neoxanthin (Neuman et al. 2014; North et al. 2007). The mutated genes in each mutant encoded different types of unknown proteins. The *Arabidopsis aba4* mutant had a defect in a gene encoding a functionally unknown chloroplastic protein with four transmembrane domains. The loss of neoxanthin in the mutant resulted in reduced levels of ABA, and the phenotype was obvious under dehydration conditions. The ABA-deficient phenotypes of the *aba4* mutant were milder than those of *aba1* since *aba4* was able to produce a small amount of ABA. The existence of 9-*cis*-violaxanthin in the *aba4* mutant could account for the mild phenotypes (North et al. 2007). Biochemical analysis of recombinant ABA4 protein had

Table 2.1 Characterized enzyme list for ABA biosynthesis and catabolism

	Gene name	Species	Gene ID	References
<i>ABA biosynthetic enzyme</i>				
Zeaxanthin epoxidase	<i>NpABA2</i>	<i>Nicotiana plumbaginifolia</i>	X95732	Marin et al. (1996)
	<i>AtABA1/LOS6</i>	<i>Arabidopsis thaliana</i>	At5g67030	Duckham et al. (1991), Xiong et al. (2002)
	<i>HP3</i>	<i>Solanum lycopersicum</i>	EU004202	Galpaz et al. (2008)
	<i>OsABA1</i>	<i>Oryza sativa</i>	Os04g0448900	Agrawal et al. (2001)
	<i>VuABA1</i>	<i>Vigna unguiculata</i>	AB030295	Iuchi et al. (2000)
Neoxanthin synthase (1)	<i>NSY</i>	<i>Solanum lycopersicum</i>	Y18297	Bouvier et al. (2000), Ronen et al. (2000)
	<i>NXS</i>	<i>Solanum tuberosum</i>	At272136	Al-Babili et al. (2000)
Neoxanthin synthase (2)	<i>AtABA4</i>	<i>Arabidopsis thaliana</i>	At1g67080	North et al. (2007)
Neoxanthin synthase (3)	<i>Nxd1</i>	<i>Solanum lycopersicum</i>	Solyc12g041880	Neuman et al. (2014)
	<i>AtNxd1</i>	<i>Arabidopsis thaliana</i>	At1g28100	Neuman et al. (2014)
9- <i>cis</i> -epoxycarotenoid dioxygenase	<i>VP14</i>	<i>Zea mays</i>	U95953	Schwartz et al. (1997b)
	<i>NOTABILIS</i>	<i>Solanum lycopersicum</i>	Z97215	Burbidge et al. (1999)
	<i>PvNCED1</i>	<i>Phaseolus vulgaris</i>	AF190462	Qin and Zeevaart (1999)
	<i>PuNCED1</i>	<i>Persea americana</i>	AF224672	Chernys and Zeevaart (2000)
	<i>PuNCED3</i>	<i>Persea americana</i>	AF224671	Chernys and Zeevaart (2000)
	<i>VuNCED1</i>	<i>Vigna unguiculata</i>	AB030293	Iuchi et al. (2000)
	<i>AtNCED2</i>	<i>Arabidopsis thaliana</i>	At4g18350	Iuchi et al. (2001)
	<i>AtNCED3</i>	<i>Arabidopsis thaliana</i>	At3g14440	Iuchi et al. (2001)
	<i>AtNCED5</i>	<i>Arabidopsis thaliana</i>	At1g30100	Iuchi et al. (2001), Toh et al. (2008)
	<i>AtNCED6</i>	<i>Arabidopsis thaliana</i>	At3g24220	Iuchi et al. (2001)
	<i>AtNCED9</i>	<i>Arabidopsis thaliana</i>	At1g78390	Iuchi et al. (2001)
	<i>CrNCED1</i>	<i>Cuscuta reflexa</i>	AY974807	Qin et al. (2008)
	<i>CrNCED2</i>	<i>Cuscuta reflexa</i>	AY974808	Qin et al. (2008)

(continued)

Table 2.1 (continued)

