PLANT HORMONE HOMEOSTASIS AND THE CONTROL OF 'HASS' AVOCADO FRUIT SIZE

by

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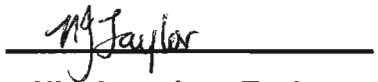
DECLARATION

I hereby declare the research work reported in this dissertation is the result of my own investigation, except where acknowledged.

Signed: 

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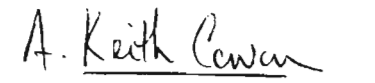
Signed: 

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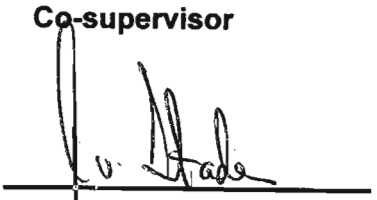
Signed: 

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Down how many roads among the stars must
man propel himself in search of the final
secret? The journey is difficult, immense,
at times impossible, yet that will not
deter some of us from attempting it.....
We have joined the caravan, you might say,
at a certain point, we will travel as far
as we can, but we cannot in one lifetime
see all that we would like to see or to
learn all that we hunger to know.

- Loren Eiseley
The Immense Journey

"Whatever I am now it is all because God poured out such kindness and grace upon me"

1 Cor. 15:10

LIST OF ABBREVIATIONS

ABA	abscisic acid
AB-alcohol	abscisic alcohol
AB-ald	abscisic aldehyde
ABA-GE	abscisic acid glucose ester
ABAMe	abscisic acid methyl ester
ACC	1-aminocyclopropane-1-carboxylic acid
ADP	adenosine diphosphate
AI	acid invertase
allo	allopurinol
allo+Mo	allopurinol plus molybdate
AMP	adenosine monophosphate
AO	aldehyde oxidase
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
d	days
DAFB	days after full bloom
DCIP	dichloroindophenol
DDC	diethyldithio carbamic acid
DHZ	dihydrozeatin
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethyl sulfoxide
DOXP	1-deoxy-D-xylulose-5-phosphate
DPA	dihydrophaseic acid
DTT	dithiothreitol
DW	dry weight
CDK	cyclin-dependent protein kinase
CK	cytokinin
CKI	cyclin-dependent kinase inhibitor
CKOX	cytokinin oxidase
4-Cl-IAA	4-chloroindole-3-acetic acid
EDTA	ethylene diamine tetra-acetic acid
FAD	flavin adenine dinucleotide
FW	fresh weight
G_1	gap 1
G_2	gap 2
GA	gibberellin
GC-EI-MS	gas chromatography-electron impact-mass spectrometry
GTP	guanosine triphosphate
h	hour
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
8'-HOABA	8'-hydroxy abscisic acid

HPLC	high performance liquid chromatography
HXK	hexokinase
IAA	indole-3-acetic acid
IA-ald	indole-3-acetaldehyde
IA-ald oxidase	indole-3-acetaldehyde oxidase
IAAMe	indole-3-acetic acid methyl ester
I-ald	indole-3-aldehyde
IAM	indole-3-acetamide
IAN	indole-3-acetonitrile
IBA	indole-3-butyric acid
IFL	interfascicular fibreless
iP	isopentenyladenine
iPA	isopentenyladenosine
IPA	indole-3-pyruvic acid
iPDP	isopentenyladenosine-5'-diphosphate
iPMP	isopentenyladenosine-5'-monophosphate
IPP	isopentenyl pyrophosphate
IPT	DMAPP:AMP isopentenyltransferase
iPTP	isopentenyladenosine-5'-triphosphate
K	kinetin
M	mitosis
MHS	Mo-hydroxylase sulfurase
min	minute
MoCo	molybdenum cofactor
MPT	molybdopterin
MTT	3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MVA	mevalonic acid
MVL	mevalonolactone
NAA	α-naphthalene acetic acid
NAD	nicotinamide adenine dinucleotide
NCED	9-Z-epoxycarotenoid dioxygenase
NR	nitrate reductase
PA	phaseic acid
PAGE	polyacrylamide gel electrophoresis
PMS	phenazine methosulfate
Polymin P	polyethylenimine
PRL1	pleiotropic regulatory locus 1
PVP	polyvinylpolypyrrolidone
REV	revoluta
RIA	radioimmunoassay
S	DNA synthesis
SDS	sodium dodecyl sulfate
sec	seconds
sis	sugar insensitive
SNF1	sucrose non-fermenting 1
SnRK1	SNF1-related protein kinase 1

SO	sulfite oxidase
SPE	solid phase extraction
SPS	sucrose phosphate synthase
SuSy	sucrose synthase
TAO	tomato aldehyde oxidase
TEMED	N, N, N', N'-tetramethylethylenediamine
TLC	thin layer chromatography
Trp	tryptophan
VLC	vacuum liquid chromatography
XAN	xanthoxal
XAN-acid	xanthoxic acid
XAN oxidase	xanthoxal oxidase
XDH	xanthine dehydrogenase
XO	xanthine oxidase
Z	zeatin
ZDP	*trans*-zeatin-riboside diphosphate
ZMP	*trans*-zeatin-riboside monophosphate
ZR	zeatin riboside
ZTP	*trans*-zeatin-riboside triphosphate

ABSTRACT

The 'Hass' avocado produces two distinct phenotypically different populations of fruit, i.e. normal and small fruit. The small fruit variant is characterized by early seed coat senescence that results in arrested growth, due to dramatically reduced cell cycle activity. This system has been used to study the metabolic control of fruit growth for two reasons. Firstly, the 'Hass' avocado is a major export crop in South Africa and unmarketable small fruit cost the industry millions of rands per season. Secondly, in the absence of evergreen tree-crop mutants with which to dissect controlling mechanisms contributing to the control of final fruit size, the 'Hass' avocado and its small fruit variant provides an ideal system to investigate the physiology, biochemistry and molecular biology of fruit growth in subtropical species. A detailed study was conducted to probe the contribution of hormones in the control of final fruit size by comparing and contrasting tissue distribution and content of hormones in developing 'Hass' avocado and its small fruit variant. In addition the proposal that changes in hormone homeostasis occur as a result of differences in the allocation of the molybdenum cofactor (MoCo) and changes in the activity of xanthine dehydrogenase (XDH) and the aldehyde oxidases (AO) involved in abscisic acid (ABA) and indole-3-acetic acid (IAA) metabolism was evaluated.

Activity of XDH, xanthoxal (XAN) oxidase, indole acetaldehyde (IA-ald) oxidase and cytokinin oxidase (CKOX) was related to tissue content and composition of IAA and ABA. Comparisons between normal and small fruit revealed that under conditions where CKOX is elevated, the increased adenine produced inhibits XDH activity, which leads to elevated activity of the AOs involved in ABA and possibly IAA biosynthesis as a result of increased MoCo allocation to these enzymes. Further analyses revealed that both cytokinin (CK) and auxin elevates CKOX activity and that adenine and CK do indeed inhibit XDH activity, which leads to increased AO activity. In addition, application of CK to normal fruit increased IAA in mesocarp tissue but reduced IAA content of seed tissue and reduced ABA in mesocarp tissue but had no effect on ABA in seed tissue. Cytokinin oxidase therefore contributes to the regulation of ABA and IAA metabolism during plant organ growth by modulating the activity of XDH.

Low XDH and IA-ald oxidase activity together with high XAN oxidase and CKOX activity early in fruit development combine to reduce both elongation and radial growth, which results in the appearance of the 'Hass' small fruit phenotype. This event was associated with high ABA and low IAA in seed tissue of small fruit, but high ABA and IAA in seed coat and mesocarp tissue of these fruit. Thus, whilst low IAA in seed tissue is associated with reduced growth the

reverse is true in seed coat and mesocarp tissue where high IAA retards tissue growth. Calculation of CK/ABA and CK/IAA ratios revealed that a decrease in these ratios was found in mesocarp tissue of small fruit. However, in seed tissue of small fruit both IAA and ABA were decreased relative to CK. The maintenance of the correct hormonal balance in avocado fruit thus ensures the continuation of cell division cycle activity, with any changes responsible for the high incidence of a small fruit variant in the 'Hass' avocado.

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1 INTRODUCTION

1.1 THE AVOCADO

1.1.1 Fruit anatomy and growth

The avocado (*Persea americana* Mill.) fruit is a berry, consisting of two distinct tissues viz. the seed and pericarp. The seed comprises three tissues, the embryo, endosperm and seed coat. Most of the embryo consists of two large cotyledons, which surround a centrally attached embryonic axis. The cotyledons are formed of undifferentiated parenchyma tissue interspersed with idioblasts. Starch is the main storage material in the cotyledons and is present in great quantities (Cummings and Schroeder 1942). A gelatinous endosperm surrounds the embryo in the early stages of fruit development, but it disappears completely approximately three months after fruit set (Blumenfeld and Gazit 1974). The seed coat surrounds the embryo and endosperm and consists of two closely associated cell layers. The vascularized part of the seed coat represents the pachychalaza and arises from basipetal, intercalary growth in the chalazal region, near the site of attachment of the integuments (Boesewinkel and Bouman 1984). This portion of the seed coat is vital to the fruit as it supplies sugars, mineral nutrients and water to the developing seed (Steyn *et al.* 1993). It also allows for exchange between the developing embryo and the rest of the fruit and tree (Blumenfeld and Gazit 1971). Later in the development of the fruit, the drying of the seed coat reduces the supply of 'food' to the embryo. This accompanies the cessation of embryo growth prior to germination, which subsequently heralds the accumulation of lipids in the mesocarp (Blumenfeld 1970). The pericarp comprises three layers: the exocarp, which is the skin or rind; the fleshy mesocarp, which is the edible part of the fruit; and the endocarp, which is the thin inner layer next to the outer seed coat (Cummings and Schroeder 1942). The mesocarp consists of large iso-diametric parenchyma cells that accumulate lipids during fruit maturation (Biale and Young 1971). It is permeated by vascular tissue that runs from the pedicel and coalesces near the chalazal apex, where it enters the seed coat (Cummings and Schroeder 1942).

Growth of the avocado fruit follows a sigmoid growth curve that consists of three distinct growth stages, with cell division occurring throughout the life of the fruit, unlike most other fruit (Schroeder 1953; 1958; Coombe 1976). Stage 1 is a slow lag phase lasting 10 weeks after full bloom, where fruit size and mass increase as a result of cell division. Stage 2 is a linear phase of rapid growth lasting 30 weeks, where cell expansion is the major process and cell division is

reduced. Finally stage 3 is a mature phase of slow growth, where cell division and cell expansion are both reduced (Schroeder 1953; Valmayor 1967; Zilkah and Klein 1987). The number and volume of cells at ripeness, and therefore fruit size at maturity, are thus influenced by the number and volume of cells at anthesis and the rate and duration of cell division and cell expansion thereafter (Coombe 1976).

In the 'Hass' avocado cultivar two distinct, phenotypically different populations of fruit, i.e. normal and small fruit, are produced (Zilkah and Klein 1987). Every 'Hass' avocado tree produces these small fruit variants and the proportion ranges between 5-20 % of the total crop (Kremer-Köhne and Köhne 1995). The incidence of small fruit increases in trees bearing heavily (Lahav and Kalmer 1977) and in stressed, older trees (Whiley *et al.* 1996; Cowan 1997) grown under warm conditions (Cutting 1993). Small fruit are regarded as less than 200 g fresh mass, or "count-size" of 20 or more fruits per standard 4 kg export carton (Köhne 1992). Up to 50 % of the crop in any one year, can be undersized.

1.1.2 The small fruit phenotype

The small fruit phenotype is characterised by early seed coat senescence, which in many instances is associated with 'pedicel ring neck'. Figure 1.1 illustrates the visible differences between normal and small fruit. Seed coat senescence can occur at any time during development and there is no pattern with respect to the distribution of the small fruit phenotype on the tree (Cowan *et al.* 1997). The earlier the seed coat senesces the smaller the fruit is likely to be (Blumenfeld and Gazit 1974; Steyn *et al.* 1993). As cell division occurs throughout the life of the avocado fruit, from fruit set to maturity (Schroeder 1953), it is important that symplastic continuity is maintained throughout this period in order to sustain the supply of sugars to the fruit and thus ensure continued growth and development (Ehlers and Kollmann 1996). The bulk movement of solutes into developing avocado fruit occurs along the following path: pedicel vasculature to mesocarp vasculature to chalaza to seed and/or seed coat vasculature to the mesocarp (Moore-Gordon *et al.* 1998). The maintenance of viability of the pachychalaza (i.e. seed coat) is therefore vital in order to assure a continued supply of sugars, mineral nutrients, water and possibly plant hormones to the developing fruit. If seed coat viability is not maintained the supply of these important compounds will be severed and the maintenance of the current rate of cell division will not be possible. The limiting factor for the growth of the phenotypically small fruit variant thus appears to be cell number and not cell size (Cowan *et al.* 1997), as found in tomato (Ho and Hewitt 1986; Bohner and Bangerth 1988a; Ho 1992), apricots (Jackson and Coombe 1966) and

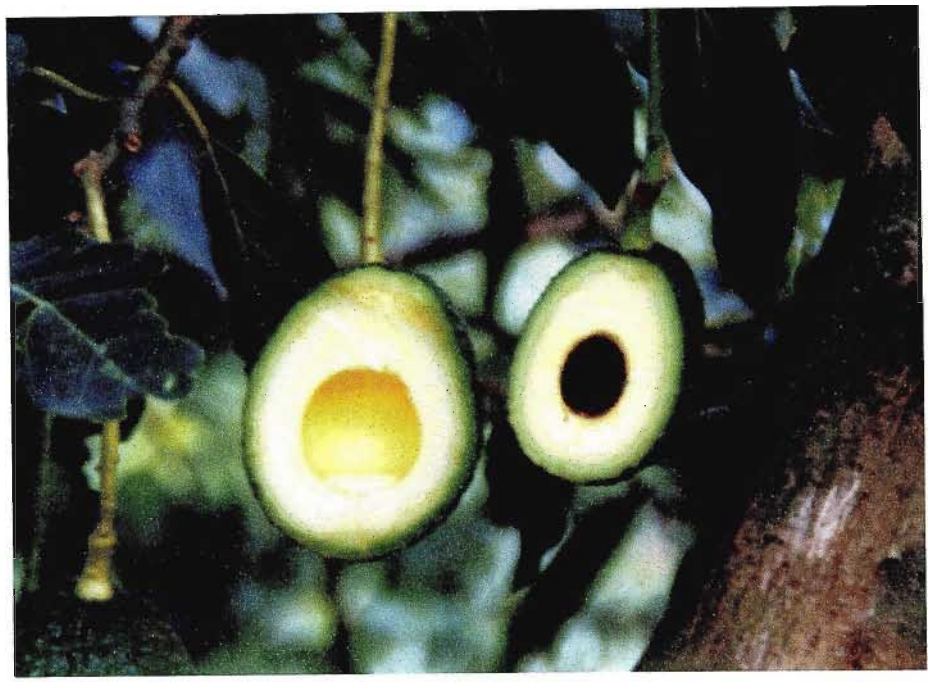


Figure 1.1 **Photograph illustrating the relationship between fruit size and seed coat viability. The normal fruit has a yellow, fleshy, healthy and functional seed coat, whereas the small fruit has a brown, senesced and non-functional seed coat.**

grapes (Harris *et al.* 1968). Cell number and size have also been found to be important factors influencing the capacity of fruit to import assimilates which emphasises the proposal that they are the determinants of final fruit size (Bohner and Bangerth 1988b). Furthermore, biochemical characterisation of the small fruit variant has revealed reduced 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34) and sucrose synthase (SuSy; EC 2.4.1.13) activity, and enhanced insoluble acid invertase (AI; EC 3.2.1.26) activity, which was associated with increased respiration, sucrose depletion, an increase in glucose as a proportion of total soluble sugars and increased abscisic acid (ABA) metabolism (Richings *et al.* 2000). In addition to this, fruit size has been found to be negatively correlated with the ABA/cytokinin (CK) ratio (Moore-Gordon *et al.* 1998). Cell division and sink strength therefore appear to be key elements in the development of the small fruit phenotype and thus attempts to elucidate this phenomenon have centred on factors controlling these two elements (Cowan *et al.* 1997; Moore-Gordon *et al.* 1998; Richings *et al.* 2000; Cowan *et al.* 2001).

