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Regulation of abscisic acid metabolism: towards a metabolic basis for abscisic acid–cytokinin antagonism

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Abstract

The penultimate step in abscisic acid (ABA) biosynthesis involves oxidation of xanthoxal (XAN) catalysed by a molybdenum-cofactor (MoCo)-containing aldehyde oxidase (AO) and represents one potential site of regulation of ABA in plant tissues. In an attempt to understand the biochemical basis for cytokinin–abscisic acid (CK-ABA) antagonism the effect of several CKs, molybdate, tungstate and allopurinol (an inhibitor of xanthine oxidase activity and purine metabolism) on the formation of XAN, ABA and related catabolites in mesocarp of ripening avocado (*Persea americana* Mill. cv. Hass) was investigated. Treatment with either adenine (Ade), isopentenyladenine (2iP) or zeatin (Z) enhanced conversion of ABA to phaseic acid (PA) and caused a reduction in the amount of radioactivity incorporated from 3*R*-[2-^{14}C] mevalonolactone (MVL) into ABA by stimulating overall ABA metabolism. Ancymidol and *N*-(2-chloro-4-pyridyl)-*N*-phenylurea (CPPU), while not affecting formation of PA and DPA, appeared to retard ABA biosynthesis which resulted in the accumulation of XAN. Tungstate caused accumulation of XAN at the expense of ABA and related acidic metabolites while molybdate and allopurinol accelerated ABA metabolism, i.e. formation of XAN, ABA, PA, and DPA. These findings are discussed in terms of the regulation of the ABA biosynthetic pathway in avocado fruit by CK-induced suppression of xanthine dehydrogenase (XDH) activity and a model illustrating the proposed metabolic interrelationship is presented.

Key words: Abscisic acid, aldehyde oxidase, allopurinol, avocado, cytokinin, molybdenum cofactor, tungstate, xanthoxal.

Introduction

Cytokinins appear to antagonize many physiological processes thought to be mediated, all or in part, by ABA. For example, ABA-induced stomatal closure, leaf senescence, and leaf and fruit abscission are reversed by exogenous application of CK while CK-mediated release of seed dormancy contrasts with ABA inhibition of germination (Salisbury, 1994). This implied antagonism may be the result of metabolic interaction particularly as CKs share, at least in part, a common biosynthetic origin with ABA. CK biosynthesis appears to involve the addition of dimethylallyl pyrophosphate to the N^6 position of AMP and subsequent conversion of N$^6\Delta$2-isopentenyl AMP to N$^6\Delta$2-isopentenyl adenosine and 2iP, followed by hydroxylation of 2iP to yield 'zeatin-like' CKs (Binns, 1994). By comparison, ABA is regarded as an apocarotenoid derived from the metabolism of isopentenyl pyrophosphate via zeaxanthin, 9′-*cis*-neoxanthin and XAN, which is then oxidized to ABA (Cowan and Richardson, 1997; Duckham *et al.*, 1991; Parry, 1993; Rock and

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Abbreviations: Ade, adenine; AMP, adenosine monophosphate; AO, aldehyde oxidase; CK, cytokinin; CPPU, *N*-(2-chloro-4-pyridyl)-*N*-phenylurea; DPA, dihydrophaseic acid; GC-EI-MS, gas chromatography-electron impact-mass spectrometry; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; 2iP, isopentenyladenine; MoCo, molybdenum cofactor; MVL, mevalonolactone; PA, phaseic acid; XAN, xanthoxal; XDH, xanthine dehydrogenase; Z, zeatin.

Zeevaart, 1991; Schwartz *et al.*, 1997*b*; Tan *et al.*, 1997; Yamomoto and Oritani, 1996). Even so, details of a possible biochemical interaction between CKs and ABA have hitherto remained obscure.

Earlier studies revealed that a range of CKs reduced the incorporation of label from 3*R*-[2-^{14}C]MVL into ABA in higher plants (Cowan and Railton, 1987*a*). Cytokinins were also shown to inhibit the production of ABA by cultures of the ABA-producing fungi *Botrytis cinerea* (Hirai *et al.*, 1986) and *Cercospora rosicola* (Norman *et al.*, 1983*a*) and, in the latter, specifically reduced incorporation of label from [1-^{14}C]farnesyl pyrophosphate into ABA. Since Ade did not retard ABA production by any of the aforementioned experimental systems, it was concluded that N^6-substitution of the purine moiety was required for inhibition of ABA biosynthesis. The CK analogue and inhibitor of gibberellin biosynthesis, ancymidol, also inhibited ABA biosynthesis in avocado mesocarp and *Cercospora rosicola* (Cowan and Railton, 1987*a*; Norman *et al.*, 1983*b*). Ancymidol appears to interact directly with cytochrome P-450 dependent mono-oxygenases by bonding of electrons from an sp^2 hybridized N atom in the heterocycle to the protohaem iron thereby displacing the molecular oxygen required for catalysis (Grossmann, 1990). Interestingly, the mode of action of CKs and ancymidol was shown to be similar with respect to the oxidation of *ent*-kaurene, i.e. interaction with cytochrome P-450 (Coolbaugh, 1984). In the absence of sufficient evidence to support the involvement of cytochrome P-450 mono-oxygenases in higher plant ABA biosynthesis, however, coupled with reports that *Cercospora rosicola* produces ABA directly from farnesyl pyrophosphate (Bennett *et al.*, 1990; Yamamoto and Oritani, 1997), it is difficult to rationalize the potential mode of action of CKs and CK analogues on ABA biosynthesis. Nevertheless, the catabolism of ABA to PA in higher plants is a cytochrome P-450-mediated reaction which is catalysed by ABA 8′-hydroxylase (Creelman and Zeevaart, 1984; Gillard and Walton, 1976). Thus, in the presence of inhibitors of cytochrome P-450 activity, an increase in endogenous ABA concentration might be expected. However, this has not been evident in the studies carried out using higher plant tissues which suggests that a different mechanism of interaction between CKs and ABA is operational.