	Gene name	Species	Gene ID	References
Xanthoxin dehydrogenase	AtABA2	<i>Arabidopsis thaliana</i>	At1g52340	Cheng et al. (2002), Gonzalez-Guzman et al. (2002)
	AAO3	<i>Arabidopsis thaliana</i>	At2g27150	Sekimoto et al. (1998), Seo et al. (2000b)
	SITIENS	<i>Solanum lycopersicum</i>	HQ317906	Harrison et al. (2011)
	PsAO3	<i>Pisum sativum</i>	EF491600	Zdunek-Zastocka and Sobczak (2013)
MoCo sulfatase	AtABA3/LOS5	<i>Arabidopsis thaliana</i>	At1g16540	Bittner et al. (2001), Xiong et al. (2001)
	FLACCA	<i>Solanum lycopersicum</i>	Soly007g066480	Sagi et al. (2002)
	NpABA1/CKR1	<i>Nicotiana plumbaginifolia</i>		Leydecker et al. (1995), Rousselin et al. (1992)
<i>ABA catabolic enzyme</i>				
ABA 8'-hydroxylase	AtCYP707A1	<i>Arabidopsis thaliana</i>	At4g19230	Kushiro et al. (2004), Saito et al. (2004)
	AtCYP707A2	<i>Arabidopsis thaliana</i>	At2g29090	Kushiro et al. (2004), Saito et al. (2004)
	AtCYP707A3	<i>Arabidopsis thaliana</i>	At5g45340	Kushiro et al. (2004), Saito et al. (2004)
	AtCYP707A4	<i>Arabidopsis thaliana</i>	At3g19270	Kushiro et al. (2004), Saito et al. (2004)
	HvABA8'OH1	<i>Hordeum vulgare</i>	DQ145932	Chono et al. (2006), Millar et al. (2006)
	HvABA8'OH2	<i>Hordeum vulgare</i>	DQ145933	Chono et al. (2006), Millar et al. (2006)
	LsABA8ox1	<i>Lactuca sativa</i>	AB235917	Sawada et al. (2008)
	LsABA8ox2	<i>Lactuca sativa</i>	AB235918	Sawada et al. (2008)
	LsABA8ox3	<i>Lactuca sativa</i>	AB235919	Sawada et al. (2008)
	LsABA8ox4	<i>Lactuca sativa</i>	AB235920	Sawada et al. (2008)
	OxABA8ox1	<i>Oryza sativa</i>	Os02g0703600	Saika et al. (2007), Yang and Choi (2006)
	OxABA8ox2	<i>Oryza sativa</i>	Os08g0472800	Saika et al. (2007), Yang and Choi (2006)
	OxABA8ox3	<i>Oryza sativa</i>	Os09g0457100	Saika et al. (2007)
	PvCYP707A1	<i>Phaseolus vulgaris</i>	DQ352541	Yang and Zeevaert (2006)
	PvCYP707A2	<i>Phaseolus vulgaris</i>	DQ352542	Yang and Zeevaert (2006)
	PvCYP707A3	<i>Phaseolus vulgaris</i>	DQ352543	Yang and Zeevaert (2006)
				(continued)

Table 2.1 (continued)

	Gene name	Species	Gene ID	References
ABA glucosyltransferases	<i>AOG</i>	<i>Vigna angularis</i>	AB065190	Xu et al. (2002)
	<i>UGT71B6</i>	<i>Arabidopsis thaliana</i>	At3g21780	Lim et al. (2005), Priest et al. (2006)
ABA specific β -glucosidases	<i>AtBG1</i>	<i>Arabidopsis thaliana</i>	At1g52400	Lee et al. (2006)
	<i>AtBG2</i>	<i>Arabidopsis thaliana</i>	At2g32860	Xu et al. (2012)

Well-recognized mutant or biochemically characterized enzymes are shown in this list

difficulty showing the NSY activity since the protein tended to be insoluble (North et al. 2007). The tomato *nxd1* mutant had a defect in an unknown protein without an obvious chloroplast targeting sequence. Although the tomato *nxd1* mutant did not produce either isomer of neoxanthin, it did not show any ABA-deficient phenotypes. The existence of 9-*cis*-violaxanthin could compensate for the loss of neoxanthin in the *nxd1* mutant (Neuman et al. 2014). *Arabidopsis* NXD1 protein expressed in *E. coli* also failed to show NSY activity even when it was coexpressed with ABA4 (Neuman et al. 2014). Further analysis will be necessary to reveal how these genes contribute to neoxanthin synthesis. The results from the analyses of neoxanthin-deficient mutants indicate that the preferred substrate of NCED could differ according to plant species and both 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin could be the in vivo substrates of NCED. In fact, the parasitic plant *Cuscuta reflexa* does not have neoxanthin but still accumulates ABA under dehydration (Qin et al. 2008).