1.2 PLANT HORMONES AND FRUIT GROWTH

1.2.1 Hormones and general fruit growth

Developing fruits are terminal sinks and require carbohydrates, other metabolites, mineral nutrients and water to sustain growth. One potential role of phytohormone signalling in fruit

growth could be to detect changes in sugar content and composition and as a consequence co-ordinate or re-direct growth. The corollary of this is that carbohydrates impact on hormone metabolism. Implicit in this is cross-talk between sugar and hormone signalling pathways. In fact, development is considered to be a result of intricate spatial and temporal interactions between resources required for growth and hormonal mediation through regulation of gene expression (Cowan *et al.* 2001).

Fruit development is governed by three genetic factors: (1) one which determines the number of carpel cells; (2) one which determines the number of cell divisions during the cell proliferation phase; and (3) one which determines the duration and enlargement rate of individual cells in the cell enlargement phase (Higashi *et al.* 1999). It is currently unknown which genetic factor plays the most important role in controlling final fruit size in higher plants (Higashi *et al.* 1999), but environmental factors, such as temperature, light, water and nutrients, can modify the expression of these genes. In addition to these environmental factors, phytohormones are also believed to be involved at most stages of fruit development, directing development from fertilization to senescence (Nitsch 1970; Coombe 1976; Gillaspy *et al.* 1993). No single growth regulator can be solely responsible for fruit morphogenesis, as phytohormones exert multiple control on organ development by alterations in concentration and as a result of changes in sensitivity of the affected tissues (Bradford and Trewavas 1994). Auxin, gibberellin (GA), CK, ABA and ethylene are all produced by fruit, often in large amounts, and often in a sequence typical for a species. Sometimes these compounds act synergistically in promoting growth, sometimes they substitute for one another and sometimes they act antagonistically. The general picture therefore involves control through interaction and balance of these hormones (Coombe 1976).

The important role that plant hormones play in fruit development is demonstrated by the fact that they enhance cell division and enlargement during different stages of development (Mapelli *et al.* 1978; Mapelli 1981; Kinet *et al.* 1985; Bohner and Bangerth 1988b). Furthermore, it is maintenance of the correct hormonal balance that ensures continuation of cell division (Nagl 1971; Nagl 1972; Barlow 1976; Nagl 1976). Both ABA and water stress are known to retard cell division cycle activity (Myers *et al.* 1990; Artlip *et al.* 1995), possibly through inhibition of nucleic acid and protein synthesis (Owen and Napier 1988). Cytokinin, on the other hand, promotes this event (Jacobs 1995). Cytokinin seems to control cell division by regulating the G_2 to M transition, i.e. the transition of cells from the stage following DNA replication to mitosis (M) and as such the withdrawal of CK causes the cessation of the cell cycle and accumulation of cells in M, S (period

of DNA replication) and G_1 (Mader and Hanke 1996). An imbalance in the CK/ABA ratio, through reduced CK synthesis or increased ABA synthesis, will therefore impact on cell division cycle activity and final fruit size, as seen in the 'Hass' avocado (Moore-Gordon *et al.* 1998). The highest levels of auxin are also found in regions of active cell division including apical meristems, cambium, developing fruit and in embryos and endosperm (Schneider and Wightman 1978). Auxin is thought to act synergistically with CK in the control of cell division and is able to control the rate of cell enlargement in fruit (Rayle and Cleland 1992; Ferreira *et al.* 1994), presumably by causing an increase in the extensibility of cell walls and by inducing uptake of water and solutes (Hackett and Thimann 1952; Cosgrove 1997). Plant hormones must therefore regulate diverse and even antagonistic signals. The decision of a cell to divide or not can therefore be perceived as the digital output of analogous signals that stimulate and inhibit the cell cycle (Grill and Himmelbach 1998).

There is good support for the hypothesis that developing seeds produce the hormones necessary for the initial growth of fruits (Crane 1969; Nitsch 1970). This is emphasised by the fact that in normal fruit development the developing embryo, or seed, controls the rate and sustenance of cell division in the surrounding tissue (Gillaspy *et al.* 1993). Thus the seed(s) of the fruit and the surrounding seed coat(s) must remain viable in order to ensure a continued and balanced supply of hormones to the fruit and therefore the continuation of cell division cycle activity and fruit growth. Implicit in this is the observation that there is a close correlation between seed size and final fruit size. In order to assess how cell division is controlled in fruit, an understanding needs to be gained of the hormones controlling this process. Since endogenous hormone concentration of tissues is a balance between biosynthesis, catabolism, import and export (Hetherington and Quatrano 1991), the determination of plant hormone levels alone is often of limited value. Such data reveals little about the site of hormone metabolism within the tissue under investigation, and very little can be inferred about the contribution of hormone metabolism to changes in net hormone levels. One way to minimize this limitation is to measure the activity of key enzymes involved in phytohormone metabolism, in addition to quantifying endogenous hormone levels.

1.2.2 Hormone content of avocado tissues

Information about the hormone content and composition of the major tissues of avocado fruit is rudimentary and most available data is based on bioassay (Fig. 1.2). Gibberellin-like activity was observed in extracts of endosperm and seed coat tissue but not in the mesocarp or embryo

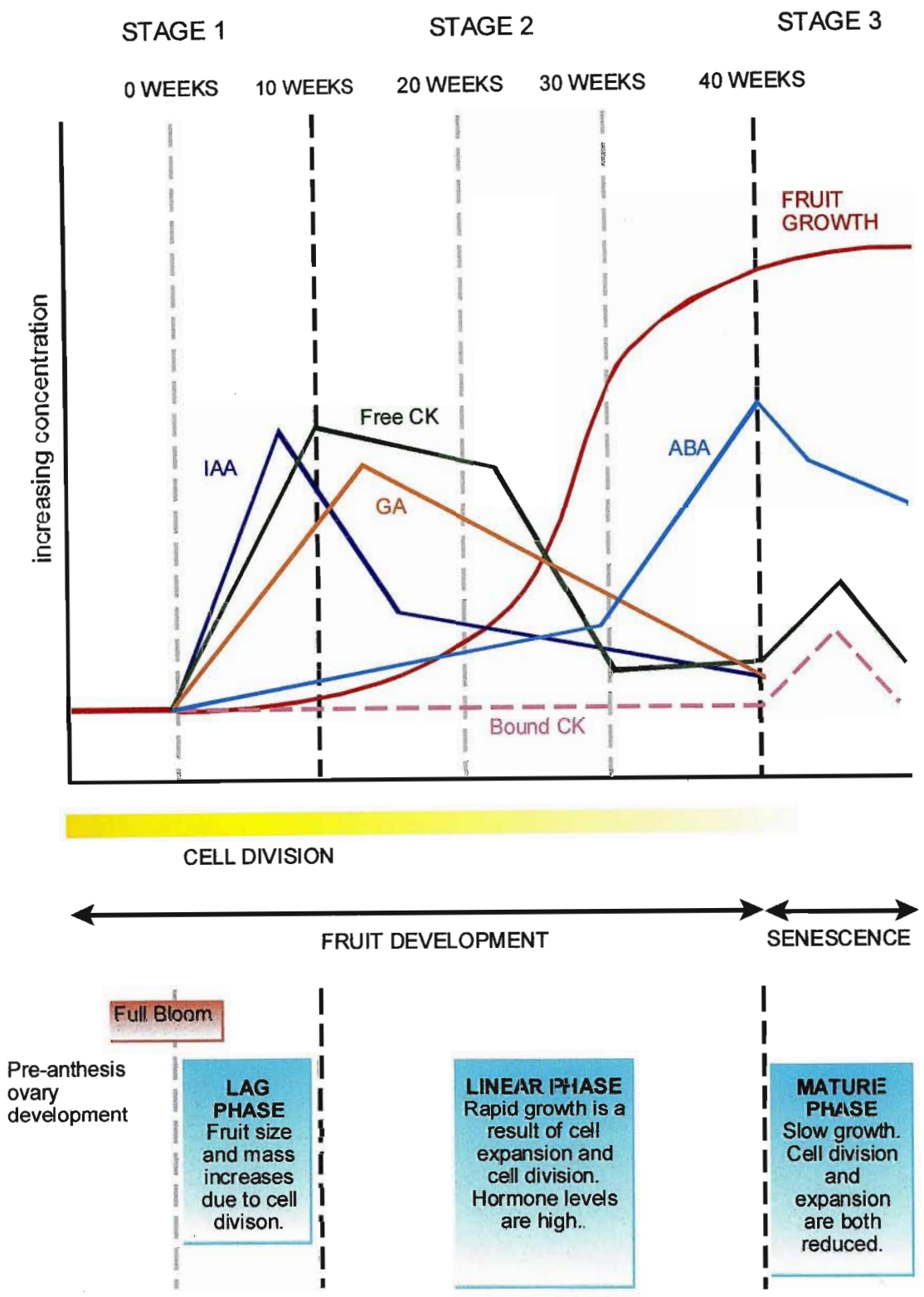


Figure 1.2 **Simplified model of avocado fruit development and corresponding physiological changes with respect to plant hormones. Trends in concentration are on a whole fruit basis and thus it does not always reflect the status of all fruit tissues at any particular time (adapted from Bower and Cutting 1988).**

(Blumenfeld and Gazit 1972). Cytokinin activity was high in the embryo, endosperm and seed coat, with levels declining over the course of fruit growth (Blumenfeld and Gazit 1970). No CK activity was detected in the mesocarp tissue (Gazit and Blumenfeld 1970). Auxin-like activity was higher in seed tissue than in mesocarp (Gazit and Blumenfeld 1972) whilst ABA, which declines as fruit approach maturity (Cowan *et al.* 1997), occurs in similar amounts in seed and mesocarp tissue (Richings *et al.* 2000; Taylor and Cowan 2001). Together, these data suggest that the seed is the primary source of hormones required for avocado fruit growth and development. This is supported by the observation that seed-bearing fruits are considerably larger than seedless fruits ('cukes') which are frequently found in the cultivars of 'Fuerte' and 'Ettinger' (Blumenfeld and Gazit 1974) and which appear to result from early seed degeneration rather than stimulative or true vegetative parthenocarpy (Tomer *et al.* 1980).

1.3. HORMONE METABOLISM

1.3.1 Cytokinin metabolism

Cytokinin affects many plant developmental processes including cell division, cellular differentiation, senescence and apical dominance (Mok 1994). They are defined by their ability to promote cell division in tissue culture in the presence of auxin (Miller *et al.* 1955; Skoog *et al.* 1965). Naturally occurring CKs are adenine derivatives with at least one substituent at the N^6-position. These substituents may be classed into three groups: (1) isoprenoid (zeatin, N^6-Δ^2-isopentenyladenine and its derivatives); (2) isoprenoid-derived (dihydrozeatin and its derivatives); and (3) aromatic CKs (Zažímalová *et al.* 1999). The level of active CK at a particular site of action is the result of several factors which include: *de novo* synthesis; oxidative degradation; formation and hydrolysis of inactive conjugates; transport into and out of particular cells; and subcellular compartmentation to or away from sites of action (Brzobohatý *et al.* 1994).

1.3.1.1 Cytokinin biosynthesis

There are two proposed biosynthetic pathways of CKs, however, in the last year Åstot and co-workers (2000) proposed a third possible route for the synthesis of *trans*-zeatin riboside. Initially it was speculated that tRNA degradation was a possible source of free, active CKs (Swaminathan and Bock 1977), but this was disproved when calculations of tRNA turnover rates revealed that this pathway contributed very little to the overall pool of CKs (McGaw and Burch 1995; Chen 1997) and that a tRNA independent *de novo* biosynthetic pathway must also be present in plants (Klämbt 1992).

A second pathway was proposed after a CK biosynthetic enzyme was found in the slime mold, *Dictyostelium discoideum* (Taya *et al.* 1978), which was able to convert adenosine monophosphate (AMP) and dimethylallyl-pyrophosphate (DMAPP) to the free CKs isopentenyladenosine-5'-monophosphate (iPMP) and the corresponding nucleoside (isopentenyladenosine; iPA) (Fig. 1.3). This finding together with studies on the metabolism of isopentenyl CKs (Miura and Miller 1969; Miura and Hall 1973) led to the proposal that iPMP is also the primary CK intermediate in plants, and that zeatin CKs are formed by hydroxylation of iPMP and its derivatives (Chen 1982; Letham and Palni 1983). 5'-Nucleotidase (Chen and Kristopeit 1981a), followed by adenosine nucleosidase (Chen and Kristopeit 1981b) are expected to be the enzymes responsible for the step-by-step conversion of iPMP to the base N^6-Δ^2-isopentenyladenine (iP). Subsequently, the product of the T-DNA gene 4 (*ipt*) of *Agrobacterium tumefaciens* was also described as a DMAPP:AMP isopentenyltransferase (ipt) (Akiyoshi *et al.* 1984; Barry *et al.* 1984; Morris *et al.* 1993). Activity of an ipt in plants has been found in crude extracts from CK-autotrophic tobacco callus (Chen and Melitz 1979) and from immature maize kernels (Blackwell and Horgan 1994), but until recently the corresponding enzyme has not been purified to homogeneity. However, Kakimoto (2001) has recently found, through Arabidopsis database searches, an *AtIPT4* gene that has unique DMAPP:ATP/ADP isopentenyltransferase activity in its purified form and is functional in plants (Fig. 1.3). Tests for substrate specificity revealed that AtIPT4 was DMAPP:ATP/ADP isopentenyltransferase and had no activity of DMAPP:AMP isopentenyltransferase, which indicates that the major function of AtIPT4 in plant cells is the isopentenylation of ATP and ADP (Kakimoto 2001).