Research into the metabolic control of avocado fruit growth has revealed several interesting aspects related to CK–ABA interaction. In summary, these include (i) final fruit size (measured as fresh weight of whole fruit) is linearly correlated with the endogenous CK : ABA ratio; (ii) mevastatin-induced retardation of avocado fruit growth occurred concomitant with a decline in HMGR activity and increased endogenous ABA concentration, responses that were negated in the presence of 2iP; (iii) ABA-induced inhibition of cell-to-cell chemical communication was negated by 2iP; and (iv) ABA-induced phenotypic variation (including a decline in growth rate and early seed coat senescence) was negated in the presence of 2iP (Cowan *et al.*, 1997; Moore-Gordon *et al.*, 1998). In addition to supporting the proposed antagonism between CKs and ABA, these observations suggest that interaction between CKs and ABA is exerted at a site some distance from HMGR in the ABA biosynthetic pathway because 2iP did not fully restore HMGR activity of ABA-treated fruit, but reversed ABA-induced retardation of fruit growth (Cowan *et al.*, 1997).

Two potential sites of regulation of ABA biosynthesis have been alluded to in recent studies. Firstly, dioxygenase-mediated cleavage of the xanthophyll precursor for ABA has been postulated to regulate the formation of XAN in an inducible manner (Parry, 1993; Cowan and Richardson, 1997). Secondly, studies using the *flacca* mutant of *Lycopersicon esculentum* (Marin and Marion-Poll, 1997) and the *aba2* and *aba3* mutants of *Arabidopsis thaliana* (Schwartz *et al.*, 1997*a*) have revealed that the reduced ability of these mutants to produce ABA is very likely due to impaired sulphurylation of the AO-MoCo.

Recently it was suggested that the pool size of MoCo may not be constant but that it varies in response to nutritional and environmental factors (Sagi *et al.*, 1997; Sagi and Lips, 1998). Thus, the activity of the MoCo-requiring enzymes, AO and XDH was enhanced in plants grown with ammonium while both salt stress and ammonium increased overall MoCo content (Omarov *et al.*, 1998; Sagi *et al.*, 1998). The increase in Mo-hydroxylases with salt stress and ammonium treatment was thus considered part of the mechanism for stress adaptation by plants which includes elevated ABA synthesis and increased ureide production (Sagi *et al.*, 1998). Nitrogen status and salinity stress also induce profound changes in CK content *in vivo* (Hare *et al.*, 1997). Since MoCo is apparently derived from GTP (Mendel, 1997; Rajagopalan, 1997), a precursor to purines, it might be anticipated that alterations in purine metabolism, including changes in CK biosynthesis, impact on XAN metabolism and ABA biosynthesis. To investigate this possibility, the effect of several CKs and CK analogues on ABA metabolism in the mesocarp of avocado was examined. In addition, the effect of molybdate, tungstate and allopurinol (an inhibitor of xanthine oxidase/dehydrogenase activity which arrests purine metabolism) on ABA biosynthesis was also studied. The results indicate that availability of MoCo for AO activity represents a potential site of interaction between CK and ABA.

Materials and methods

Chemicals

3*R*-[2-^{14}C]MVL (2.11 GBq mmol^{-1}) was purchased from Amersham International, Buckinghamshire, UK. All other chemicals were from Sigma Chemical Co., St Louis, MO, USA.

Authentic standards of PA and DPA were prepared as previously described except that 21-d-old greenhouse-cultivated *Lycopersicon esculentum* plants were used to prepare these catabolites from applied (±)-ABA (Cowan and Railton, 1986).

Plant material and application of chemicals

Mature avocado (*Persea americana* Mill. cv. Hass) fruit were harvested from trees cultivated on clonal Duke 7 rootstocks in orchards in the KwaZulu-Natal Midlands, South Africa, and allowed to ripen in darkness at 25 °C for 8–10 d.