All-*trans*-epoxycarotenoids, violaxanthin and neoxanthin, are then converted into 9-*cis* isomers by an unknown isomerase (Fig. 2.2). It has been a long-standing question that gene encodes the 9-*cis*-epoxycarotenoid-forming isomerase in ABA biosynthesis. Recently, Alder et al. (2012) (Alder et al. 2012) found that the rice strigolactone biosynthetic enzyme D27 had isomerase activity converting all-*trans*- β -carotene into 9-*cis*- β -carotene and that the conversion activity was reversible. The structural similarity between β -carotene and epoxycarotenoids evokes the possible involvement of D27 in ABA biosynthesis. Rice has two additional genes homologous to D27, and *Arabidopsis* has three genes showing similarity to D27. Further analyzes of these genes and the corresponding mutants will help to discover the isomerase. Identification of the isomerase is the final piece for establishing the main framework of the ABA biosynthetic pathway in plants.

9-*cis* isomers of violaxanthin and neoxanthin are then cleaved into C15 and C25 compounds to produce the C15 xanthoxin, the direct precursor of ABA (Fig. 2.2). The first discovery of 9-*cis*-epoxycarotenoid dioxygenase came in the study of the viviparous, ABA-deficient, maize *vp14* mutant (Schwartz et al. 1997b; Tan et al. 1997). *VP14* encodes a non-heme iron (II)-dependent dioxygenase. Recombinant VP14 recognized the 9-*cis* configuration of C40 carotenoids and was able to cleave 9-*cis*-violaxanthin, 9'-*cis*-neoxanthin, and 9-*cis*-zeaxanthin at the 11–12 position but not all-*trans* isomers (Schwartz et al. 1997b). After the cleavage reaction in vitro, C25 compounds such as epoxy-apo-aldehyde or allenic-apo-aldehyde were generated as by-products with C15 xanthoxin. Whereas xanthoxin was detectable in plants, neither of these two C25 by-products could be detected (Parry and Horgan 1991; Schwartz and Zeevaart 2010). After the cloning of *VP14*, *NCED* genes were isolated from various plant species (Table 2.1). Overexpression of *NCED* genes resulted in higher accumulation of ABA in the transgenic plants, indicating that NCED catalyzes the rate-limiting step of ABA biosynthesis. *NCED* genes form a small gene family in various plant species. Most plants have a drought-inducible *NCED* gene whose expression levels are correlated with the accumulation of ABA in response to drought. Carotenoids including the substrate of NCED are abundantly localized at the thylakoid membrane in

the chloroplast (Parry and Horgan 1991). The PvNCED1 protein is also predominantly localized at the thylakoid membrane in the chloroplast (Qin and Zeevaart 1999). The N-terminal amphipathic sequence of VP14 was necessary to bind to the thylakoid membrane (Tan et al. 2001). The crystal structure of VP14 revealed how it accesses the substrate in the thylakoid membrane via its N-terminal amphipathic region (Messing et al. 2010). In *Arabidopsis*, drought-inducible AtNCED3 was detected in two forms with different molecular weights in the chloroplast (Endo et al. 2008). The larger form was localized in the thylakoid membrane and the smaller existed in the stroma. A similar distribution was observed in a chloroplast import assay of AtNCED proteins (Tan et al. 2003). The meaning of the differential localization of NCED in the chloroplast remains to be resolved.

After the oxidative cleavage of 9-*cis*-epoxycarotenoids by NCED, xanthoxin (Xan) is translocated from the plastid to the cytosol and then converted into abscisic aldehyde (ABAld) (Fig. 2.2). The *Arabidopsis aba2* mutant was supposed to be impaired in this step since a cell-free extract from the *aba2* mutant did not show conversion activity from Xan to ABAld (Schwartz et al. 1997a). The *aba2* mutants have a defect in a member of the short-chain dehydrogenase/reductase (SDR) gene family, which includes 56 members in the *Arabidopsis* genome, and ABA2 was identified as a cytosolic NAD-dependent oxidoreductase with xanthoxin dehydrogenase (XD) activity (Cheng et al. 2002; Gonzalez-Guzman et al. 2002). Intragenic complementation of *aba2* mutant alleles suggested that the activity of mutated ABA2 enzymes could be recovered through multimer formation (Merlot et al. 2002; Rook et al. 2001). *Arabidopsis aba2* mutants were originally isolated based on their ability to germinate in the presence of inhibitors of gibberellin biosynthesis (LeonKloosterziel et al. 1996; Nambara et al. 1998). Different alleles of *aba2* such as *gin1*, *sis4*, *isi4*, *sre1*, and *san3* were isolated by various kinds of screening that utilized their ability to germinate and grow in toxic concentrations of glucose, sucrose, or NaCl (Cheng et al. 2002; Gonzalez-Guzman et al. 2002; Laby et al. 2000; Rook et al. 2001), or by using thermal imaging screening (Merlot et al. 2002). These facts indicate that *aba2* mutants always have a short supply of ABA during their life cycle. In other words, there are no functionally redundant genes for ABA2 in the *Arabidopsis* genome. Hwang et al. (2012) reported that neither AtSDR2 nor AtSDR3, the closest homologs of ABA2, was likely to substitute the function of ABA2, supporting the uniqueness of ABA2 in the *Arabidopsis* genome. Although orthologs of *Arabidopsis* ABA2 are likely to be conserved in various plant species (Hanada et al. 2011), there have been no reports of additional XDs other than *Arabidopsis* ABA2.