Recently, a third pathway has been demonstrated in *Arabidopsis thaliana* and, although the exact reactions constituting this pathway have yet to be identified, it is proposed that *trans*-zeatin-riboside monophosphate (ZMP) is synthesised independently of iPMP and that two different precursors are used by the ipt to synthesise ZMP and iPMP (Åstot *et al.* 2000) (Fig. 1.3). The iPMP-independent pathway is thought to use a precursor that is derived from the acetate/mevalonic acid (MVA) pathway of terpenoid biosynthesis, as there was a significant reduction in deuterium incorporation into ZMP in the presence of metyrapone and mevastatin, inhibitors of HMGR. This iPMP-independent pathway has been demonstrated to exist in both the transgenic *A. thaliana* expressing the *A. tumefaciens ipt* gene and as part of normal wild-type plant metabolism (Åstot *et al.* 2000), suggesting that the relative contributions of the iPMP-dependent and -independent pathways may vary in a tissue- and time-dependent manner. These two recent discoveries concerning CK biosynthesis are not necessarily mutually exclusive and

can be linked together to form an iPMP-independent pathway of CK biosynthesis in which one of the methyl groups of isopentenyladenosine-5'-triphosphate (iPTP) and isopentenyladenosine-5'-diphosphate (iPDP) are hydroxylated to produce *trans*-zeatin-riboside triphosphate (ZTP) and *trans*-zeatin-riboside diphosphate (ZDP), respectively, followed by dephosphorylation to produce ZMP (Kakimoto 2001).

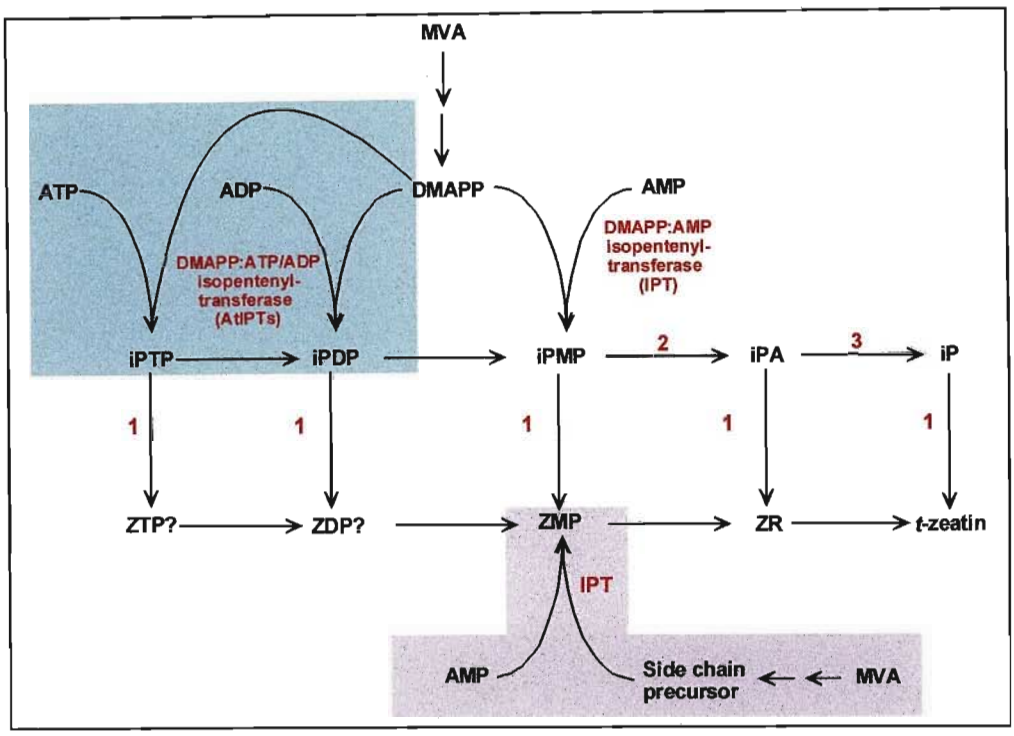


Figure 1.3 **A model for CK biosynthesis in plants (adapted from Kakimoto 2001). The blue highlighted block indicates the proposed model by Kakimoto (2001), where CK biosynthesis is initiated by the addition of the isopentenyl side chain to ATP and ADP. The pink highlighted block indicates the iPMP-independent pathway proposed by Åstot *et al.* (2000). Enzymes catalysing steps in CK biosynthesis are 1) hydroxylase, 2) 5'-nucleotidase and 3) adenosine nucleosidase. ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; DMAPP, dimethylallyl pyrophosphate; iP, isopentenyladenine; iPA, isopentenyladenosine; iPDP, isopentenyladenosine-5'-diphosphate; iPMP, isopentenyladenosine-5'-monophosphate; IPT, isopentenyltransferase; iPTP, isopentenyladenosine-5'-triphosphate; MVA, mevalonic acid; ZDP, *trans*-zeatin-riboside diphosphate, ZMP, *trans*-zeatin-riboside monophosphate; ZTP, *trans*-zeatin-riboside triphosphate.**

Assuming that CKs are derived *in situ* in fruit by the isoprenylation of purines (Binns 1994), the inhibition of isoprenoid biosynthesis will limit the availability of DMAPP, and other side chain donors of terpenoid origin, needed for CK biosynthesis. These precursors are formed from MVA, which is derived from acetate in the cytosol, as demonstrated by Åstot *et al.* (2000), or from triose phosphates (e.g. glyceraldehyde-3-phosphate) derived from the 1-deoxy-D-xylulose-5-phosphate

(DOXP) pathway in plastids (Lichtenthaler 1999). In the acetate/MVA pathway the formation of isopentenyl pyrophosphate (IPP; an interconvertible isomer of DMAPP) is catalysed by the enzyme HMGR (Chappell 1995) and this implies that any factor affecting the activity of this enzyme will impact on CK biosynthesis. In fact, Crowell and Salaz (1992) have suggested that CK biosynthesis is more sensitive to HMGR inhibition than the biosynthesis of any other essential isoprenoids. It will also impact on other plant hormones, including ABA, GA and brassinosteroids, as IPP also serves as a precursor for these compounds. The same is true for the DOXP pathway, in that any factor limiting flow through this pathway will limit IPP synthesis and thus CK and other hormone biosynthesis. This common metabolic origin could serve as one possible explanation for the known metabolic interaction between plant hormones, eg. ABA inhibition of germination and CK-mediated release of seed dormancy (Salisbury 1994).

1.3.1.2 Cytokinin catabolism

Cytokinin catabolism includes mutual conversions among CK bases, ribosides and ribotides (i.e. riboside-5'-monophosphate), conjugation (*N*-glucosylation, *N*-alanyl conjugation, *O*-glucosylation and *O*-acetylation), conjugate-hydrolysing reactions and degradative (i.e. oxidation) reactions (Zažímalová *et al.* 1999). It is believed that the oxidative degradation of CKs is the principle point of inactivation, as the dominant CKs accumulated in plants are substrates of the degradative enzyme (Jones and Schreiber 1997).

Cytokinin degradation occurs via the cleavage of the N^6 side chain, through the action of the enzyme CK oxidase (CKOX; EC 1.4.3.6) (Kamínek *et al.* 1997), releasing adenine or its derivatives and the corresponding side chain aldehyde, in the presence of molecular oxygen. It will only degrade CKs bearing an unsaturated side chain (Hare and van Staden 1994), with the preferred substrate for many CKOXs being iP (Pačes and Kamínek 1976; McGaw and Horgan 1983; Chatfield and Armstrong 1986; Armstrong and Firtel 1989; Laloue and Fox 1989; Auer *et al.* 1999; Bilyeu *et al.* 2001). It can, however, also degrade zeatin and its ribonucleosides (Kamínek and Armstrong 1990). Ultimately, the result of this degradation is the irreversible loss of CK structure and thus biological activity. In this way CKOX is thought to play an important part in controlling the internal pool of CK in plants. A CKOX enzyme has been partially purified from maize, beans, poplar, wheat and *Vinca rosea* crown gall tissues (Armstrong 1994; Hare and van Staden 1994; Jones and Schreiber 1997). The wide occurrence of this oxidase is matched by its diversity. They vary in mass from 25 kDa in *Vinca rosea* to 94 kDa for maize (McGaw and Horgan 1983; Armstrong 1994). Most, but not all, of the enzymes are glycoproteins, with pH optima

ranging from 6.0 to 9.0 (Kamínek and Armstrong 1990; Armstrong 1994). Initially classified as a copper-containing amine oxidase (Burch and Horgan 1989), there is new evidence to suggest that this is incorrect (Galuszka *et al.* 1998) and that it is in fact a FAD-containing flavoprotein (Houba-Hérin *et al.* 1999; Morris *et al.* 1999) and thus it could belong to the large family of FAD amine oxidases (Rinaldi and Comandini 1999). However, the diverse properties of CKOXs indicate that there may be more than one class of CK degradative enzyme (Mok and Mok 2001).

Available evidence suggests that the activity of CKOX and the degradative metabolism of CKs can be mediated by four principle mechanisms: (1) CK supply; (2) application of phenylurea compounds; (3) auxin levels; and (4) glycosylation and/or isozyme variation (Jones and Schreiber 1997). Both substrate and non-substrate CKs (Kamínek *et al.* 1997) and auxin (Coenen and Lomax 1997) appear to stimulate activity, whilst phenylurea-type CKs inhibit activity (Laloue and Fox 1989). Glycosylation, on the other hand, is thought to play a role in the compartmentation and activity of the enzyme (Jones and Schreiber 1997).

1.3.2 Abscisic acid metabolism

Abscisic acid is a small, lipophilic plant hormone that is involved in the control of a wide variety of physiological and developmental processes in plants (Walton 1980; Addicott 1983; Zeevaart and Creelman 1988). It is known to play a role in plant development, seed dormancy, germination, cell division and in responses to stress such as drought, cold, salt, pathogen attack and UV radiation (Addicott and Carns 1983; Zeevaart and Creelman 1988; Sánchez-Serrano *et al.* 1991; McCarty 1995; Rock and Quatrano 1995; Ueno 1998; Albinsky *et al.* 1999). Levels of ABA can rise and fall dramatically in several types of tissue in response to environmental and developmental changes and thus the manner in which its levels are controlled is particularly interesting.

1.3.2.1 Abscisic acid biosynthesis

Since ABA is a sesquiterpene ($C_{15}H_{20}O_4$) it was initially believed that the molecule was synthesised via the condensation of three molecules of IPP, with farnesyl pyrophosphate as an intermediate, the so-called *direct* pathway. Subsequently Taylor and Smith (1967) and later Taylor and Burden (1972) obtained supportive evidence that ABA can be synthesised via an *indirect* route in which ABA was proposed as a cleavage product of certain C_{40} xanthophylls. In both cases the ultimate precursor is the same molecule viz. IPP. The most probable pathway of ABA biosynthesis in plants appears to be confined to the chloroplasts (Milborrow and Lee 1998), where pyruvate and glyceraldehyde phosphate are combined and rearranged, via DOXP, to give

IPP (Cutler and Krochko 1999; Lichtenthaler 1999). Eight IPP residues are combined to form the C_{40} uncyclized carotenoid phytoene. This carotenoid is subsequently converted into β-carotene, which is hydroxylated to zeaxanthin followed by epoxidation to violaxanthin and rearranged to give 9'-Z-neoxanthin (Milborrow 2001) (Fig. 1.4). 9'-Z-neoxanthin is then cleaved by the 9-Z-epoxycarotenoid dioxygenase (NCED) cleavage enzyme to give the C15 xanthoxal (XAN) (Schwartz *et al.* 1997b; Tan *et al.* 1997), which undergoes oxidation via a molybdenum cofactor (MoCo) requiring aldehyde oxidase (AO) (Walker-Simmons *et al.* 1989), to yield xanthoxic acid (XAN-acid) (Lee and Milborrow 1997). Xanthoxic acid undergoes further oxidation and rearrangement to form ABA (Cowan and Richardson 1997; Milborrow *et al.* 1997; Cowan 2001; Milborrow 2001).

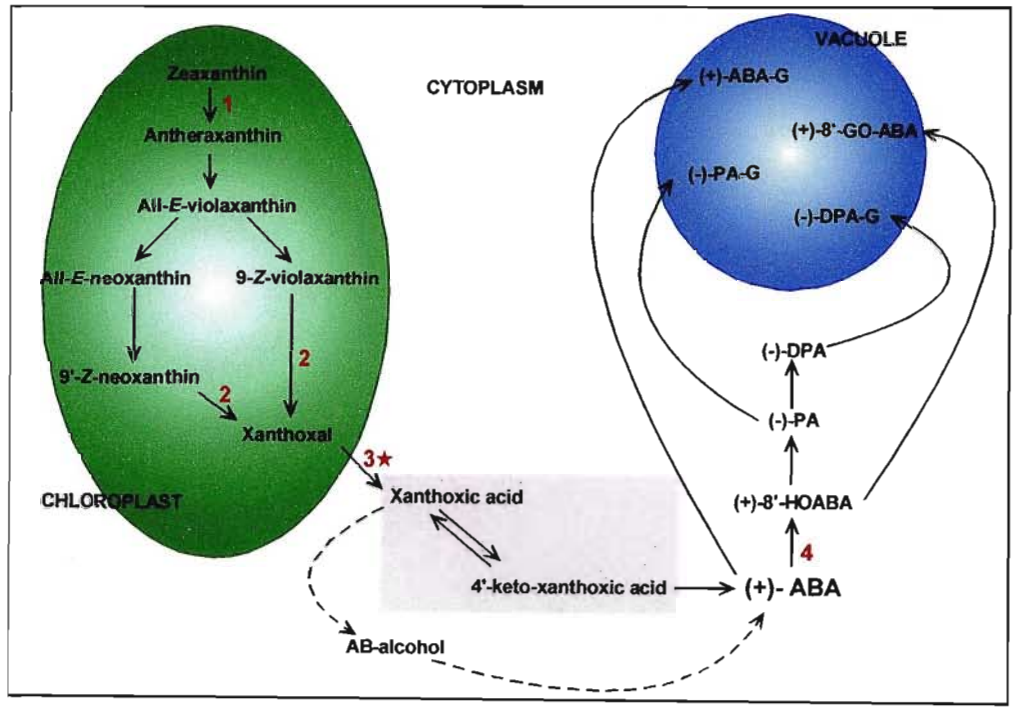


Figure 1.4 **Simplified scheme of ABA metabolism (adapted from Cutler and Krochko 1999; Cowan 2001). The pink highlighted block indicates the hypothetical scheme of the conversion of xanthoxal to ABA (Cowan 2001). Dotted arrows indicate a minor pathway. Enzymes catalysing steps in ABA biosynthesis are 1) zeaxanthin epoxidase, 2) NCED, 3) XAN oxidase and 4) ABA-8'-hydroxylase. The position of a MoCo-requiring enzyme is indicated by (★). Glucose esters, glucosides and other conjugates are all represented by G. AB-alcohol, abscisic alcohol; DPA, dihydrophaseic acid; 8'-HOABA, 8'-hydroxy abscisic acid; PA, phaseic acid.**

This last step in ABA biosynthesis, i.e. the conversion of XAN to ABA, is highly debated and is largely thought to occur via abscisic aldehyde (AB-ald). However, to date there has been no conclusive evidence of the natural occurrence of AB-ald and its metabolism in plant tissues. This

currently accepted view has been refuted in two studies which suggest that XAN, and not AB-ald, is the substrate for the MoCo-requiring enzyme AO (Lee and Milborrow 1997; Cowan *et al.* 1999). Lee and Milborrow (1997) demonstrated tungstate-induced accumulation of XAN in avocado and through the addition of cinchonine, which forms an insoluble complex with tungstate, they were also able to show restoration of ABA production in the presence of this alkaloid. Similarly, Cowan *et al.* (1999) showed a reduction in climacteric-induced accumulation of ABA and a concomitant rise in XAN in mesocarp from ripening avocado fruit treated with tungstate. These results strongly suggest that the pathway from XAN to ABA in avocado involves the oxidation of the C-1 aldehyde group of XAN by an oxidase that is sensitive to tungstate, as it is extremely unlikely that the inhibition of AO by tungstate, if its function is to convert AB-ald to ABA, would cause the accumulation of previous intermediates in the pathway, via product inhibition, as far back as xanthoxal (Milborrow 2001). If the C-1 aldehyde group of XAN is the first part of the molecule to be oxidized then AB-ald cannot be an intermediate (Cowan 2001; Milborrow 2001). The restoration of ABA biosynthesis in extracts of ABA deficient mutants through the addition of Na_2S plus dithionite (Schwartz *et al.* 1997a; Akaba *et al.* 1998) and stimulation in extracts from wild type plants tissues in the presence of substrates for MoCo sulfuration (Sagi *et al.* 1999), taken together with the finding of tungstate inhibition of XAN metabolism, makes it distinctly possible that XAN oxidase is the MoCo-containing AO. As a consequence the next intermediate *en route* to ABA must be XAN-acid. Xanthoxic acid is readily oxidized to ABA *in vivo* (Milborrow *et al.* 1997) and has been identified as a product of XAN metabolism *in vitro* (Cowan and Richardson 1997). The remaining steps to ABA must therefore involve oxidation at C-4' and isomerisation of the 1',2'-epoxide to a 1'-hydroxyl and a 2'-ene (Milborrow 2001).