Blocks of mesocarp tissue were excised from the equatorial region of soft ripe fruits and supplied various combinations of chemicals in the presence or absence of radiolabelled substrate, at the concentrations specified in the Results. For each experimental treatment, blocks of mesocarp tissue (20 g fresh weight) were prepared from the same fruit, and an equivalent sized tissue sample freeze-dried immediately for determination of basal metabolite concentration. Chemicals were formulated in Tween 20 : acetone : water (1 : 1 : 8, by vol.) and infiltrated via a series of cuts in the surface of the tissue. A total volume of 0.3 ml of solution was administered in this way and the blocks of mesocarp incubated for 24 h in a water-saturated environment at 25 °C.

Determination of XAN, ABA and related metabolites

Freeze-dried mesocarp tissue together with insoluble PVP (10%, w/w) was homogenized in ice-cold methanol:ethyl acetate (50 : 50, v/v), containing butylated hydroxytoluene (100 mg l^{-1}) and diethyldithiocarbamate (200 mg l^{-1}), and extracted in darkness at 2 °C. Homogenates were filtered under vacuum, the filter-cake washed with further ice-cold methanol : ethyl acetate (50 : 50, v/v) and the filtrate reduced to dryness *in vacuo* at 35 °C. The residue was resuspended in 0.5 M potassium phosphate buffer (pH 8.5) and partitioned six times against equal volumes of *n*-hexane to remove pigments and other lipophilic impurities. To extract XAN, the pH of the aqueous fraction was adjusted to 7.0 and partitioned twice against equal volumes of ethyl acetate. To extract ABA and related acids, the pH of the aqueous phase was then adjusted to 2.5 and these compounds partitioned into ethyl acetate four times using equal volumes of solvent. Water was removed from the XAN and ABA-containing ethyl acetate fractions by freezing to −20 °C followed by filtration under vacuum and the organic phase reduced to dryness *in vacuo* at 35 °C.

XAN, and ABA and related acids were purified by thin layer chromatography using silica gel GF_{254} plates (20 × 20 cm, 0.5 mm thick) developed to 15 cm, once in *n*-hexane : ethyl acetate (3 : 2, v/v) and three times in toluene:ethyl acetate:acetic acid (25 : 25 : 2, by vol.), respectively. The UV-absorbing zones corresponding to XAN (R_F 0.2) and the region from DPA to ABA (R_F 0.43–0.76) were recovered into water-saturated ethyl acetate and analysed by reversed-phase HPLC. Chromatography was carried out using either a 5 µm C_{18} column (250 × 4.6 mm i.d. ODS 2, Spherisorb, Phase Separations Inc., Clwyd, UK) eluted isocratically with methanol : water : acetic acid (40 : 60 : 0.5, by vol.) at a flow rate of 1.5 ml min^{-1} (System A) or, a 5 µm C_{18} column (250 × 10 mm i.d. ODS 1, Sphereclone, Phenomenex, Torrance, CA) eluted using a linear gradient of 20–100% methanol in either 0.5% aqueous acetic acid (System B) or water (System C) at a flow rate of 2 ml min^{-1}. Compounds were quantified at 260 nm by peak integration following calibration with authentic standards using a Spectra System UV3000 rapid scanning detector or a Spectra System UV/VIS 1000 detector (Thermo Separation Products, Freemont, CA, USA). For analysis of [^{14}C]-labelled products, the HPLC eluate was fractionated into 0.4 ml aliquots and the amount of radioactivity determined by liquid scintillation spectrometry.

GC-EI-MS

HPLC-purified acidic metabolites co-eluting with authentic standards were collected, methylated at room temperature with ice-cold ethereal diazomethane, repurified on thin layers of silica gel GF_{254} in *n*-hexane : ethyl acetate (1 : 1, v/v) and the methyl ester derivatives recovered into diethyl ether. Unequivocal identification was achieved by GC-EI-MS using a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard quadrupole MS system. Samples were analysed using a fused-silica capillary column (12 m × 0.32 mm i.d.) of OV-1 (Supelco Inc., Bellefonte, USA) programmed from 120 °C at 5 °C min^{-1} with He as carrier gas (1.5–2.0 ml min^{-1}) and EI spectra recorded at 70 eV.

Results

Effect of CKs and CK analogues on ABA metabolism

Detailed analysis of the neutral, ethyl acetate fraction generated from mesocarp of ripening avocado fruit by a combination of TLC followed by reversed phase HPLC yielded a major component (Rt = 16.7 min, System A; and Rt = 37.6 min, System C) which, after derivatization, was subsequently identified by GC-EI-MS as *o*-acetyl-XAN [M^+292 (2), 232 (21), 203 (3), 189 (2), 176 (12), 150 (12) 149 (100), 133 (23), 121 (12), 105 (8), 95 (16), 43 (75)]. Similar biochemical scrutiny of the acidic ethyl acetate soluble acid fraction followed by GC-EI-MS analysis of the methyl ester derivatives confirmed the identity of ABA [M^+278 (1), 260 (4), 190 (100), 162 (54), 134 (49), 125 (54), 112 (14), 91 (28)], PA [M^+294 (3), 276 (3), 262 (1), 244 (3), 233 (3), 177 (11), 167 (23), 154 (23), 139 (38), 135 (31), 125 (100), 122 (80), 121 (64), 109 (29), 94 (50)] and DPA [M^+296 (3), 278 (12), 246 (6), 220 (13), 189 (10), 188 (19), 154 (52), 122 (100), 121 (58), 109 (53), 95 (48)].