After the conversion of Xan to ABAld, ABA is produced by the oxidation of ABAld. Two enzymes are involved in this oxidation step, abscisic aldehyde oxidase (ABAO) and MoCo sulfurase (Fig. 2.2). The conversion activity from ABAld to ABA was diminished in cell-free extracts of two wilted tomato mutants, *flacca* and *sitiens* (Sindhu and Walton 1988; Taylor et al. 1988). The enzyme that oxidizes ABAld to ABA was supposed to be an aldehyde oxidase (AO) (Leydecker et al. 1995; Walkersimmons et al. 1989) and four *Arabidopsis aldehyde oxidase* genes (*AAO1-4*) were isolated (Table 2.1) (Sekimoto et al. 1998). In-gel activity staining

of AAOs revealed that the homodimer of AAO3, could utilize ABAlD as a substrate, and recombinant AAO3 effectively converted ABAlD into ABA (Seo et al. 2000a). In addition, the transcript level of AAO3 was increased by dehydration but transcripts of the other AAOs were not (Seo et al. 2000a). The *Arabidopsis v11* mutant impaired in AAO3 could not synthesize ABA under drought stress, resulting in a wilted phenotype (Seo et al. 2000b). While AAO3 is the major ABAO it was reported that, the other members of the AAO family have minor contributions to ABA biosynthesis in seeds (Seo et al. 2004). In addition, ectopic expression of AAO3 successfully rescued the ABA-deficient tomato mutant *sitiens* (Okamoto et al. 2002). *sitiens* was supposed to be impaired in a newly identified *AO* gene other than previously reported *TAOs* (Table 2.1) (Harrison et al. 2011; Min et al. 2000). Recently, three *AO* genes were isolated from *Pisum sativum* (Zdunek-Zastocka 2008). Recombinant PsAO3 protein showed abscisic aldehyde oxidase activity and the levels of *PsAO3* transcript were increased by progressive drought stress in leaves and roots (Zdunek-Zastocka and Sobczak 2013).

Aldehyde oxidase (AO), xanthine dehydrogenase (XDH), sulfite oxidase (SO), and nitrate reductase (NR) are MoCo-containing enzymes in plants (Mendel and Hänsch 2002). Whereas the activities of SO and NR need a dioxo Mo-center in the MoCo, AO and XDH require the sulfuration of MoCo to generate a mono-oxo Mo-center for their activity. The tomato *flacca*, tobacco *Npaba1/CKR1*, and *Arabidopsis aba3/los5* mutants were isolated as ABA-deficient phenotypes because of the loss of activities in AO and XDH but not NR (Leydecker et al. 1995; Rousselin et al. 1992; Sagi et al. 1999; Schwartz et al. 1997a). The *aba3/los5* and *flacca* mutants are impaired in the gene encoding the MoCo sulfurase responsible for sulfuration of MoCo (Table 2.1) (Bittner et al. 2001; Sagi et al. 2002; Xiong et al. 2001). This is the reason why the biochemical defects of *flacca* and *aba3* mutants are similar to those of *sitiens* and *aoa3* with respect to their inability to convert ABAlD into ABA.

2.4 Indirect Pathway—Possible Minor Routes After the Production of Xanthoxin

As mentioned above, Xan is first converted to ABAlD, which is then converted to ABA. On the other hand, Xan has been supposed to be converted to ABA via two possible minor routes in plants (Cowan 2000; Nambara and Marion-Poll 2005; Seo and Koshiba 2002).