Recently, Cowan (2001) has suggested that XAN and ABA biosynthesis might represent independent processes in plants. The author argues that ABA biosynthesis *in planta* is a constitutive process and distinct from XAN metabolism, which is probably enhanced under stress. This argument is partly based on the observation that ABA biosynthesis *in planta* appears to be more complex than oxidative cleavage of a xanthophyll 'precursor' and subsequent oxidation of XAN. In this proposed ABA biosynthetic pathway, two precursor pools are proposed for ABA, viz. carotenoids (plastid-localised and stress-induced) and *E-Z*-farnesol (cytosolic and stress and developmentally regulated). In both cases ABA arises through metabolism of the intermediates 2-*cis*-abscisic alcohol and 4'-keto-xanthoxic acid, via ABA-adduct (Cowan 2001).

1.3.2.2 Abscisic acid catabolism

The main route of degradation of *cis*-ABA is through oxidative catabolism to 8'-hydroxy-ABA (8'-HOABA) (Walton 1980; Loveys and Milborrow 1984), which subsequently cyclizes spontaneously (or enzymatically) to form *S*-(-) phaseic acid (PA) (Fig. 1.4). (-)Phaseic acid is further reduced, in some tissues, at the 4' position to form dihydrophaseic acid (DPA) (Zeevaart and Creelman 1988; Parry 1993; Walton and Li 1995; Zeevaart 1999). As these latter metabolites are less biologically active than ABA, the result of these conversions is a reduction in ABA effectiveness in the tissue (Walton and Li 1995; Walker-Simmons *et al.* 1997). The conversion of ABA to PA is therefore an important determinant of ABA levels and evidence is accumulating which suggests that this conversion is an important control point.

The conversion of ABA to HOABA is catalysed by ABA-8'-hydroxylase (systematic name: (+)-ABA, NADPH: oxygen oxidoreductase [8'-hydroxylase]) (Gillard and Walton 1976; Creelman and Zeevaart 1984; Gergs *et al.* 1993; Babiano 1995), which is a membrane associated cytochrome P450 monooxygenase (Krochko *et al.* 1998). The appearance of ABA-8'-hydroxylase activity, in some tissues, is dependent on the presence of ABA and the level to which this enzyme is induced is clearly dependent on ABA concentration (Cutler *et al.* 1997). The appearance of PA is also blocked by the addition of cycloheximide, indicating *de novo* protein synthesis of ABA-8'-hydroxylase in the presence of ABA (Cutler *et al.* 1997). The level of PA formation thus increases with ABA treatment (Uknes and Ho 1984; Railton and Cowan 1987; Gergs *et al.* 1993; Babiano 1995) indicating that ABA stimulates its own catabolism. ABA-8'-hydroxylase is expressed at high levels in plant tissues recovering from abiotic stresses such as water stress (Creelman and Zeevaart 1984; Walton and Li 1995), in tubers and roots (Zhang and Davies 1987; Vreugdenhil *et al.* 1994) and in leaves, developing seeds and seedlings (Gillard and Walton 1976; Babiano 1995; Garello and Lepage-Degivry 1995; Jia *et al.* 1996; Zhang *et al.* 1997; Qi *et al.* 1998). Abscisic acid may also be removed by sugar conjugates, which are stored in the vacuole (Lehmann and Glund 1986). Of the sugar conjugates, ABA glucose ester (ABA-GE) appears to be the most widespread. Conjugation of ABA to ABA-GE is irreversible (Zeevaart 1983; Zeevaart and Boyer 1984), with the conjugate being sequestered in the vacuole (Bray and Zeevaart 1985; Lehmann and Glund 1986).

1.3.2.3 Regulation of ABA biosynthesis

It is believed that there may be two potential sites for the regulation of ABA biosynthesis (Zeevaart and Creelman 1988; Koornneef *et al.* 1998; Cowan *et al.* 1999). 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). Characterization of the *Vp14* viviparous mutant from maize revealed that it exhibited a defect in ABA biosynthesis (Tan *et al.* 1997) and it was found that recombinant VP14 protein catalysed the cleavage of 9-*Z*-xanthophylls to form XAN (Schwartz *et al.* 1997b). Furthermore, mRNA of *vp14* was induced by water stress and accompanied a rise in ABA levels (Tan *et al.* 1997). Nomenclature has designated homologous *Vp14* genes as 9-*Z*-epoxycarotenoid dioxygenase genes or *NCED* and it is suggested that there are a family of differentially regulated *NCED* genes that contribute to environmental and developmental control of ABA biosynthesis in plants (Qin and Zeevaart 1999). Chernys and Zeevaart (2000) isolated two *NCED* genes in avocado fruit, *PaNCED1* and *PaNCED3*, that encode proteins that are capable of *in vitro* synthesis of XAN and whose mRNA levels rise in concert with ABA levels. Carotenoid levels in fruit appear to be high enough so as not to be limiting for ABA biosynthesis and therefore the cleavage of xanthophylls, from a C_{40} to C_{15} molecule, appears to be the rate-limiting step in ABA biosynthesis.

The second potential site for regulation of ABA biosynthesis is the conversion of XAN to ABA, which is catalysed by a MoCo-containing AO. Studies using the *flacca* mutant of *Lycopersicon esculentum* (Marin and Marion-Poll 1997), the *aba1* mutant of *Nicotiana plumbaginifolia* (Leydecker *et al.* 1995) and the *aba3* mutant of *Arabidopsis thaliana* (Schwartz *et al.* 1997a) have found that the reduced ability of these mutants to produce ABA is most probably due to impaired sulfuration of the MoCo required for the activity of the enzyme catalysing the conversion of XAN to ABA, viz. aldehyde oxidase. This second potential site of regulation is of particular interest in hormone interaction studies, as the MoCo is apparently derived from guanosine triphosphate (GTP) (Mendel 1997; Rajagopalan 1997) a precursor of purines, and thus changes in purine metabolism, including changes in CK biosynthesis, might impact on AO activity and hence XAN metabolism and ABA biosynthesis (Cowan *et al.* 1999).

1.3.3 Auxin metabolism

The role of auxin in plants is manifold and includes the stimulation of cell division and elongation, stimulation of shoot growth, inhibition of root growth, control of vascular system differentiation, control of apical dominance, promotion of flowering and fruit setting and ripening. The major natural auxin is indole-3-acetic acid (IAA) (Schneider and Wightman 1978). A number of related compounds also exist in plants including indole-3-butyric acid (IBA) and indole-3-acetonitrile (IAN), but these compounds are active primarily when converted to IAA (Schneider and

Wightman 1978). A group of IAA conjugates with sugars and amino acids also exist (Cohen and Bandurski 1982). The steady state of auxin in a particular tissue or cell is determined by inputs to and outputs from the IAA pool (Cohen *et al.* 1986; Bandurski *et al.* 1995). Inputs to the pool include: *de novo* synthesis; hydrolysis of IAA conjugates to release free IAA; and transport of the hormone, or a conjugate thereof, from one part or organ of the plant to the site under consideration. Known outputs include: transport of the hormone away from the site under consideration; conjugation of the hormone into an inactive form; and irreversible oxidation of the hormone or a conjugate thereof.

1.3.3.1 Auxin biosynthesis

Indole-3-acetic acid is derived either from tryptophan (Trp) or from a precursor of Trp (Bartel 1997) (Fig. 1.5). Tryptophan-dependent pathways are proposed to predominate during early embryogenesis and seed germination, whereas Trp-independent pathways are proposed to predominate during late embryogenesis and vegetative growth (Normanly 1997; Cohen and Slovin 1999). However, little is known about which pathway a plant uses for a specific physiological process or why one pathway is used and not the other (Slovin *et al.* 1999)

Several pathways have been proposed for the conversion of Trp to IAA (Fig. 1.5), with the general scheme being successive deamination and oxidative decarboxylation (Slovin *et al.* 1999). If indole-3-pyruvic (IPA) is the first intermediate, then Trp is thought to participate in transamination (Forest and Wightman 1972) catalysed by Trp aminotransferase. Indole-3-pyruvic acid is subsequently converted to the aldehyde indole-3-acetaldehyde (IA-ald) (Moore and Shaner 1968; Gibson *et al.* 1972; Purves and Brown 1978), which is then converted to IAA via a MoCo-requiring AO that has been identified in several plant species (Sekimoto *et al.* 1998).

For some plants the importance of Trp as an IAA precursor is minor (Baldi *et al.* 1991) and plants incapable of making Trp are still able to synthesize IAA *de novo* (Wright *et al.* 1991; Normanly *et al.* 1993). The exact pathway for this Trp-independent synthesis of IAA has yet to be elucidated. However, through the use of *Arabidopsis* mutants, Normanly and co-workers (1993) suggest that the branch point for Trp-independent IAA synthesis is at indole or its precursor, indole-3-gycerol phosphate (Fig. 1.5).

1.3.3.2 Auxin catabolism

Current understanding is that most of the IAA in plants is conjugated via ester-linkages to sugars and *myo*-inositol or via amide-linkages to amino acids, peptides or proteins (Fig. 1.5). A generalization is that all plants examined to date contain more conjugated than free IAA and that all plants are capable of forming conjugates (Sztein *et al.* 1995). Conjugate formation and hydrolysis is tissue-specific and developmentally regulated (Kleczkowski and Schell 1995), with conjugates having several fates including: storage; transport; protection from peroxidation; and catabolism (Sembdner *et al.* 1994; Kleczkowski and Schell 1995; Bartel 1997). Conjugation is reversible and is regarded as a homeostatic system for storing IAA and regulating levels of free IAA (Slovin *et al.* 1999). Multiple IAA conjugate-hydrolyzing enzymes exist that exhibit a differential specificity, activity and localization (Normanly 1997).

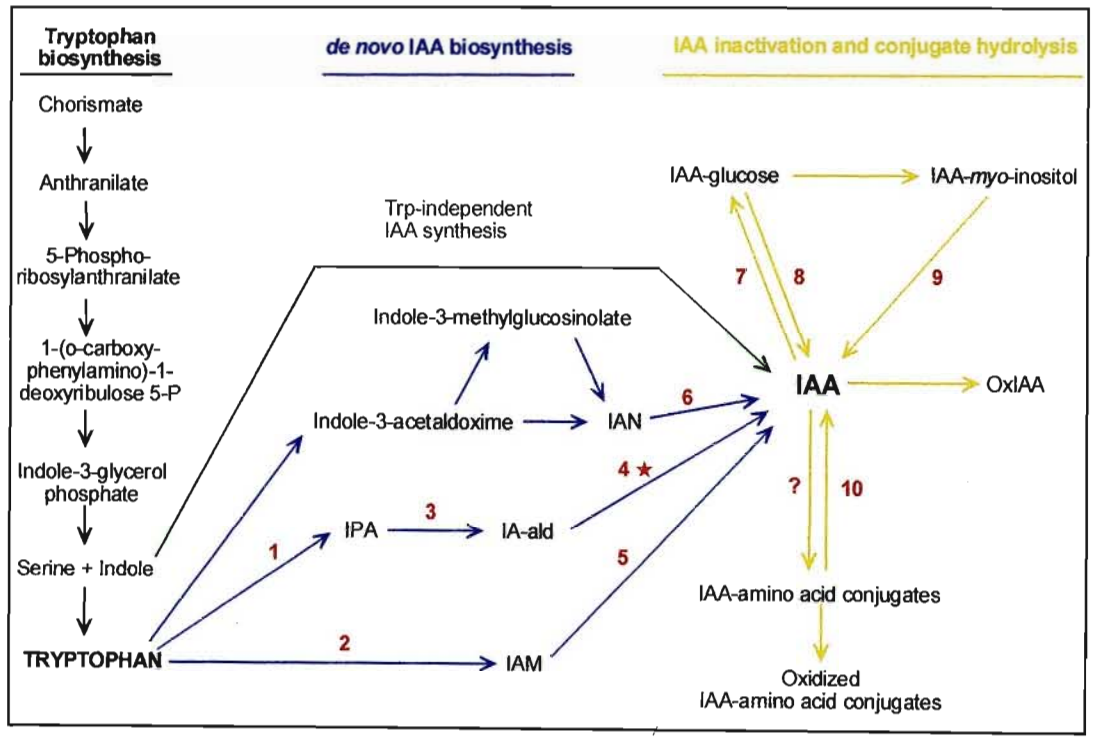


Figure 1.5 **Simplified diagram of IAA metabolism (Normanly and Bartel 1999). Indole-3-acetic acid can be synthesized via several Trp-dependent (blue arrows) or Trp-independent pathways (green arrow). Tryptophan synthesis is represented by black arrows and IAA conjugation and inactivation are represented by yellow arrows. Enzymes catalyzing steps in IAA metabolism are 1) Trp aminotransferase, 2) Trp monooxygenase, 3) IPA decarboxylase, 4) IA-ald oxidase, 5) IAM hydrolase, 6) nitrilase, 7) IAA-glucose synthase, 8) IAA-glucose hydrolase, 9) IAA-inositol hydrolase, and 10) IAA-amino acid hydrolases. The position of a MoCo-requiring enzyme is indicated by (★). IA-ald, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IPA, indole-3-pyruvic acid; OxIAA, oxindole-3-acetic acid.**

Indole-3-acetic acid is also able to undergo irreversible oxidation of the side chain (decarboxylation) or the indole ring (without decarboxylation of the side chain) (Bandurski *et al.* 1995; Östin 1995). Ubiquitous peroxidase enzymes have been shown to decarboxylate IAA *in vitro*. It is unknown whether this oxidation serves any physiological role other than destroying IAA (Slovin *et al.* 1999). It may, however, be linked to the growth-promoting reaction, either as an essential part of the growth-promoting mechanism, or as a means of preventing repetitive use of the hormone (Cohen and Bandurski 1978; Bandurski *et al.* 1985). A more general role for IAA oxidation may involve the scavenging of the aromatic ring (Slovin *et al.* 1999).