ABA accumulation by mesocarp tissue is characteristic of ripening avocado fruit (Adato *et al.*, 1976). To determine the effect of CK and CK analogues on this process, the rate of change in ABA content was measured throughout the ripening period to establish the linear phase of ABA accumulation (data not shown). Mesocarp tissue was treated with or without CK during this phase and the effect on ABA production examined. The results presented in Table 1 show that Ade, CPPU and Z had no apparent effect on ABA production. By comparison, ancymidol decreased ABA formation 2-fold whereas 2iP stimulated ABA production 1.5-fold. To elucidate these contrasting effects the change in endogenous concentration of XAN, ABA, PA, and DPA in response to CK application was determined and the results are presented in Table 2. Ade and 2iP caused an increase in the endogenous concentration of these metabolites whereas ancymidol

Table 1. *Effect of CK and CK analogues on the rate of increase in mesocarp ABA concentration during ripening of avocado fruit*

Mesocarp of ripening avocado fruit was treated at different times during the linear stage of ABA accumulation with solutions of Tween 20/acetone/water (1:1:8, by vol.) containing 400 μg of either adenine, ancymidol, CPPU, iP or zeatin via the cut surface and incubated in a water-saturated environment at 25 °C for 24 h. Tissue was extracted and analysed as described in Materials and methods. Rates were calculated by subtracting the initial ABA concentration, of mesocarp of the same fruit, from the value obtained after a 24 h incubation period. Data are the mean of three replicates ± SE.

Treatment	Rate of ABA formation (nmol g^{-1} DW h^{-1})
Control	0.351 ± 0.062
Adenine	0.383 ± 0.025
Ancymidol	0.161 ± 0.037
CPPU	0.355 ± 0.028
iP	0.503 ± 0.041
Zeatin	0.364 ± 0.012

reduced the tissue content of XAN + ABA + PA + DPA. All the compounds tested increased mesocarp PA and DPA concentration indicating that CK treatment did not adversely affect the cytochrome P-450-mediated oxidation of ABA to PA and the further reduction of PA to DPA. In contrast, ancymidol reduced ABA concentration by 82% which, when coupled with the observed increase in XAN concentration, suggested that ancymidol inhibited the aldehyde oxidase-catalysed conversion of XAN to ABA. Treatment with 2iP increased ABA concentration substantially. In Z-treated mesocarp, less XAN remained at the conclusion of the incubation period than was present at the start, which might suggest that Z stimulated the conversion of XAN to ABA, PA and DPA.

Radiolabelling studies on the incorporation of [^{14}C] from 3*R*-[2-^{14}C]MVL into XAN, ABA and DPA did not wholly support the aforesaid results (Table 3). Contrary to expectation, label associated with XAN did not increase in response to ancymidol (or any of the other CKs tested) although incorporation of radioactivity into ABA and DPA was substantially reduced by this treatment. Incorporation of label into ABA was lower in the presence of ancymidol, CPPU and Z, but was stimulated by 2iP. Only ancymidol and CPPU reduced the apparent incorporation of radioactivity into DPA. The specific activity of XAN increased in response to Ade and 2iP whereas activity in ABA and DPA was unaffected by these treatments. Only

Table 2. *Effect of CK and CK analogues on ABA metabolism in mesocarp of ripening avocado*

Avocado mesocarp was supplied solutions of Tween 20/acetone/water (1:1:8, by vol.) containing either adenine (2.96 μmol), ancymidol (1.56 μmol), CPPU (1.61 μmol), zeatin (1.82 μmol) or iP (1.97 μmol) via the cut surface and incubated in a water-saturated environment at 25 °C for 24 h. Metabolites were extracted and analysed as described in Materials and methods. Data are expressed as net change (i.e. $t_{24} - t_0$) and are the mean of four individual experiments.

Treatment	XAN nmol g^{-1} DW (% of control)	ABA nmol g^{-1} DW (% of control)	PA nmol g^{-1} DW (% of control)	DPA nmol g^{-1} DW (% of control)	Total nmol g^{-1} DW (% of control)
Control	0.25 (100)	13.87 (100)	1.03 (100)	4.23 (100)	19.38 (100)
Adenine	0.40 (160)	13.37 (96)	2.09* (203)	9.12* (216)	24.98 (129)
Ancymidol	2.45* (980)	2.47* (18)	1.32* (148)	8.43* (199)	14.67* (76)
CPPU	1.15* (460)	9.29 (69)	1.49* (135)	7.92* (187)	19.85 (102)
iP	0.16 (64)	21.78* (157)	1.64* (159)	7.38* (174)	30.96* (160)
Zeatin	—	9.91 (71)	1.41* (137)	8.49* (200)	19.81 (102)

Values followed by * are significantly different ($P \leq 0.01$) from the control.