One pathway might operate via xanthoxic acid (Fig. 2.2). In ripening avocado mesocarp, inhibition of AO activity by tungstate, a potent inhibitor of the molybdo-enzymes in plants, results in the accumulation of xanthoxin, suggesting that xanthoxin is a substrate of AO (Lee and Milborrow 1997). When Xan was utilized as a substrate of AO, xanthoxic acid might be produced by the oxidation of xan by AO. Although xanthoxic acid is thought to then be converted into ABA

by an enzyme having activity like *Arabidopsis* ABA2, AtABA2 did not show conversion activity from xanthoxic acid to ABA (Cheng et al. 2002). While xanthoxic acid was not effectively converted to ABA in a cell-free conversion assay, it could be converted to ABA (Sindhu and Walton 1988). These results suggest that xanthoxic acid could be a precursor in ABA biosynthesis.

The other pathway, via abscisic alcohol, might be activated in some mutants (Fig. 2.2). When ABAlD was supplied to *flacca* or *sitiens* mutants, it was converted to abscisic alcohol, showing that ABAlD is reduced to abscisic alcohol and then oxidized to ABA via a shunt pathway (Rock et al. 1991; Taylor et al. 1988). This phenomenon was also observed in the *Npabal/CKR1* mutant in *Nicotiana plum-baginifolia* (Parry et al. 1991). The shunt pathway appears to be a minor source of ABA in wild-type plants but might play a significant role in mutants defective in the oxidation of ABAlD to generate ABA directly. One might speculate on the possibility of the minor ABA biosynthetic pathways operating in an organ and/or developmental stage-dependent manner.

2.5 ABA Catabolism

In contrast to biosynthesis, ABA is catabolized through several pathways in plants. ABA catabolism is largely categorized into two types of reaction, hydroxylation and conjugation (Fig. 2.3). Among them, ABA 8'-hydroxylation is a key step in the major ABA catabolic route in several plant species (Nambara and Marion-Poll 2005). Hydroxylation at C-8' of ABA is catalyzed by cytochrome P-450 type mono-oxygenases, and unstable 8'-hydroxy-ABA is then isomerized spontaneously to phaseic acid (PA). Although PA has faint ABA-like activity (Kepka et al. 2011; Walton 1983), substantial PA activity is observed in specific tissues such as barley aleurone layers (Hill et al. 1995; Todoroki et al. 1995). PA is further metabolized by an unidentified reductase to form dihydrophaseic acid (DPA) or *epi*-DPA, which have almost no biological activity (Walton 1983). ABA 8'-hydroxylases are encoded by the CYP707A family (Kushiro et al. 2004; Saito et al. 2004) (Table 2.1). The ABA 8'-hydroxylation reaction catalyzed by CYP707A requires both NADPH and P450 reductase (Kushiro et al. 2004; Saito et al. 2004). CYP707A selectively catalyzes the naturally occurring (+)-S-ABA enantiomer, but not the unnatural type (–)-R-ABA (Kushiro et al. 2004; Saito et al. 2004). Since ABA 8'-hydroxylase activity was observed in the microsomal fraction of suspension-cultured corn cells and CYP707A-green fluorescent protein (GFP) fusion protein localizes to the endoplasmic reticulum (ER), the ABA 8'-hydroxylation reaction is thought to take place in the ER (Krochko et al. 1998; Saika et al. 2007). Multiple mutants of CYP707A in *Arabidopsis* accumulate a large amount of ABA, whereas overexpression of CYP707A effectively reduces endogenous ABA (Millar et al. 2006; Okamoto et al. 2006, 2010, 2011; Umezawa et al. 2006). Thus, CYP707A plays a major regulatory role in controlling the level