1.4 THE MOLYBDENUM COFACTOR REQUIRING ENZYMES

1.4.1 Molybdenum cofactor biosynthesis

Molybdenum is an essential element for plants as it is involved in many vital metabolic functions and thus soil deficiencies or a mutational block of cellular ability to use molybdenum can lead to the death of the plant. Molybdenum is complexed by a novel pterin with a four-carbon alkyl side chain containing a Mo-coordinating dithiolene group and a terminal phosphate ester (Johnson and Rajagopalan 1982). This novel pterin is different to all other pterin compounds classified so far and has been named molybdopterin (MPT). The term 'MoCo' is used for the complete cofactor unit, i.e. MPT plus molybdenum. Without molybdenum, the molybdo enzymes are inactive (Coughlan 1980) and, conversely, molybdenum on its own is biologically inactive.

Most of the present knowledge concerning MoCo biosynthesis has been obtained from *Escherichia coli*. Based on the finding of homology in amino acid sequence between *E. coli* and *Arabidopsis* MoCo biosynthetic proteins and the suggested function of these encoded proteins in *E. coli* (Rajagopalan 1996), the following MoCo biosynthetic pathway in plants has been proposed (Mendel and Schwarz 1999). According to International convention, the seven cloned MoCo genes from higher plants are designated *cnx* followed by a number (Caboche *et al.* 1994). As found in *E. coli* the MoCo biosynthetic pathway in plants can be subdivided into 3 stages (Fig. 1.6). During the first stage a guanosine-X-phosphate (where X is mono-, di- or tri-) derivative is transformed by an unknown mechanism into a sulfur-free pterin compound possessing the MoCo-typical four-carbon side chain, which is referred to as precursor Z. This reaction is catalysed by the products of the *cnx2* and *cnx3* genes (Hoff *et al.* 1995), but little is known about the functions of these proteins in this step. In the second stage, sulfur is incorporated into precursor Z to form a dithiolene group and is converted to MPT. Molybdopterin synthase is proposed to catalyse this reaction (Pitterle *et al.* 1993) and consists of two subunits. The large subunit is encoded by the

gene *cnx6* and the small subunit is proposed to be encoded by *cnx7*, although this gene has not yet been cloned in plants (Mendel and Schwarz 1999). In *E. coli* it has been shown that the small subunit of MPT synthase serves as the donor of dithiolene sulfur for MPT synthesis and has to be resulfurated by the MoeB protein (MPT synthase sulfurase) (Pitterle *et al.* 1993; Pitterle and Rajagopalan 1993). A homologous gene has been found in *Arabidopsis* and has been designated *cnx5* (Nieder *et al.* 1997). In order to form the MoCo, Mo is transferred to MPT in the third stage (Rajagopalan 1996). This requires firstly, the uptake of Mo, about which little is known in plants and secondly, the intracellular processing of molybdate. *Cnx1* plays a role in this last stage of MoCo biosynthesis. The Cnx1 protein consists of two domains, the E-domain and the G-domain (Schwarz *et al.* 1997a). The E-domain is proposed to function in Mo insertion/transfer to MPT (Schwarz *et al.* 1997b). The G-domain, on the other hand, appears to play a role in the stabilization and transfer of the active MoCo (Mendel and Müller 1985; Schwarz 1997; Schwarz *et al.* 1997b). Structural analyses have revealed that: (1) the cofactor is located deep within the interior of the enzyme protein; and (2) the pterin-ring system could participate in the electron transfer to or from the Mo-atom (Mendel 1997). Wray and Filner (1970) and Coughlan (1980) also showed that elevated amounts of the Mo-antagonist, tungstate, inhibit Mo-enzymes by replacing molybdenum as the ligand in MPT.

Three groups of MoCo-requiring enzymes have been described in plants, which are aldehyde oxidase (AO; EC 1.2.3.1), xanthine dehydrogenase (XDH; EC 1.1.204, formerly EC 1.2.1.37) and nitrate reductase (NR; EC 1.6.6.1-3). A fourth group, sulfite oxidase (SO; EC 1.8.3.1), also exists in the completely sequenced Arabidopsis genome (Eilers *et al.* 2001). Pateman *et al.* (1964) proposed that these three Mo-enzymes share a common cofactor based upon observations that certain mutations had a pleiotropic effect on both NR and XDH in *Aspergillus nidulans*. Similar MoCo mutants with pleiotropic deficiencies have been isolated in bacteria, fungi and higher plants (Kleinhofs *et al.* 1986; Wray 1986). Subsequently, the *aba1* mutant in tobacco and *aba3* mutant in *Arabidopsis thaliana* were isolated, which are impaired in AO and XDH activity, but overexpresses NR (Leydecker *et al.* 1995; Schwartz *et al.* 1997a). This led to the conclusion that an additional step is required in the synthesis of the MoCo for AO and XDH and it is at this step that the genetic lesion of the *aba1* and *aba3* mutants occur. This was confirmed based on the ability of Na_2S plus dithionite to restore AO activity in extracts from this mutant by sulfurating the dioxo form of the MoCo (Schwartz *et al.* 1997a; Akaba *et al.* 1998). Recently, the *Arabidopsis ABA3* gene has been cloned (Bittner *et al.* 2001; Xiong *et al.* 2001), with the recombinant ABA3 protein shown to have MoCo sulfurase activity (Bittner *et al.* 2001). It has been found that

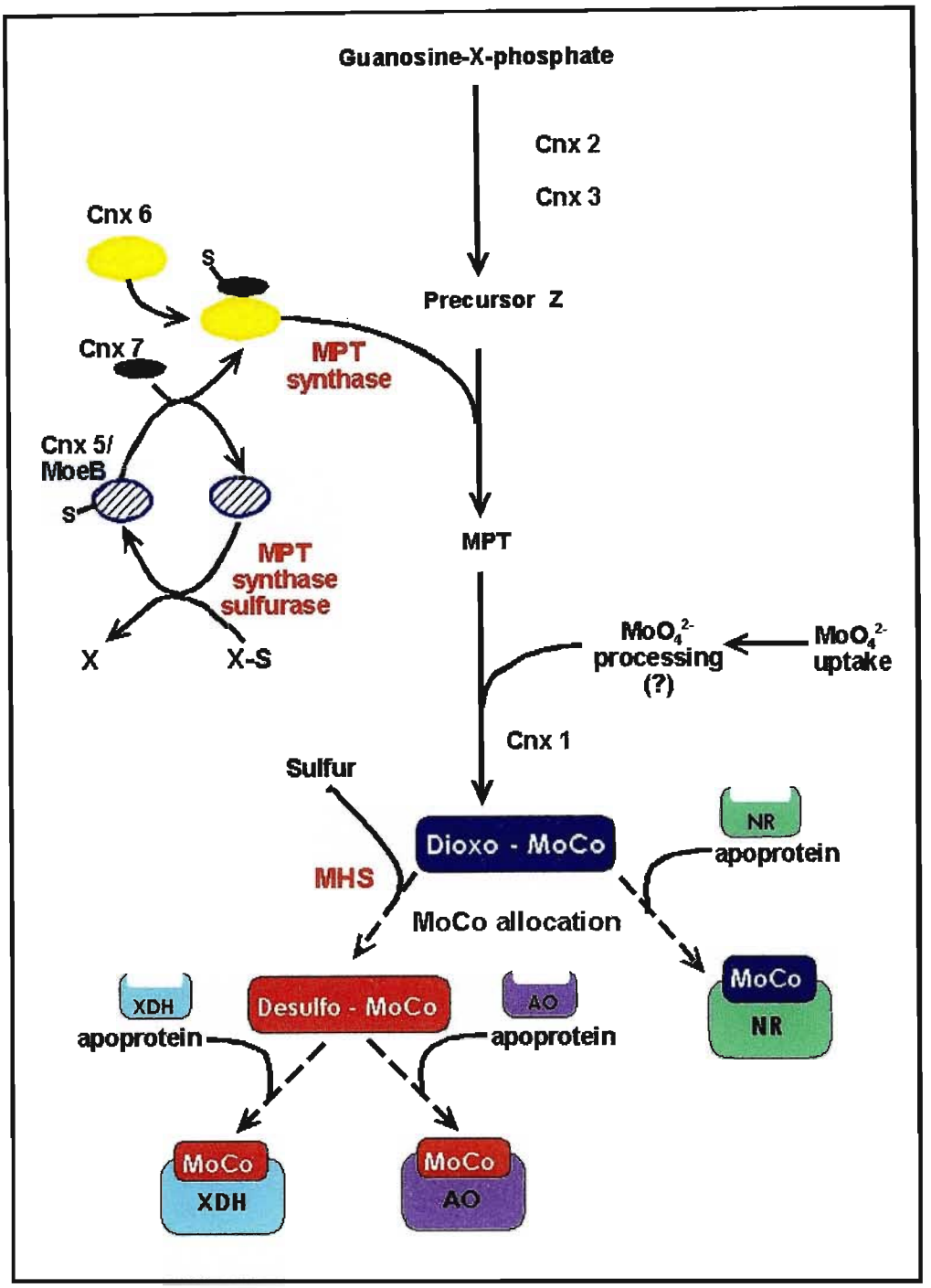


Figure 1.6 **Model for MoCo synthesis and allocation to Mo-enzymes in *Arabidopsis thaliana* based on homologies to the corresponding *E. coli* proteins (adapted from Lips 1997; Mendel 1997). The plant proteins are given in the *cnx* nomenclature (Caboche *et al.* 1994). Under stressful conditions it is expected that there will be increased MoCo allocation into the desulfo branch allowing for the increased expression of XDH and AO. AO, aldehyde oxidase; MHS, Mo-hydroxylase sulfurase; MPT, molybdopterin; NR, nitrate reductase; XDH, xanthine dehydrogenase.**

reductive dehydroxylases, such as NR and SO, utilize a dioxo form of the cofactor (Johnson and Rajagopalan 1982), whereas AO and XDH use a desulfo form (Wahl and Rajagopalan 1982). The formation of the desulfo form of the enzyme involves the replacement of the oxygen atoms binding molybdenum, in the dioxo form, by a sulfur atom. This reaction is catalysed by the enzyme Mo-hydroxylase sulfurase (MHS) (Sagi *et al.* 1999). This latter group of molybdo-enzymes also contain FAD and two types of iron-sulfur centres (Wootton *et al.* 1991). The relative allocation of the MoCo to these three enzymes (Fig. 1.6) can determine their relative activities, e.g. under stressful conditions it is proposed that there will be increased allocation of the MoCo to AO and XDH, resulting in decreased allocation to NR (Lips 1997).

1.4.2 Nitrate reductase

Nitrate reductase is a homodimeric protein, with an apparent molecular weight of 200 kDa that is cytoplasmically localised (Mendel and Schwarz 1999). The protein monomer binds heme-iron, FAD and MoCo in a 1:1:1 ratio. Nitrate reductase catalyses the first step in the nitrate assimilation pathway, reducing nitrate to nitrite, which is then assimilated into amino acids and is the rate-controlling step of nitrate assimilation into organic nitrogen compounds. It is an important enzyme in plants as it can be a limiting factor for growth and development and protein synthesis (Solomonson and Barber 1990), particularly under unfavourable environmental conditions, as the enzyme is very sensitive to stress and is influenced by a number of environmental factors (Huffaker *et al.* 1970; Hueur *et al.* 1979; Crawford 1995). Nitrate assimilation regulation is therefore part of a complex regulatory network that responds to a diverse range of environmental and internal signals, such as nitrate, light, carbon dioxide, phytohormones and carbon and nitrogen metabolites.

1.4.3 Aldehyde oxidase

Aldehyde oxidase contains redox-active iron, not as heme-iron, but in the form of Fe-S centres localised on the N-terminal two domains (Mendel and Schwarz 1999). The protein monomer of the homodimeric enzyme binds Fe-S, FAD and MoCo in a ratio of 4:1:1. The homodimer has an apparent molecular weight of 360 kDa (Rothe 1975) and is localised in the cytoplasm. Aldehyde oxidase in plants shows a broad substrate specificity, being responsible for the oxidation of a considerable number of aldehydes and N-containing heterocyclic compounds in the presence of O_2 or certain redox dyes (Rajagopalan and Handler 1966; Hall and Krenitsky 1986; Yoshihara and Tatsumi 1986). However, it is the action of AO at the final steps of the biosynthesis of two plant hormones that is of great interest, i.e. the conversion of XAN to ABA (Walker-Simmons *et al.*

1989; Sindhu *et al.* 1990; Leydecker *et al.* 1995; Cowan 2001; Milborrow 2001) and IA-ald to IAA (Koshiba *et al.* 1996; Lips *et al.* 1999). It is due to the action of AO at the final step in the synthesis of these two plant hormones that it has been ascribed an important role in plant development and adaptation to environmental stress (Sagi *et al.* 1998).

Different isoforms of the AO enzyme have been found in plants, which show tissue-specific expression and different substrate preferences (Rothe 1974; Koshiba *et al.* 1996; Ori *et al.* 1997; Sekimoto *et al.* 1998; Seo *et al.* 1998; Omarov *et al.* 1999; Seo *et al.* 2000a; b). It is possible that these isoforms have different physiological functions, which could include ABA and IAA biosynthesis (Sekimoto *et al.* 1998). Three organ- and substrate-specific AO activity bands were detected in *Arabidopsis* seedlings, namely AOα, AOβ and AOγ (Seo *et al.* 1998). AOα was abundant in roots, whilst AOγ was most abundant in cotyledons and leaves. In terms of substrate specificity, AOα showed a strong preference for indole-3-aldehyde (I-ald) and IA-ald, while AOγ efficiently oxidised 1-naphthaldehyde. AOβ exhibited properties intermediate between AOα and AOγ in terms of its mobility in native-PAGE and substrate preference. Thus, of the three isoforms, AOα seems most likely to be involved in IAA biosynthesis due to its high affinity for IA-ald and its high expression in the IAA overproducing *sur1* mutant of *Arabidopsis* compared to the wild type (Seo *et al.* 1998). Preliminary studies with *Arabidopsis* revealed that two AO genes, *AAO3* and *AAO4* (formerly called *atAO-3* and *atAO-4*), were rapidly induced after desiccation, suggesting that these genes encoded an AB-ald oxidase (Seo *et al.* 1999). Subsequently, *AAO3* has been found to encode the AO isoform AOδ, which has high specificity for AB-ald and is expressed mainly in rosette leaves of *Arabidopsis* (Seo *et al.* 2000a;b). High AO-type activity has also been detected in tomato fruit, and its expression seems to be related to the biosynthetic capacity required for typical plant metabolic "sink" tissues. Alternatively, it may however catalyse the final step in IAA and ABA biosynthesis (Ori *et al.* 1997). In the latter case, the tissue-specific expression of the *TAO1* (tomato aldehyde oxidase 1) gene, detected by the TAO1 antibody in fruit, may reflect the role ABA plays in seed maturation and dormancy, whilst its expression in apical meristems could reflect the role this tissue plays in auxin biosynthesis (Ori *et al.* 1997).