Table 3. *Effect of CK and CK analogues on the incorporation of label from 3*R*-[2^{14}C]MVL into XAN, ABA and DPA in mesocarp of ripening avocado fruit*

Avocado mesocarp was supplied solutions of Tween 20/acetone/water (1:1:8, by vol.) containing 3*R*-[2-^{14}C]MVL (180 kBq) with or without adenine (2.96 μmol), ancymidol (1.56 μmol), CPPU (1.61 μmol), zeatin (1.82 μmol) and iP (1.97 μmol) via the cut surface and incubated in a water-saturated environment at 25 °C for 24 h. Metabolites were extracted and analysed as described in Materials and methods. Data are the mean of three individual experiments.

Treatment	XAN Bq (specific activity)[a]	ABA Bq (specific activity)[a]	DPA Bq (specific activity)
Control	9.16 (2.78)	83.00 (0.79)	43.20 (0.78)
Adenine	9.56 (3.21*)	84.16 (0.82)	47.16 (0.76)
Ancymidol	7.40 (0.59*)	26.32* (0.54*)	31.08* (0.55*)
CPPU	8.04 (1.49*)	49.40* (0.73)	29.92* (0.65)
iP	8.24 (4.45*)	95.92 (0.66)	45.84 (0.85)
Zeatin	8.72 —	52.04* (0.70)	45.96 (0.77)

[a]Specific activity expressed as Bq $nmol^{-1}$.
Values followed by * are significantly different ($P \leq 0.01$) from the control.

in response to ancymidol was the specific activity of XAN, ABA and DPA markedly reduced. Since the endogenous concentration of PA and DPA did not decline in response to ancymidol treatment, the data indicate dilution of label by non-labelled product. Taken together, these results suggest that CK increased the formation of PA and DPA by exerting an influence on the oxidation of XAN to ABA. Thus, ancymidol and CPPU appeared to retard conversion of XAN to ABA, resulting in depletion of ABA via sustained catabolism, whereas Ade, 2iP and Z stimulated overall ABA metabolism.

Modulation of ABA metabolism by tungstate, molybdate and allopurinol

The above results suggested that CK and CK analogues may exert an effect on ABA metabolism by influencing the oxidation of XAN to ABA and its further conversion to PA and DPA. In order to investigate this possibility in more detail, the effect of molybdate and tungstate (which are both incorporated into the MoCo) and allopurinol (an inhibitor of xanthine oxidase activity which arrests purine metabolism) on formation of XAN, ABA and its major acidic catabolites in the mesocarp of ripening avocado fruit was determined. In addition to an increase in ABA concentration, allopurinol and molybdate stimulated formation of PA and DPA (Table 4). By comparison, treatment with tungstate caused accumulation of XAN at the expense of ABA, PA and DPA. To elucidate this result further two approaches were adopted. First, the effect of increasing concentrations of molybdate, tungstate and allopurinol on XAN and ABA formation during the linear phase of ABA accumulation by avocado mesocarp, was determined. Second, the effects of molybdate, tungstate and allopurinol on the incorporation of label from 3*R*-[2-^{14}C]MVL into XAN, ABA and DPA were investigated.

Increasing concentrations of tungstate and allopurinol caused accumulation of XAN whereas only allopurinol caused a concomitant increase in ABA, indicating that this inhibitor of purine metabolism was capable of enhancing ABA formation (Fig. 1). Molybdate had little or no effect on the formation of XAN, but stimulated ABA production and accelerated both XAN and ABA metabolism when applied together with allopurinol causing a marked increase in the specific activity of ABA and DPA (Table 5). At all concentrations tested, tungstate reduced ABA formation. Similarly, the incorporation of radioactivity from 3*R*-[2-^{14}C]MVL into ABA and DPA was inhibited by tungstate but stimulated by allopurinol and molybdate (Table 5). Although allopurinol treatment increased incorporation of label into XAN, ABA and DPA the specific activity of ABA and DPA was approximately 50% that of the control.

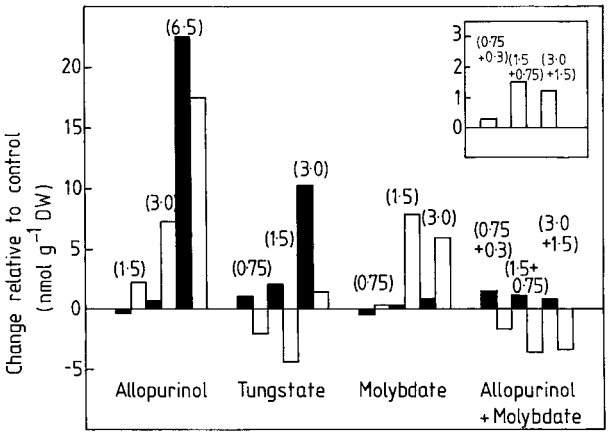

Fig. 1. Effect of increasing concentrations of allopurinol, molybdate and tungstate on mesocarp XAN (solid bars) and ABA (open bars) concentration of ripening avocado fruit. Inset; content of DPA in mesocarp supplied increasing amounts of allopurinol plus molybdate. Mesocarp was supplied solutions of Tween 20/acetone/water (1:1:8, by vol.) containing either allopurinol and/or potassium molybdate, or potassium tungstate via the cut surface and incubated for 24 h at 25 °C. XAN and ABA were extracted and quantified as described in Materials and methods. Data are expressed as net XAN, ABA or DPA following subtraction of the basal values (t_0), obtained by extracting a known amount of mesocarp from the same fruit at the start of the experiment. Values in parentheses represent the mass of applied compound in μmol.