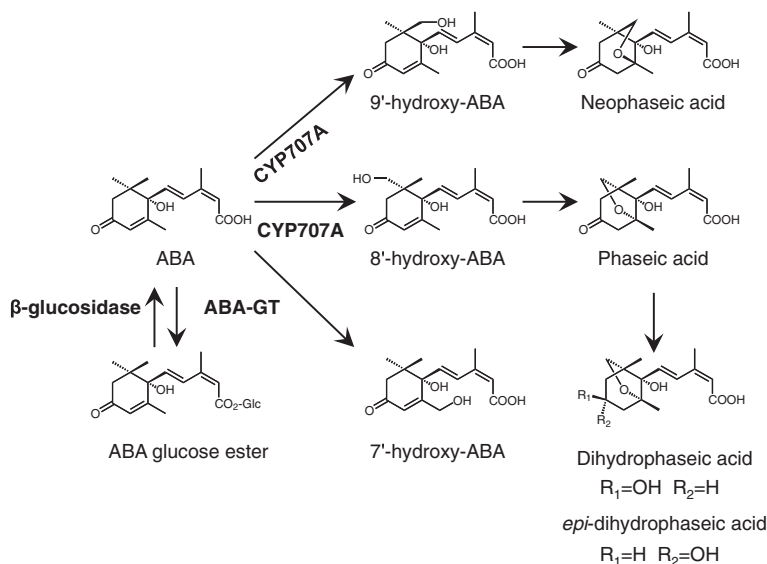


Fig. 2.3 ABA catabolic pathways in higher plants. In hydroxylation pathways, ABA 8'-hydroxylation is thought to be a major ABA catabolic route. ABA is also inactivated to ABA glucosyl ester (ABA-GE) by ABA-glucosyltransferase (ABA-GT), but ABA-GE is converted to free ABA by β -glucosidase

of ABA. In addition to the ABA 8'-hydroxylation pathway, ABA is also hydroxylated at C-7' and C-9' to form 7'- and 9'-hydroxy-ABA, respectively (Hampson et al. 1992; Zhou et al. 2004). These hydroxylated ABA catabolites have substantial biological activity (Hill et al. 1995; Zhou et al. 2004). Unstable 9'-hydroxy-ABA is spontaneously isomerized to form neophaseic acid (NeoPA) like PA (Zhou et al. 2004). NeoPA is a minor catabolite, but exists in a wide range of plant species (Zhou et al. 2004). The levels of neoPA accumulate substantially in restricted tissues such as developing seeds of *Arabidopsis thaliana* and *Brassica napus* (Kanno et al. 2010; Zhou et al. 2004). *Arabidopsis* CYP707As have minor activity for the 9'-hydroxylation of ABA in vitro (Okamoto et al. 2011). Additionally, endogenous neoPA levels in the *Arabidopsis cyp707a* mutant are lower than in the wild type, as with PA (Okamoto et al. 2011). Therefore, neoPA is thought to be produced by CYP707A as a side reaction. On the other hand, the gene encoding 7'-hydroxylase has not been identified yet. Hydroxylated catabolites of ABA are further conjugated with glucose (Cutler and Krochko 1999; Nambara and Marion-Poll 2005).

Among several conjugated catabolites, ABA glucosyl ester (ABA-GE) is the most widespread (Hartung et al. 2002). ABA-GE has been thought to be an inactive pool of ABA and accumulate in the vacuole or apoplast. However, ABA-GE is considered a storage or long-distance transport form of ABA that functions as a mobile stress signal from roots to shoots (Hartung et al. 2002; Jiang and Hartung

2008; Sauter et al. 2002). Glucosylation at the carboxyl group of ABA is catalyzed by glucosyltransferase (Lim et al. 2005; Xu et al. 2002). Eight ABA glucosyltransferases have been reported in *Arabidopsis*, although only one (UGT71B6) selectively recognizes (+)-ABA, but not (–)-R-ABA (Lim et al. 2005) (Table 2.1). The single knockout mutant of UGT71B6 does not show a significant difference in the levels of ABA and its metabolites compared with the wild type (Priest et al. 2006). In contrast, overexpression of UGT71B6 (UGT71B6OX) causes the accumulation of a large amount of ABA-GE (Priest et al. 2006). Nevertheless, UGT71B6OX displays only minor changes in ABA levels (Priest et al. 2006). This might be because the ABA 8'-hydroxylation pathway is predominantly involved in homeostatic regulation of ABA.

β -glucosidase hydrolyzes ABA-GE to form active ABA. There are two homologous β -glucosidases in *Arabidopsis*, AtBG1 and AtBG2, which localize to ER and vacuole, respectively (Lee et al. 2006; Xu et al. 2012) (Table 2.1). The enzymatic activity of AtBG1 is enhanced by dehydration-induced polymerization (Lee et al. 2006). It was reported that the purine metabolite allantoin promotes polymerization of AtBG1 and increases endogenous ABA levels (Watanabe et al. 2014). In contrast, the AtBG2 protein exists in high molecular weight complexes through polymerization under non-stress conditions and is protected from degradation under dehydration stress (Xu et al. 2012). However, the details of how allantoin regulates the polymerization of AtBG1 and how protein degradation of vacuole-localized AtBG2 is inhibited under stress remain unknown. The *atbg2* mutant does not affect endogenous ABA levels, whereas the *atbg1* mutant shows markedly reduced ABA levels in the extracellular space, but not in the intracellular space or xylem sap (Lee et al. 2006; Xu et al. 2012). Additionally, the *atbg1 atbg2* double mutant shows a more sensitive phenotype to drought stress (Xu et al. 2012). Therefore, β -glucosidase-mediated ABA production from ABA-GE is also considered a key pathway for regulating the local ABA concentration in response to environmental stimuli.