1.4.4 Xanthine dehydrogenase

Nguyen (1986) classified XDH, in plants, as a dehydrogenase which is NAD^+ dependent and catalyses the first oxidative step in purine catabolism. Like AO it is a homodimeric enzyme binding Fe-S, FAD and MoCo in the ratio of 4:1:1 per monomer (Mendel and Schwarz 1999) and is thought to be a microbody-associated enzyme (Nguyen 1986; Corpas *et al.* 1997). It is a

ubiquitous enzyme and ensures that purine compounds, originating from CK and nucleic acid degradation, are irreversibly committed into purine catabolism (Wasternack 1982; Nguyen 1986). Xanthine dehydrogenase catalyses the formation of uric acid from xanthine and hypoxanthine and is thus necessary for the synthesis of ureides in higher plants (Nguyen 1986). Although plant XDH shows highest affinities for xanthine and hypoxanthine as substrates, it also accepts purines and pterines, but at much lower rates (Nguyen 1986). Xanthine dehydrogenase is inhibited by excess substrate (Bray 1963) and product (Nguyen 1979; Boland 1981). In addition to this, substrate and product analogues are inhibitory (Nguyen 1986), for example adenine and guanine inhibit XDH in cowpea (Woo *et al.* 1981) and *P. vulgaris* nodules (Boland 1981). A particularly potent inhibitor of XDH is allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine) (Weir and Fischer 1970; Bray 1975) as well as SH group inhibitors (like *p*-hydroxymercuribenzoate) and non-heme iron complexing agents (like salicylhydroxamic acid) (Mendel and Schwarz 1999).

1.4.5 The potential role of MoCo enzymes in the control of hormone homeostasis

The XDH and AO enzymes are of particular importance in developing a model for the metabolic control of fruit size as they are intimately related to the metabolism of CK, ABA and IAA. As such these enzymes serve as potential control points for the regulation of hormone homeostasis and the processes controlled thereby. Furthermore, it is the shared MoCo that is proposed to be the site of regulation of these enzymes. It has been suggested that the MoCo pool size is not consistent, but varies in response to nutritional and environmental factors (Sagi *et al.* 1997; Sagi and Lips 1998). An increase in the activity of the Mo-hydroxylases (i.e. AO and XDH), with salt stress and ammonium treatment, was thus considered to be part of the mechanism for stress adaptation in plants, which includes elevated ABA synthesis and increased ureide production (Sagi *et al.* 1998). It is thus hypothesized that, under conditions where nitrate assimilation is reduced and/or XDH inhibited, more MoCo might be expected to be available for AO required for ABA and IAA biosynthesis. This "shift" in MoCo allocation and activity of the respective enzymes is further demonstrated by the finding of reduced ABA and IAA levels in plants in which NR has been induced by its substrate (Omarov *et al.* 1999). The availability of MoCo for AO activity may therefore represent a potential site of interaction between the CK, ABA and IAA biosynthetic pathways (Fig. 1.7) and therefore the control of the hormonal balance, which is often the deciding factor determining whether plants will tolerate or be susceptible to imposed stress (Fedina *et al.* 1994).

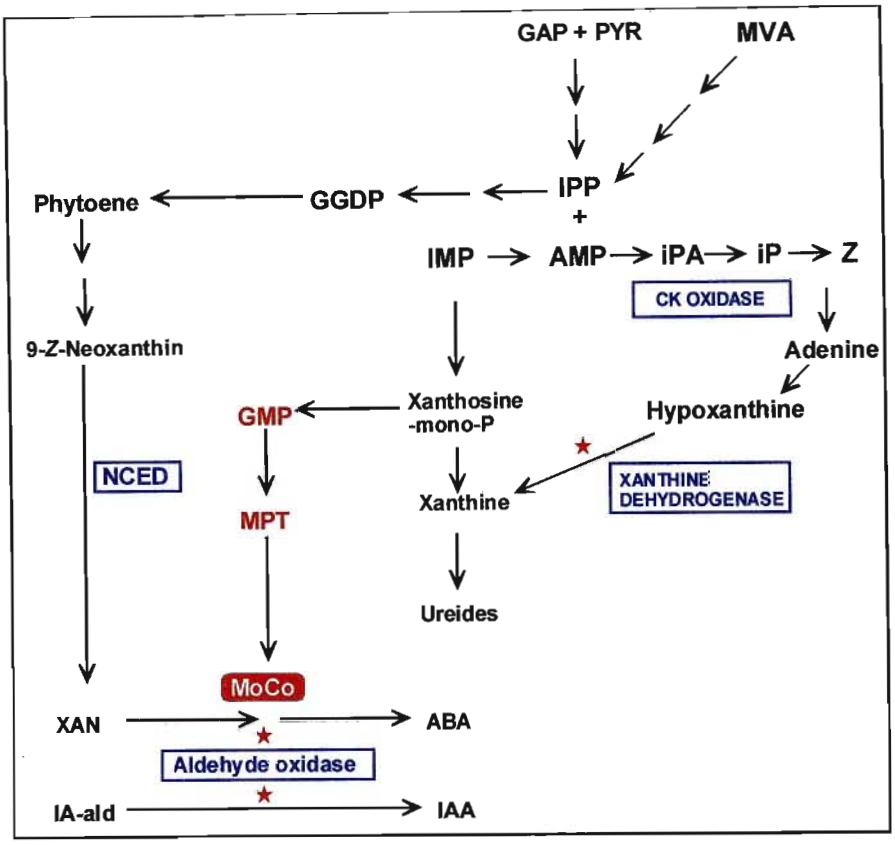


Figure 1.7 **Proposed model illustrating the relationship between CK and ABA biosynthetic pathways (Cowan *et al.* 1999). The position of a MoCo-requiring enzyme is indicated by (★). AMP, adenosine monophosphate; GAP, glyceraldehyde phosphate; GGDP, geranylgeranyl diphosphate; GMP, guanosine monophosphate; IMP, inosine monophosphate; iP, isopentenyladenine; iPA, isopentenyladenosine; IPP, isopentenyl pyrophosphate; MPT, molybdopterin; MVA, mevalonic acid; NCED, neoxanthin cleavage enzyme; PYR, pyruvate; XAN, xanthoxal; Z, zeatin.**

In addition to the role of MoCo enzymes in the IAA, ABA and CK biosynthesis, it is possible that a MoCo-requiring AO may be involved either directly or indirectly in GA metabolism (Fig. 1.8). Conversion of GA_{12}-aldehyde in GA biosynthesis involves oxidation at C7 to yield a dicarboxylic acid and this reaction is catalysed by either a dioxygenase or monooxygenase (Hedden and Kamiya 1997). The gene for GA_{12}-aldehyde 7-oxidase has been cloned from pumpkin by functional screening and in this system it is a 2-oxoglutarate-dependent dioxygenase, whereas in other systems the enzyme is believed to be a cytochrome P450 monooxygenase (Lange 1997; Hedden and Phillips 2000). However, unequivocal evidence for this is still awaited. The further oxidation of GA_{12}, and its 13-hydroxylated analog GA_{53}, to bioactive GAs is catalysed by soluble dioxygenases, one of which is GA 20-oxidase, whose expression is enhanced by the auxin 4-chloroindole-3-acetic acid (4-Cl-IAA) in pea fruit (van Huizen *et al.* 1997) (Fig. 1.8). 4-chlorindole-3-acetic acid is proposed to originate in seed tissue from where it is transported to the pericarp, where it in turn regulates the conversion of GA_{19} to GA_{20} by increasing the level and/or stability of

GA 20-oxidase mRNA (van Huizen *et al.* 1997). The response is specific for 4-Cl-IAA, as the synthetic auxin 2,4-D had little effect on GA 20-oxidase expression in unpollinated pea ovaries (García-Martínez *et al.* 1997). It has also recently been shown that normal levels of IAA are required to maintain normal levels of bioactive GA (GA_1) in pea stems (Ross *et al.* 2000). Key evidence for this hypothesis was the observation that auxin transport inhibitors, applied just below the apical bud of intact plants, markedly reduced GA_1 biosynthesis in pea stems (Ross 1998). Subsequently it was found that expression of the pea gene *LE* (GA_{20} to GA_1) in stem internodes requires IAA from the shoot apex and that the expression of *PsGA2ox1* (deactivation of GA_{20} to GA_{29} and GA_1 to GA_8) is reduced by IAA (Ross *et al.* 2000).

Two possibilities are therefore evident, either one or both of these enzymes has a MoCo requirement or AO-induced IAA formation impacts GA biosynthesis directly to promote formation of bioactive GAs and to reduce deactivation. Aldehyde oxidases in plants have been shown to exhibit wide substrate specificities (Rajagopalan and Handler 1966; Hall and Krenitsky 1986; Yoshihara and Tatsumi 1986) and different isoforms of the AO enzyme have been found, which show tissue-specific expression and different substrate preferences (Rothe 1974; Koshiba *et al.* 1996; Ori *et al.* 1997; Sekimoto *et al.* 1998; Seo *et al.* 1998; Omarov *et al.* 1999; Seo *et al.* 2000a; b). The possibility therefore exists that an AO isoform may be involved in GA metabolism.

1.5 THE SMALL FRUIT PROBLEM IN PERSPECTIVE

The CK to ABA ratio in 'Hass' avocado fruit appears to be crucial in the control of cell division and fruit growth, as evidence has been found that an imbalance in this ratio is pivotal in seed coat senescence and retardation of fruit growth (Moore-Gordon *et al.* 1998). These workers also showed that the mesocarp ABA concentration of mature fruit was negatively correlated with fruit size and that application of ABA during the linear phase of rapid fruit growth resulted in seed coat senescence and the retardation of fruit growth. Consequently, it was found that ABA-induced retardation of fruit growth could be negated by co-injection of iP. Thus there seems to be a relationship between CK and ABA in the control of 'Hass' avocado fruit growth and this interaction is thought to be mediated at the level of their biosynthetic pathways, considering they share a common biosynthetic origin in the isoprenoid or DOXP pathway (Chappell 1995; Lichtenthaler 1999).

A second possible site of interaction lies in the allocation of the molybdenum cofactor to the AO enzyme required for ABA and IAA biosynthesis (Fig. 1.7). This was demonstrated in a study of

the response of ABA to CK, allopurinol and adenine in ripening avocado tissue, which indicated the involvement of a MoCo-containing AO (Cowan *et al.* 1999). These studies showed that allopurinol and adenine, a product of CKOX activity and inhibitor of XDH, promoted ABA metabolism. Since CKOX is a substrate-inducible enzyme it was suggested that CK-induced CKOX activity contributed to the regulation of endogenous ABA during plant organ growth

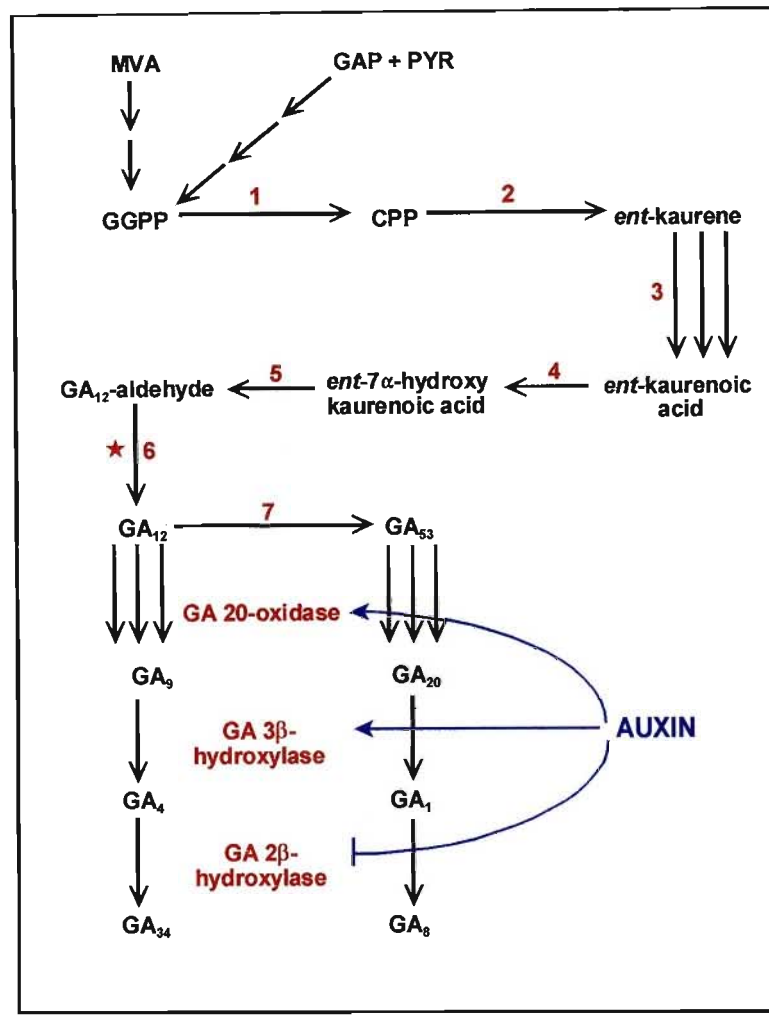


Figure 1.8 **Simplified model of GA biosynthesis (Hedden and Kamiya 1997; Hedden and Proebsting 1999). The possible position of a MoCo-requiring AO is indicated by (★). Enzymes catalysing steps in GA biosynthesis are 1) *ent*-copalyl diphosphate synthase, 2) *ent*-kaurene synthase, 3) *ent*-kaurene oxidase, 4) *ent*-kaurenoic acid 7β-hydroxylase, 5) GA_{12}-ald synthase, 6) GA_{12}-ald 7-oxidase, 7) GA 13-hydroxylase. Auxin regulation of GA biosynthesis is indicated in blue, with large arrowheads denoting enhanced gene expression and bars denoting suppressed expression. CPP, copalyl diphosphate; GAP, glyceraldehyde phosphate; GGPP, geranylgeranyl diphosphate; MVA, mevalonic acid; PYR, pyruvate.**

(Cowan *et al.* 1999). A change in CK metabolism is thus postulated to impact on ABA levels through changes in XDH activity and altered MoCo allocation to AO. As accumulation of ABA in the mesocarp is correlated with reduced fruit size, it is hypothesised that this accumulation would be associated with elevated AO activity and a corresponding decrease in XDH activity, resulting from CK-induced CKOX activity. As an isoform of AO is also thought to catalyse the last step of IAA biosynthesis, changes in MoCo synthesis and allocation will therefore also impact on auxin production. This lends further credence to the proposal that the MoCo enzymes are intimately linked with hormone homeostasis and thus the control of avocado fruit size.

Although the exact role of GA in fruit growth is currently poorly understood, it is proposed that it plays a role in the stimulation of cell division and maintenance of cell expansion (Gillaspy *et al.* 1993). As such, this hormone is likely to play a role in the appearance of the 'Hass' avocado small fruit phenotype, acting in concert with CK, IAA and ABA. It is plausible that GA will interact with CK, IAA and ABA through the sharing of a common biosynthetic precursor in the isoprenoid pathway or through the availability of MoCo for the MoCo-requiring enzymes. As alluded to above, the possibility exists that a step in GA biosynthesis might be catalysed by a MoCo-requiring AO, which would further extend the hypothesis that MoCo enzymes play an important role in controlling hormone homeostasis in plant tissues.