Table 4. *Effect of allopurinol, molybdate and tungstate on ABA metabolism in mesocarp of ripening avocado*

Mesocarp of ripening avocado was supplied solutions Tween 20/acetone/water (1:1:8, by vol.) containing either allopurinol (1.5 μmol), potassium molybdate (0.75 μmol) or potassium tungstate (0.75 mol) via the cut surface and incubated in a water-saturated environment at 25 °C for 24 h. XAN, ABA, PA, and DPA were extracted and quantified as described in Materials and methods. Data are presented as net change (i.e. $t_{24}-t_0$) in metabolite concentration and are representative of four individual experiments.

Treatment	XAN nmol g^{-1} DW (% of control)	ABA nmol g^{-1} DW (% of control)	PA nmol g^{-1} DW (% of control)	DPA nmol g^{-1} DW (% of control)	Total nmol g^{-1} DW (% of control)
Control	0.78 (100)	2.04 (100)	0.02 (100)	0.71 (100)	3.55 (100)
Allopurinol	0.64 (82)	4.23* (207)	0.20* (1000)	3.62* (510)	8.69* (245)
Molybdate	0.46 (59)	2.25 (110)	0.09* (450)	1.98* (279)	4.78* (135)
Tungstate	1.64* (210)	0.03* (<2)	ND[a]	ND[a]	1.67* (47)

[a]ND, Not detected.
Values followed by * are significantly ($P \leq 0.01$) different from the control.

Table 5. *Effect of allopurinol, molybdate and tungstate on incorporation of 3*R*-[2-^{14}C]MVL into XAN, ABA and DPA*

Mesocarp of ripening avocado fruit was supplied solutions of Tween 20/acetone/water (1 : 1 : 8, by vol.) containing 3*R*-[2-^{14}C]MVL (180 kBq) with or without allopurinol (1.5 μmol), potassium molybdate (0.75 μmol) and potassium tungstate (0.75 μmol) via the cut surface and incubated in a water-saturated environment at 25 °C for 24 h. XAN, ABA and related acids were extracted and quantified as described in Material and methods. Data are representative of two individual experiments.

Treatment	XAN Bq (specific activity)[a]	ABA Bq (specific activity)[a]	DPA Bq (specific activity)[a]
Control	10.80 (0.89)	84.32 (2.52)	59.84 (2.52)
Allopurinol	14.72* (1.38)	97.56* (1.34*)	74.84* (1.25*)
Molybdate	8.12 (1.08)	106.92* (2.75)	72.04* (2.24)
Tungstate	16.24* (0.72)	4.92	4.86
Allopurinol + molybdate	ND[b]	24.60* (—)	81.49* (19.88*)

[a]Specific activity is expressed as Bq nmol^{-1}.
ND = not detected.
Values followed by * are significantly ($P \leq 0.01$) different from control.

Discussion

XAN is oxidized by an aldehyde oxidase

Although mesocarp of ripening avocado fruit has been used extensively to study aspects of ABA biosynthesis (Milborrow, 1976, 1978; Milborrow and Robinson, 1973) the pathway from MVA to ABA and the identity of the endogenous intermediates, particularly those post farnesyl pyrophosphate, have remained elusive. Even so, evidence in support of XAN as the first free C_{15} compound in ABA biosynthesis in avocado mesocarp has been presented (Milborrow and Garmston, 1973; Milborrow *et al.*, 1997). These authors also suggest that XAN is the substrate for the MoCo-containing AO involved in ABA biosynthesis based on observations that 16% of label from the proposed product, xanthoxic acid, was incorporated into ABA (Milborrow *et al.*, 1997) and that inhibition of ABA biosynthesis by tungstate resulted in accumulation of XAN (Lee and Milborrow, 1997*a*, *b*).

There is increasing evidence to suggest that the AO involved in ABA biosynthesis is a MoCo-containing enzyme (Leydecker *et al.*, 1995; Marin and Marion-Poll, 1997; Schwartz *et al.*, 1997*a*; Walker-Simmons *et al.*, 1989). MoCo-containing enzymes require a mononuclear molybdenum (or tungsten) atom co-ordinated via a disulpho moiety to the MoCo (Kisker *et al.*, 1997). The present investigation has shown that tungstate retarded metabolism of ABA by causing accumulation of XAN whereas molybdate and allopurinol increased the production of XAN and ABA. Thus, these results concur with those of Milborrow and co-workers (Milborrow *et al.*, 1997; Lee and Milborrow, 1997*a*, *b*) that XAN is the substrate for AO and that the pathway for ABA biosynthesis in all probability proceeds from XAN via xanthoxic acid. The identical pathway has recently been proposed based on the characterization of products derived from the metabolism of 9′-*cis*-neoxanthin in a cell-free system prepared from citrus peel (Cowan and Richardson, 1997).