2.6 Chemical Inhibitors of ABA Metabolism

Inhibitors of ABA metabolism have been utilized in studies of the physiological roles of ABA in plants. They are especially useful for analyzing ABA physiology in plant species that are difficult to perform genetic analyses on.

Many researchers have used fluridone and norflurazon as inhibitors of ABA biosynthesis (Fig. 2.4). These inhibitors target phytoene desaturase, which functions in carotenoid biosynthesis, and cause concurrent bleaching of plants due to the destruction of chlorophyll resulting in loss of ABA biosynthesis (Gamble and Mullet 1986). This approach cannot distinguish the effects of ABA from the consequences of depleting most carotenoids and their derivatives (Taylor et al. 2005). As mentioned above, carotenoid deficiency causes a pleiotropic phenotype including ABA deficiency. Therefore, we need to use these inhibitors carefully.

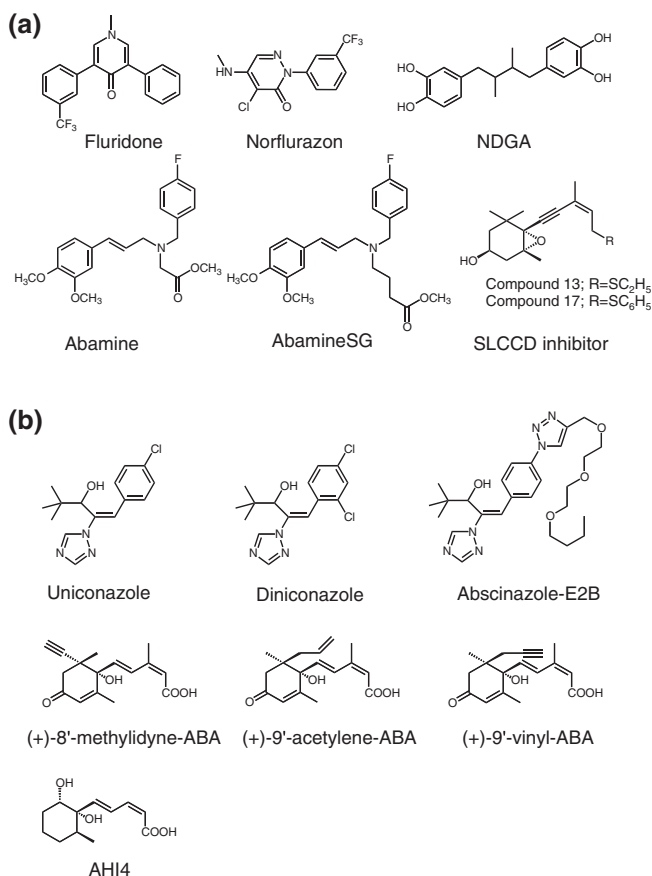


Fig. 2.4 Inhibitors of ABA biosynthetic and catabolic pathways. **a** ABA biosynthetic inhibitors. These chemicals inhibit phytoene desaturase or NCED activity. Abbreviation: NDGA nordihydroguaiaretic acid. **b** ABA catabolic inhibitors. Both the azole types (uniconazole, diniconazole, and abscinsazole-E2B) and ABA analogues shown in this figure inhibit CYP707A activity

Nordihydroguaiaretic acid (NDGA) is an inhibitor of lipoxygenase, which catalyzes dioxygenation of polyunsaturated fatty acids (Fig. 2.4). NDGA is supposed to inhibit carotenoid cleavage dioxygenases including NCED (Creelman et al. 1992). The application of NDGA to osmotically stressed soybean suspension cells inhibited the accumulation of ABA (Creelman et al. 1992). Abamine was first developed as an NCED-specific inhibitor based on the structure of NDGA (Han et al. 2004). Abamine inhibited ABA production in osmotically stressed spinach leaf disks more effectively than NDGA (Han et al. 2004). Kitahata et al. (2006) further improved abamine to generate abamineSG, which has increased specificity for NCED without the growth retardation effect seen in abamine. Abamine and abamineSG inhibited ABA accumulation by 35 and 77 %, respectively, and acted as inhibitors of cowpea NCED in vitro with K_i values of 38.8 and 18.5 μM ,

respectively (Kitahata et al. 2006). Another group developed several kinds of sesquiterpene-like carotenoid cleavage dioxygenase (SLCCD) inhibitors against *Arabidopsis* NCED3, based on the structures of 9-*cis*-epoxycarotenoid and xanthoxin (Boyd et al. 2009). Compounds 13 and 17 inhibited the activity of AtNCED3 in vitro with K_i values of 93 and 57 μ M, respectively (Fig. 2.4) (Boyd et al. 2009). Compound 13 had a greater inhibitory effect on ABA biosynthesis *in planta* than compound 17 and abamineSG (Boyd et al. 2009).