1.6 OBJECTIVES

In the absence of evergreen tree-crop mutants with aberrant fruit growth, the 'Hass' avocado small fruit phenotype provides an ideal system with which to probe more detailed aspects of the control of final avocado fruit size. One such aspect of control centres on phytohormones, which have been found to play an interactive role in controlling avocado fruit size. Following observations which established that the CK to ABA ratio was negatively correlated with final avocado fruit size (Moore-Gordon *et al.* 1998) and that CK impacted antagonistically on ABA metabolism in avocado fruit by apparently influencing activity of MoCo enzymes (Cowan *et al.* 1999), this study was initiated:

1) to probe the contribution of hormones in the control of final fruit size by comparing tissue distribution and content of CK, ABA and IAA in developing 'Hass' avocado and its small fruit phenotype; and
2) to evaluate the hypothesis that alterations in hormone homeostasis occur as a result of differences in the allocation of the MoCo, and changes in activity of XDH (or NR) and the AO's involved in IAA and ABA metabolism.

2 MATERIALS AND METHODS

2.1. CHEMICAL AND LABORATORY SUPPLIES

2.1.1 Radioactive and stable isotopes

N^6-isopent-2-enyl[2-^{3}H]adenine (sp. act. 1.25 TBq mmol^{-1}) and *trans*-[2-^{3}H]zeatin (sp. act. 1.3 TBq mmol^{-1}) were purchased from The Institute of Experimental Botany, Academy of Sciences of the Czech Republic Isotope Laboratory IEB, Prague, Czech Republic. DL-*cis, trans*-[G-^{3}H]ABA (sp. act. 1.11 TBq mmol^{-1}) and 3-[5(n)-^{3}H]IAA (sp. act. 1.11 TBq mmol^{-1}) were both obtained from Amersham International, Buckinghamshire, UK.

2.1.2 Fine chemicals and cofactors

Butylated hydroxytoluene (BHT; 2,6-Di-t-butyl-p-cresol, $C_{15}H_{24}O$), diethyldithio carbamic acid (DDC; sodium salt, $C_5H_{10}NS_2Na$), reduced glutathione ($C_{10}H_{17}N_3O_6S$), phenazine methosulphate (PMS; N-methyldibenzopyrazine methyl sulphate salt, $C_{13}H_{11}N_2{\cdot}CH_3SO_4$), dichloroindophenol (DCIP; $C_{12}H_6Cl_2NO_2Na$), indole-3-aldehyde (I-ald; C_9H_7NO), hypoxanthine (6-hydroxypurine, $C_5H_4N_4O$), indole-3-acetaldehyde (IA-ald; sodium bisulfite addition, $C_{10}H_9NO{\cdot}NaHSO_3$), linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid, $C_{18}H_{32}O_2$), *p*-aminophenol (4-hydroxyanilin, $H_2NC_6H_4OH$), Coomassie Brilliant Blue G ($C_{47}H_{48}N_3O_7S_2Na$), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; $C_{18}H_{16}N_5SBr$), bovine serum albumin (BSA), adenine (6-aminopurine, free base, $C_5H_5N_5$), allopurinol (allo; 4-hydroxypyrazolo[3,4-d]pyrimidine, $C_5H_4N_4O$), polyethylenimine (Polymin P; 50 % (w/v) aqueous solution, a branched chain polymer), L-cysteine hydrochloride ($C_3H_7NO_2S{\cdot}HCl$), N-nitroso-N-methylurea ($C_2H_5N_3O_2$), lipoxidase (from Soybean, EC 1.13.11.12) and N,N-dimethyloctylamine ($CH_3(CH_2)_7N(CH_3)_2$) were all purchased from Sigma, St Louis, MO, USA.

Nicotinamide adenine dinucleotide (NAD; free acid, $C_{21}H_{27}N_7O_{14}P_2$), flavin adenine dinucleotide (FAD; disodium salt, $C_{27}H_{31}N_9O_{15}P_2Na_2$) and tris (2-amino-2-(hydroxymethyl)-1,3-propandiol, $C_4H_{11}NO_3$) were purchased from Boehringer Mannheim GmbH, Mannheim, Germany. Potassium molybdate (K_2MoO_4) was purchased from Aldrich Chemical Company Inc., Milwaukee, WI, USA. Sodium molybdate ($Na_2MoO_4.2H_2O$), trichloroacetic acid (CCl_3COOH) and di-potassium hydrogen orthophosphate (K_2HPO_4) were purchased from Associated Chemical Enterprises, Glenvista, RSA. Magnesium sulphate (hepthydrate, $MgSO_4.7H_2O$) was purchased from Holpro Analytics (Pty) Ltd, Johannesburg, RSA. Ammonium sulphate ($(NH_4)_2SO_4$) and sodium formate (HCOONa) were obtained from Merck Laboratory Supplies (Pty) Ltd, Midrand, RSA. Ethylene diamine tetra-acetic acid (EDTA; disodium salt, $C_{10}H_{14}O_8N_2Na_2.2H_2O$) and ammonium hydroxide (25 % (w/v) solution, NH_4OH)

was purchased from Saarchem, Krugersdorp, RSA. Potassium dihydrogen phosphate (KH_2PO_4) was purchased from Riedel-de Haën GmbH, Seelze, Germany. Ammonium dihydrogen phosphate ($(NH_4)H_2PO_4$) was purchased from Merck KGaA, Darmstadt, Germany. 1,4-Dithiothreitol (DTT; $C_4H_{10}O_2S_2$) and Complete® protease inhibitor tablets were obtained from Roche Diagnostics GmbH, Mannhein, Germany. Insoluble polyvinylpolypyrrolidone (PVP; Polyclar AT) and calcium chloride ($CaCl_2$) were obtained from BDH Laboratory Supplies, Poole, England. Heptaldehyde ($C_7H_{14}O$), citral ($C_{10}H_{16}O$) and 3-methyl-2-butenal (3,3-dimethylacrolein, C_5H_8O) were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Benzaldehyde (C_7H_6O) and acetaldehyde (C_2H_4O) were purchased from Fluka Chemie Ltd, Buchs, Switzerland. Pico-Fluor™ 40 (Universal LSC cocktail) was purchased from Packard Bioscience Company, Groningen, The Netherlands.

2.1.3 Electrophoresis reagents

Acrylamide (C_3H_5NO), bromophenol blue (3', 3", 5', 5"-tetrabromophenol-sulfonephthalein, $C_{19}H_9Br_4O_5SNa$) and glycine ($C_2H_5NO_2$) were purchased from BDH Laboratory Supplies, Poole, England. Bis-acrylamide (N,N'-methylene-bis-acrylamide, $C_7H_{10}N_2O_2$) and sodium thiosulphate (pentahydrate, $Na_2S_2O_3.5H_2O$) were obtained from ICN Biomedicals Inc., Aurora, Ohio. Ammonium persulphate ($(NH_4)_2S_2O_8$) was obtained from Biosolve Ltd, Valkenswaard, The Netherlands. N,N,N',N'-Tetramethylethylenediamine (TEMED; $C_6H_{16}N_2$), urease molecular weight markers for native-PAGE (Jack Bean, approx. mol. wt 272 000 (trimer) and 545 000 (hexamer)) and formaldehyde (37 % (w/v) solution, CH_2O) were purchased from Sigma, St Louis, MO, USA. Silver nitrate ($AgNO_3$) was purchased from Merck Laboratory Supplies (Pty) Ltd, Midrand, RSA. Sodium carbonate (anhydrous, Na_2CO_3) was purchased from Saarchem, Krugersdorp, RSA.

2.1.4 Tissue culture reagents

Agar Bacteriological (Agar No. 1) was purchased from Oxoid Ltd, Basingstoke, Hampshire, England. Myo-inositol ($C_6H_{12}O_6$) and nicotinic acid (niacin, pyridine-3-carboxylic acid, $C_6H_5NO_2$) were purchased from Sigma, St Louis, MO, USA. Pyrodixine HCl (vitamin B_6, $C_8H_{11}NO_3 \cdot HCl$) and thiamin HCl (vitamin B_1, $C_{12}H_{17}N_4OSCl \cdot HCl$) were purchased from BDH Laboratory Supplies, Poole, England. All other chemicals for the basal medium were obtained from Merck Laboratory Supplies (Pty) Ltd, Midrand, RSA and BDH Laboratory Supplies, Poole, England.

2.1.5 Growth regulators

Isopentenyladenine (iP; 6-(γ,γ-dimethylallylamino)-purine, $C_{10}H_{13}N_5$), isopentenyladenosine (iPA; 6-(γ,γ-dimethylallylamino)-purine riboside, $C_{15}H_{21}H_5O_4$), DL-zeatin (Z; 6-[4-hydroxy-3-

methylbut-2-enylamine]purine, $C_{10}H_{13}N_5O$), *trans*-zeatin riboside (ZR; 9-[β-D-ribofuranosyl]-trans-zeatin, $C_{15}H_{21}N_5O_5$), DL-dihydrozeatin (DHZ; $C_{10}H_{15}N_5O$), kinetin (K; 6-furfurylamino purine, $C_{10}H_9N_5O$), indole-3-acetic acid (IAA; $C_{10}H_9NO_2$), indole butyric acid (IBA; $C_{12}H_{13}NO_2$), α-naphthalene acetic acid (NAA; $C_{12}H_{10}O_2$), *cis-trans* abscisic acid (ABA; $C_{15}H_{20}O_4$) and authentic methylated abscisic acid (ABAMe) and indole-3-acetic acid (IAAMe) standards were obtained from Sigma, St Loius, MO, USA. 6-chloropurine, 2,6-dichloropurine, 2-methylthio-6-chloropurine and 2-methylthio-6-chloro-9-methylpurine were obtained from OlChemIm Ltd, Olomouc, Czech Republic. Authentic phaseic acid (PA) and dihydrophaseic acid (DPA) standards were kindly supplied by Professor A.K. Cowan, Research Centre for Plant Growth and Development, University of Natal, Pietermaritzburg.

2.1.6 Solvents

High performance liquid chromatography (HPLC) grade solvents were obtained from Burdick and Jackson, AlliedSignal Inc., Muskegon, MI, USA. Analytical grade solvents and polyoxyethylene (20) sorbitan monooleate (Tween 80®) were supplied by Merck Laboratory Supplies (Pty) Ltd, Midrand, RSA and Associated Chemical Enterprises (Pty) Ltd, Southdale, RSA. Dimethyl sulphoxide (DMSO) was purchased from Sigma, St Loius, MO, USA. Polyoxyethylene (20) sorbitan monolaurate (Tween 20®) was purchased from Saarchem (Pty) Ltd, Krugersdorp.

2.1.7 Purification of indole-3-acetaldehyde

Commercially purchased IA-ald comes as a bisulfite addition compound. The bisulfite was removed prior to activity staining, as it is a strong reducing agent and will thus reduce MTT to formazan. Free IA-ald was prepared according to a modified method of Bower *et al.* (1978). A known amount of IA-ald bisulfite was dissolved in 1 mL water and the pH adjusted to 10 with 0.5 N NaOH, which resulted in the precipitation of free IA-ald. The free IA-ald was removed by partitioning three times against an equal volume of hexane/ethyl acetate (9:1, v/v). The hexane/ethyl acetate fraction was dried *in vacuo* at 35 °C, using a Büchi Rotavapor® R110 (Büchi Laboratoriums-Technik AG, Flawil, Switzerland), resuspended in 0.5 mL acetone and diluted to 20 mL with 0.1 M Tris-HCl buffer (pH 8). The final concentration of free IA-ald was determined by measuring the absorbance at 280 nm in an Anthelie Advanced Spectrophotometer (Secomam CE, Domont Cedex, France) and applying Beer's law with ε_A^{280} = 5 400 L mol^{-1} cm^{-1} (Brown and Purves 1976).

2.1.8 Preparation of xanthoxal

Xanthoxal was produced enzymatically according to the method of Firn and Friend (1972), by coupling violaxanthin oxidation to the enzymatic oxidation of linoleic acid.

Violaxanthin was extracted from orange peel and purified chromatographically using a modified method of Taylor and Burden (1970) and Davies (1976). The outer layer of the flavedo of orange fruit was grated from the fruit and a total of 750 g macerated with 1.5 L methanol in an Osterizer® blender. This extract was filtered under vacuum and the residue re-extracted twice with diethyl ether (total volume 4 L). The methanol and diethyl ether extracts were combined and partitioned against water containing NaCl, to prevent an emulsion from forming (Davies 1976). The organic fraction was dried *in vacuo* at 30 °C, using a Büchi Rotavapor® R110. Sufficient methanol was then added to dissolve the residue and 1 M KOH added in a ratio of 2 mL KOH to 10 mL solvent. The extract was saponified for 12 h at room temperature, in the dark. The methanol fraction was reduced to the aqueous phase *in vacuo* at 35 °C and a small volume of water was added. This fraction was subsequently partitioned against diethyl ether, until the ether fraction was colourless. The diethyl ether fractions were pooled, dried *in vacuo* at 30 °C and resuspended in a small volume of hexane. This fraction was then subjected to vacuum liquid chromatography (VLC) on a 6 cm x 13 cm silica gel (150 g; Type 60, particle size 0.063-0.2 mm) column. The sample was dissolved in 5-6 g silica gel, placed on top of the column and covered with glass wool. The column was eluted (400 mL solvent/fraction) with increasing concentrations of ethyl acetate in hexane. The fractions eluted with 50:50 (v/v) hexane/ethyl acetate; 40:60 (v/v) hexane/ethyl acetate and 30:70 (v/v) hexane/ethyl acetate were pooled and dried *in vacuo* at 35 °C. The final concentration of violaxanthin was determined by measuring the absorbance at 450 nm in an Anthelie Advanced Spectrophotometer and applying Beer's law with $\varepsilon_{\lambda}^{450} = 2\ 500\ L\ mol^{-1}\ cm^{-1}$.

A solution of 10 mg violaxanthin and 10 mg Tween 80® in 10 mL 0.2 M Tris-HCl buffer (pH 7.6) was mixed with a solution of 20 mg linoleic acid and 20 mg Tween 80® in 10 mL 0.2 M Tris-HCl buffer (pH 7.6). The mixed solution was divided into four equal aliquots and 2 mL soybean lipoxidase (60 µg mL^{-1}) in 0.2 M Tris-HCl buffer (pH 7.6) was added to each aliquot. These aliquots were then incubated at 30 °C for 30 min, after which the reaction was terminated by the addition of 1 g ammonium sulphate. This solution was then partitioned three times against ethyl acetate. The ethyl acetate fractions were pooled and dried *in vacuo* at 35 °C. These fractions were loaded onto silica gel plates (GF_{254}) and developed to 15 cm in hexane/ethyl acetate (1:1, v/v). The zone corresponding to XAN ($R_f = 0.21$) was removed and eluted with water-saturated ethyl acetate. This was subsequently filtered through glass wool, dried *in vacuo* at 35 °C and re-purified on silica gel (GF_{254}) plates to a height of 15 cm in petroleum ether/acetone (3:1, v/v). The XAN zone was removed, eluted in water-saturated ethyl acetate and dried *in vacuo* at 35 °C. This fraction was resuspended in 20 % methanol, filtered through a 0.2 µm syringe filter (Lida Manufacturing Corp., Kenosha, WI, USA) and

further purified by HPLC (see section 2.9.7 for HPLC method (System B)). Xanthoxal was quantified following calibration with an authentic ABAMe standard.