Purines as mediators of ABA metabolism

Initially, the effect of a range of CK and CK analogues on ABA metabolism in ripening avocado mesocarp was examined. Previous work demonstrated CK retardation of incorporation of labelled MVL into ABA in avocado, indicative of CK-inhibition of ABA synthesis (Cowan and Railton, 1987*a*). By comparison, the present study strongly suggests that CK stimulates ABA catabolism to PA and DPA rather than inhibiting XAN oxidation and ABA biosynthesis. Depletion of XAN in Z-treated mesocarp, coupled with the increase in specific activity of [^{14}C]XAN in Ade- and 2iP-treated tissue suggested that CK was exerting an effect specifically on the oxidation of XAN. Partial support for this conclusion was obtained from experiments in which mesocarp had been treated with ancymidol. Ancymidol, was the only compound tested that significantly retarded ABA accumulation, apparently by increasing the concentration of XAN. Both ancymidol and Z increased formation of XAN at the expense of ABA in a cell-free system capable of synthesizing ABA from β-carotene (Richardson and Cowan, 1996). Interestingly, ancymidol stimulated the catabolism of ABA causing an increase in PA and DPA to levels similar to those observed in CK-treated mesocarp. Since conversion of ABA to PA is a cytochrome P-450-mediated reaction (Gillard and Walton, 1976; Cutler *et al.*, 1997) these observations indicate a mechanism contrary to the proposed metabolic influence of CKs, i.e. inhibition of cytochrome P-450 mono-oxygenase-catalysed reactions (Coolbaugh, 1984; Grossmann, 1990, 1992). A similar, albeit less pronounced response was observed with the CK analogue, CPPU. Thus, CKs appeared to affect ABA metabolism firstly, by stimulating the oxidation of XAN and, secondly, by enhancing the conversion of ABA to PA and DPA.

ABA enhances its own conversion by inducing ABA 8′-hydroxylase activity (Uknes and Ho, 1984; Cutler *et al.*, 1997). Since CKs enhanced oxidation of XAN, an overall

stimulation of ABA metabolism in response to CK treatment might be expected. Support for this proposal comes from the observation that the CK-resistant *aba1* mutant of *Nicotiana plumbaginofolia* which is MoCo-deficient, is both ABA-deficient and wilty (Blonstein *et al.*, 1991), due specifically to impairment in the oxidation of XAN (Parry *et al.*, 1991). These authors obtained similar results for the *flacca* mutant of tomato, an observation that has recently been confirmed (Marin and Marion-Poll, 1997). Since the *aba1* mutation is clearly involved in the last step of the pathway leading to synthesis of the MoCo for the AO required in ABA biosynthesis (Leydecker *et al.*, 1995), it seems plausible that the biochemical basis for CK–ABA antagonism in higher plants is the result of metabolic interaction at the level of MoCo biosynthesis.

Purines and XAN molybdo-aldehyde oxidase activity

The purine, allopurinol, a potent inhibitor of mammalian xanthine oxidase (plant XDH, see below) but relatively weak substrate for AO, stimulated incorporation of label from 3*R*-[2-^{14}C]MVL into XAN, ABA and DPA and increased the endogenous concentration of ABA, PA and DPA in avocado mesocarp. In humans, allopurinol exerts its effect in a manner consistent with suicide inhibition, i.e. xanthine oxidase converts allopurinol to oxypurinol which inactivates the enzyme (Dollery, 1991). Inhibition of xanthine oxidase causes accumulation of hypoxanthine and xanthine at the expense of ureides. By feedback, overall *de novo* purine formation is decreased. Likewise, allopurinol is converted to oxypurinol in plants and accumulation of xanthine results (Montalbini and Torre, 1995). Since application of allopurinol to nodulated roots of ureide-forming legumes also inhibited XDH and ureide synthesis (Atkins *et al.*, 1992) the biochemical effect of this purine in plants may be similar to that reported for mammals. Thus, accumulation of XAN in the presence of either allopurinol or tungstate and its depletion in response to molybdate confirms that the activity responsible for oxidation of XAN is a MoCo-containing AO.

The CKs, 2iP and Z appeared to influence XAN oxidation to a greater extent than Ade. In fact, the specific activity of [^{14}C]XAN, derived from 3*R*-[2-^{14}C]MVL in the presence of CKs, was: Ade < 2iP < Z. Similarly, in experiments on the incorporation of label from 3*R*-[2-^{14}C]MVA into ABA in a cell-free system from barley embryos the effect was Z > 2iP > Ade (Cowan and Railton, 1987*b*).