Most reported ABA catabolic inhibitors are chemicals targeting CYP707A. The structural features of CYP707A inhibitors are categorized into two types, azole types and ABA analogues (Fig. 2.4). Since CYP707A is a P-450 monooxygenase, several P450 inhibitors containing an azole group are considered candidate inhibitors of ABA catabolism. Uniconazole impairs the conversion of ABA to PA and inhibits the activity of the CYP707A enzyme (Kitahata et al. 2005; Saito et al. 2006). Although uniconazole effectively confers drought stress tolerance to plants, it was originally developed as a gibberellin biosynthesis inhibitor and consequently induces dwarfism (Izumi et al. 1985; Saito et al. 2006). Diniconazole functions as a fungicide, and its chemical structure is similar to that of S-uniconazole (Fletcher et al. 1986). Therefore, diniconazole also inhibits the activity of CYP707A (Kitahata et al. 2005). However, these azole P450 inhibitors arrest plant growth because of the broad inhibition spectrum of azole compounds for P450 enzymes (Rademacher 2000; Yokota et al. 1991). The structure of uniconazole was modified, and several inhibitors were synthesized to develop a more specific inhibitor against CYP707A (Okazaki et al. 2012; Todoroki et al. 2009a, b, 2010). Unlike uniconazole, abscinazole-E2B selectively inhibits CYP707A, but not CYP701A, which is involved in gibberellin biosynthesis. Application of abscinazole-E2B increases endogenous ABA levels during dehydration and confers drought tolerance with less growth arrest.

Among non-azole-type ABA catabolic inhibitors, ABA analogues designed based on the structure of ABA have the potential to function as specific inhibitors. Several ABA 8'- or 9'-derivatives have been synthesized and tested for their effect on the activity of the CYP707A enzyme, because C-8' and its neighboring position in ABA are thought to be the possible influence groups for the hydroxylation reaction by CYP707A. (+)-8'-methylidyne-ABA, (+)-9'-acetylene-ABA, (–)-9'-acetylene-ABA, and (+)-9'-vinyl-ABA have been reported as suicide substrates that irreversibly inhibit the activity of ABA 8'-hydroxylase (Cutler et al. 2000). Among these, (+)-9'-acetylene-ABA functions as the most effective inhibitor of ABA 8'-hydroxylase (Cutler et al. 2000). There is no clear explanation why 9'-derivatives show potent inhibition effects despite the fact that CYP707A mainly catalyzes hydroxylation at the C-8' of ABA. It is possible that the catalytic site of CYP707A might recognize both C-8' and -9' methyl groups for the hydroxylation reaction, since CYP707A catalyzes both 8'- and 9'-hydroxylation of ABA. In addition to the suicide effect, ABA 8'- or 9'-derivatives are thought to possess ABA agonist activity. Indeed, ABA-responsive genes regulated by (+)-8'-methylidyne-ABA widely overlap with ABA (Huang et al. 2007). To overcome the ABA agonist activity of ABA analogues, several specific inhibitors have been synthesized

and developed by integrating designs based on the structural features of ABA analogues and the biochemical activity of CYP707A (Araki et al. 2006; Ueno et al. 2005). AHI4 does not exhibit ABA activity such as growth arrest and germination inhibition, but it strongly inhibits the activity of ABA 8'-hydroxylase (Araki et al. 2006). Therefore, co-treatment of ABA and AHI4 enhances ABA's activity, and application of AHI4 confers drought stress tolerance (Araki et al. 2006). Specific inhibitors are useful not only for understanding ABA metabolic regulation, but also as agrochemicals that can control the action of ABA in crops.

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Absciscic Acid: Metabolism, Transport and Signaling

Zhang, D.-P. (Ed.)

2014, XII, 465 p. 49 illus., 23 illus. in color., Hardcover

ISBN: 978-94-017-9423-7