In higher plants, CK homeostasis is regulated by positive feedback and/or CK-induced CK-oxidase activity (Kaminek *et al.*, 1997). CK-oxidase cleaves the N^6 side chain of isoprenoid CKs with the release of Ade. Commonly occurring nucleobases including Ade, are readily oxidized by mammalian xanthine oxidase, but not by AO (Hall and Krenitsky, 1986; Krenitsky *et al.*, 1972, 1986). Treatment with Ade routinely enhanced ABA metabolism in the present study and stimulated ABA biosynthesis in ABA-producing fungi (Norman *et al.*, 1983*a*).

The *aba3* mutant of *Arabidopsis* lacks the AO activity to produce ABA from AB-aldehyde whereas nitrate reductase activity remains unimpaired (Schwartz *et al.*, 1997*a*). Since treatment with Na_2S plus dithionite restored AB-aldehyde oxidase activity, these authors presumed that the lack of AO-MoCo sulphurylation represented the genetic lesion in the *aba3* mutant. Similarly, Marin and Marion-Poll (1997) suggested that the *flacca* mutant of tomato is deficient in the MoCo needed for AO activity and that additional steps in the synthesis of the MoCo are required to facilitate ABA formation. Implicit in these arguments is the requirement for sulphurylation of MoCo required for activity of XDH/AO. As outlined above, Ade and allopurinol are efficient substrates for XDH not AO. Thus, in response to allopurinol, Ade and N^6 substituted Ade-type CKs the activity of AO relative to XDH is enhanced. Although the subject of our current investigations, partial support for this proposal includes the recent observation that AO activity and ABA levels of roots of barley grown with ammonium are higher, and nitrate reductase activity lower, than in nitrate-fed plants (Omarov *et al.*, 1998).

Molybdenum cofactor biosynthesis and ABA metabolism: a proposal

MoCo biosynthesis utilizes either the mono-, di-, or triphosphate derivative of guanosine, a product of inosine monophosphate metabolism, as the precursor of the pterin heterocycle in a pathway that is similar for animals, plants, fungi, algae, and bacteria (Mendel, 1997; Rajagopalan, 1997). Recent studies on MoCo biosynthesis in mutant strains and wild-type *E. coli* have revealed formation of an intermediate, termed precursor Z, which is derived from guanosine and incorporated into MPT with retention of all ten carbon atoms (Rajagopalan, 1997). In the presence of molybdenum, MPT is converted to MoCo by the attachment of molybdenum to the dithiolene to give an inactive dioxo-MoCo which is then inserted into the AO/XDH apoproteins and activated by sulphurylation. Based on results presented in this paper, it is proposed that accumulation of inosine monophosphate and/or guanosine monophosphate due to CK-induced retardation of xanthine oxidase-type enzyme activity, increases the availability or level of MoCo required for oxidation of XAN in ABA biosynthesis. The proposed relationship between CK (and allopurinol) and purine metabolism, and MoCo and ABA biosynthesis is illustrated in Fig. 2. According to this scheme, CK homeostasis which is regulated by positive feedback and/or CK-induced CK oxidase activity (Kamínek *et al.*,

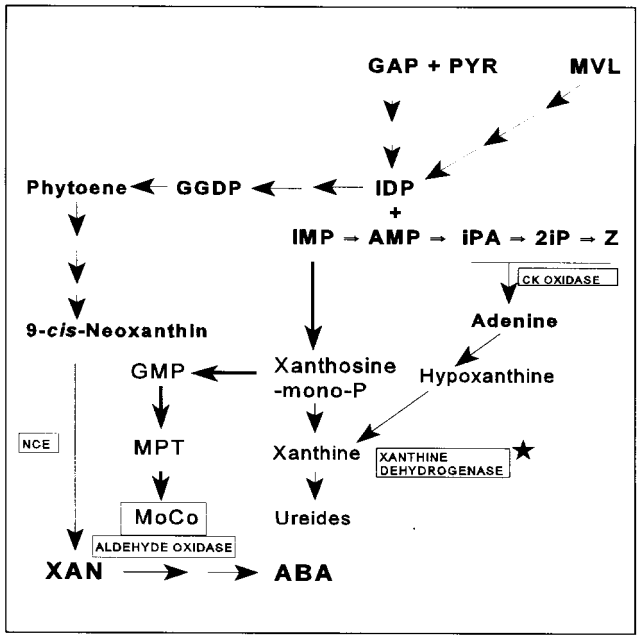

Fig. 2. Proposed model illustrating the mechanism of action of allopurinol in relation to purine metabolism and CK production, MoCo biosynthesis, and conversion of XAN to ABA (see text for details). (✱) Site of action of allopurinol and N^6-substituted purines. AMP, adenosine monophosphate; GAP, glyceraldehyde phosphate; GGDP, geranylgeranyl diphosphate; GMP, guanosine monophosphate; IDP, isopentenyl diphosphate; IMP, inosine monophosphate; iPA, isopentenyladenosine; MPT, molybdopterin; NCE, neoxanthin cleavage enzyme.

1997), contributes to the modulation of purine metabolism at the level of xanthine and hypoxanthine oxidation. Consequently, the endogenous pool of nucleotide phosphates and MoCo is increased. In a feedforward mechanism, sulphurylation of MoCo is facilitated which enhances the AO-catalysed conversion of XAN to ABA.

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