2.2 CHROMATOGRAPHIC MEDIA

C_{18} solid phase extraction (SPE) columns and nitrile (SPE) cartridge columns were obtained from Isolute, International Sorbent Technology Ltd, Glamorgan, UK. Waters Sep-Pak® Plus †C_{18} cartridges and Waters Oasis® MCX 6cc (150 mg) extraction cartridges were purchased from Waters Corporation, Milford, MASS, USA. For thin layer chromatography (TLC) and VLC, silica gel 60 F_{254} plates (layer thickness 0.2 mm) and Type 60 silica gel (particle size 0.063 mm-0.2 mm) were purchased from Merck KGaA, Darmstadt, Germany. Whatman® No. 1 chromatography paper was purchased from Whatman International Ltd, Maidstone, England.

For HPLC, the analytical columns were as follows: 1) For ABA and IAA analysis a 5 µm C_{18} ODS1 Sphereclone column (250 mm x 10 mm i.d.) was purchased from Phenomenex, Torrance, CA, USA; 2) for purine analysis a 5 µm C_{18} Nucleosil 100-5 (250 mm x 4 mm i.d.) column with a 100-5 guard column (8 mm x 4 mm i.d.) was purchased from Macherey-Nagel GmbH and Co., Düren, Germany.

DEAE-Sephadex A-25 for anion exchange chromatography was purchased from Amersham Pharmacia Biotech Inc., Piscataway, New Jersey, USA. Sephadex G-25 for size exclusion chromatography and diatomaceous earth (acid washed) for reagent purification were obtained from Sigma, St Loius, MO, USA. Dowex 50W-X8 cation exchange resin (H+ form: 200-400 mesh) was purchased from BDH Chemicals Ltd, Poole, England. Activated charcoal for reagent purification was purchased from Merck Laboratory Supplies (Pty) Ltd, Midrand, RSA.

2.3 PREPARATION OF REAGENTS

2.3.1 Bradford reagent

The Bradford dye-binding reagent was prepared by dissolving Coomassie Brilliant Blue G-250 (500 mg) in 250 mL ethanol (99.9 %, w/v) and 500 mL concentrated phosphoric acid (85 %, w/v). The solution was made up to 1 L with distilled water and stirred overnight at 4 °C. The resulting solution was filtered through Whatman® No. 1 filter paper and stored in an amber-coloured bottle at 4 °C for up to six months. Prior to use the reagent was diluted five times, such that the final concentrations in the reagent were 0.01 % (w/v) Coomassie Brilliant Blue G-250, 5 % (w/v) ethanol and 8.5 % (w/v) phosphoric acid.

2.3.2 *p*-Aminophenol reagent

The *p*-aminophenol reagent is used to estimate CKOX activity as, under acidic conditions, it reacts with 3-methyl-2-butenal, a product of irreversible CK degradation, to form a highly coloured Schiff base with an absorbance maximum at 352 nm. This is based on the protocol for detection of 2,3-unsaturated aldehydes (Pesez and Bartos 1974). The reagent is prepared as a 3 % (w/v) *p*-aminophenol in 6 % (w/v) trichloroacetic acid solution. In order to decolourise the reagent it was passed through a charcoal column (1 cm x 5 cm) overnight at 4 °C. The reagent was prepared daily and protected from light at all times.

2.3.3 Ethereal diazomethane preparation

Ethereal diazomethane was generated, without co-distillation, on ice, by hydrolysis of N-nitroso-N-methylurea with 5 N NaOH in a Wheaton Diazomethane Generator (Pierce Chemical Co., Rockford, ILL, USA) using the small scale technique described by Fales *et al.* (1973). N-nitroso-N-methylurea (133 mg) and 0.5 mL water (for dissipation of generated heat) were placed in the inner tube, whilst 3 mL dry diethyl ether was placed in the outer tube. Subsequently to the unit being cooled on ice for 15 min, 0.6 mL 5 N NaOH was injected through the teflon rubber septum. The reaction was allowed to proceed for approximately 45 min or until the ether developed a deep yellow colour.

Dry diethyl ether was prepared by passing diethyl ether through a charcoal/diatomaceous earth (50:50, v/v) column (1 cm x 5 cm), upon which the dry diethyl ether was stored in a bottle containing iron filings, to remove peroxides. This is important as water and peroxides in the diethyl ether can cause an explosion during ethereal diazomethane generation.

2.4 PLANT MATERIAL

Avocado (*Persea americana* Mill. cv Hass) fruit were harvested from 8-year-old trees cultivated on clonal Duke 7 rootstocks in orchards in the KwaZulu-Natal Midlands, South Africa (Philips Bioclimatic group 3 – cool subtropical, summer rainfall area). Fruit was harvested in the early morning and transported to the laboratory where the seed, seed coat and mesocarp tissue was dissected into liquid nitrogen and freeze-dried immediately using a Xerotec Freeze Drier (University of Natal, Pietermartizburg, RSA) or extracted immediately for selected enzymes or plant hormones.

2.5 APPLICATION OF CHEMICALS

2.5.1 Excised whole fruit

For studies on the effect of various compounds on hormone metabolism in whole fruit harvested during the linear phase of growth, fruit pedicels were re-cut under water and the

fruit supplied with solutions via the transpiration stream. A total volume of 0.5 mL was pulsed into fruit via the pedicel, which was subsequently incubated at 25 °C, prior to extraction and analysis for the time period specified in Results.

Whole fruit was treated with CK (iP, iPA, zeatin and adenine), auxin (IAA, NAA and IBA) and allopurinol and molybdate, at the concentrations specified in Results. Cytokinin and allopurinol and molybdate were dissolved in 2 % (v/v) DMSO and formulated in water. Auxin was dissolved in 2 % (v/v) ethanol and formulated in water. Controls consisted of a fruit treated with either 0.5 mL 2 % (v/v) DMSO or 0.5 mL 2 % (v/v) ethanol.

2.5.2 Mesocarp from ripe fruit

Alternatively, for studies on the effect of various compounds on hormone metabolism in ripe fruit, mature fruit were allowed to ripen in darkness at 25 °C for 8-10 days and excised mesocarp tissue was infiltrated with 0.5 mL of the various compounds, via a series of cuts in the surface of the fruit. The fruit was then incubated in a water-saturated environment at 25 °C prior to extraction and analysis. For each experiment mesocarp blocks (ca. 15 g FW) were from the same fruit.

Ripe fruit were treated with cytokinin (iP, zeatin, adenine, 6-chloropurine, 2,6-dichloropurine, 2-methylthio-6-chloropurine and 2-methylthio-6-chloro-9-methylpurine) and allopurinol and molybdate, at the concentrations specified in Results. These treatments were dissolved in 2 % (v/v) DMSO and formulated in Tween 20®/acetone/water (1:1:8, v/v/v). Controls consisted of a mesocarp block treated with 5 mL 2 % (v/v) DMSO in Tween 20®/acetone/water (1:1:8, v/v/v). An equivalent sized tissue sample from each fruit was extracted immediately for determination of basal metabolic concentration.

2.6 PREPARATION OF ENZYME EXTRACTS

Tissue was extracted in buffer in the presence of 10 % (w/w) insoluble PVP (Polyclar AT), unless otherwise stated, and removed prior to analysis by filtration or centrifugation. Polyvinylpolypyrrolidone binds phenolic compounds by hydrogen bonding (Andersen and Sowers 1968) and is very useful when extracting plant material that contains significant quantities of phenols, such as avocado seed tissue, which is a rich source of polyphenolic compounds (Biale and Young 1971).

All freeze dried avocado tissue was milled to a fine powder using a IKA Analytical mill A10 (Janke and Kunkel GmbH and Co., IKA-Laboratory Technology, Staufen, Germany) and stored at -20 °C. When extracting fresh tissue, the tissue was finely chopped and kept on ice,

prior to being homogenised. Extracts were homogenised using an IKA Ultra-Turrax® top-drive tissue disperser (Janke and Kankel GmbH and Co., IKA-Laboratory Technology, Staufen, Germany).

2.6.1 Aldehyde oxidase and xanthine dehydrogenase

2.6.1.1 In vitro assay extraction

Crude extracts for assays of AO and XDH *in vitro* were prepared according to the procedure described by Triplett *et al.* (1982). Milled freeze-dried tissue (0.5 g), together with PVP (Polyclar AT, 10 % w/w), was homogenised on ice, in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM DTT, using an Ultra-Turrax top-drive tissue disperser. Extracts were allowed to stand on ice for 20 min prior to centrifugation at 30,000 g for 15 min at 2 °C, using a Hitachi Himac automatic high speed refrigerated centrifuge (Model CR20B2) with a RPR 20-2 rotar (Hitachi Koki Co. Ltd, Tokyo, Japan). The resulting supernatant was brought to 60 % saturation with solid ammonium sulphate. After stirring for 30 min the mixture was re-centrifuged at 40,000 g for 20 min at 2 °C. The pellet was resuspended in 2 mL of 50 mM potassium phosphate buffer (pH 7.8) and desalted on a 1 cm × 3 cm Sephadex G-25 column, equilibrated with 50 mM potassium phosphate buffer (pH 7.8). The column was centrifuged at 1,500 g for 2 min in a Hermle Z510 BHG (swinging bucket) centrifuge (Berthold Hermle GmbH and Co., Gosheim, Germany) to remove excess buffer used for equilibration. The enzyme extract was then loaded onto the column and centrifuged at 1,500 g for 2 min. The resulting eluant was used for subsequent assays.

2.6.1.2 Native-PAGE assay extraction

Crude avocado tissue extracts for assays of XDH and AO activity following native polyacrylamide gel electrophoresis (PAGE) were prepared according to a modified method of Sagi *et al.* (1999). Fresh tissue (5g) together with PVP (Polyclar AT, 10 % w/w) was homogenised on ice in 50 mM Tris-HCl buffer (pH 8.5) containing 1 mM DTT, 5 mM L-cysteine, 80 µM sodium molybdate, 0.03 mM FAD, 10 mM reduced glutathione and Complete® protease inhibitor tablets (1 tablet 50 mL^{-1}). Extracts were allowed to stand on ice for 20 min prior to centrifugation at 30,000 g for 15 min at 2 °C. The supernatant was retained for subsequent assays.

2.6.2 Cytokinin oxidase

2.6.2.1 Spectrophotometric assay extraction

Milled freeze-dried tissue (0.5 g) together with insoluble PVP (Polyclar AT, 10 % w/w) was homogenised on ice in 50 mM potassium phosphate buffer (pH 7.4), containing 2 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM DTT, Complete® protease inhibitor tablets (1 tablet 50 mL^{-1}) and 1 % (v/v)

Polymin P (50 % (w/v) aqueous solution) and allowed to stand for 20 min on ice. The suspension was centrifuged at 20,000 g for 20 min at 2 °C and the resulting supernatant was retained for subsequent analysis.

2.6.2.2 Radioactive assay extraction

Extracts for the radioactive assay of CKOX activity were prepared according to the method of Motyka and Kamínek (1994). Fresh tissue (5 g) together with insoluble PVP (Polyclar AT, 10 % w/w) was homogenised on ice in 10 mL 0.1 M Tris-HCl buffer (pH 7.5) containing Complete® protease inhibitor tablets (1 tablet 50 mL^{-1}) and allowed to stand for 20 min on ice. The homogenate was filtered through two layers of Miracloth (Calbiochem, Biosciences Inc., La Jolla, CA, USA) and the retained solids washed with two 5 mL aliquots of 0.1 M Tris-HCl buffer (pH 7.5). The resulting combined filtrate was centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was retained and 1 % (v/v) Polymin P (40 µL mL^{-1}; adjusted to pH 7.5) was added dropwise while stirring. After stirring for 10 min, the precipitates were removed by centrifugation at 10,000 g for 10 min at 4 °C. Solid ammonium sulphate was then added to the supernatant to give 80 % saturation. After stirring for 30 min, the ammonium sulphate precipitates were collected by centrifugation at 20,000 g for 20 min at 4 °C. The resulting pellet was resuspended in 0.05 M Tris-HCl buffer (pH 8.0) and desalted on a 1 cm x 3 cm Sephadex G-25 column equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) (see section 2.6.1.1 for procedure).

2.7 PROTEIN DETERMINATION

Protein in enzyme extracts was determined using Bradford's dye-binding assay (1976). This method of protein determination is rapid, sensitive, relatively inexpensive and specific for proteins, working in the range of 25 µg mL^{-1} to 200 µg mL^{-1} protein in solution (Bradford 1976; Read and Northcote 1981). Protein extract (100 µL) was added to 5 mL of the diluted Bradford reagent (see section 2.3.1 for preparation), vortexed and allowed to stand for 5 min to allow colour to develop. Absorbance was read at 595 nm in an Anthelie Advanced Spectrophotometer. Samples were assayed in triplicate and interpolated from a standard curve prepared using BSA as a protein standard. Assays for the standard curve were performed using nine replicates at five concentrations of BSA (0.2 mg mL^{-1} to 1 mg mL^{-1}). Blanks for protein determination were prepared using the corresponding extraction buffer.

2.8 ASSAY PROCEDURES

2.8.1 Spectrophotometric enzyme assays

2.8.1.1 Aldehyde oxidase in vitro assay

Aldehyde oxidase activity was determined spectrophotometrically by monitoring the decrease in absorbance of DCIP at 600 nm (Courtright 1967) in an Anthelie Advanced

Spectrophotometer. The reaction mixture consisted of 0.2 mL enzyme extract and 1.8 mL 0.1 mM PMS and either 2 mM I-ald (for XAN oxidase activity) or IA-ald (for IA-ald oxidase activity) in 50 mM potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of 0.002 % (w/v) DCIP in 50 mM potassium phosphate buffer (pH 7.4) and allowed to proceed for a maximum of 10 min. The final concentration of reduced DCIP was determined by applying Beer's law $\varepsilon_{\lambda}^{600} = 2.2 \times 10^4$ L mol^{-1} cm^{-1} and specific activity of AO is expressed as nmol DCIP reduced mg^{-1} protein min^{-1} (Sagi *et al.* 1998).

2.8.1.2 Xanthine dehydrogenase in vitro assay

For the determination of XDH activity, the reaction mixture included 1 mM hypoxanthine and 1 mM DTT in 50 mM Tris-HCl buffer (pH 6.5). The reaction was initiated by the addition of either 2.5 mM NAD^+ or 0.002 % (w/v) DCIP in 50 mM Tris-HCl buffer (pH 6.5) followed by incubation at 30 °C (Sagi *et al.* 1998). Xanthine dehydrogenase activity was monitored spectrophotometrically, in an Anthelie Advanced Spectrophotometer, by following the production of NADH at 340 nm (Triplett *et al.* 1982) or the decrease in absorbance of DCIP at 600 nm (Pérez-Vicente *et al.* 1988), for a maximum of 10 min. The final concentration of NADH or DCIP was determined by applying Beers law $\varepsilon_{\lambda}^{340} = 6.333 \times 10^3$ L mol^{-1} cm^{-1} for NADH and $\varepsilon_{\lambda}^{600} = 2.2 \times 10^4$ L mol^{-1} cm^{-1} for DCIP and is expressed as nmol DCIP reduced (NADH) mg^{-1} protein min^{-1}.

For the determination of the kinetic properties of AO and XDH, assays were conducted with 1.0 to 5.0 mM substrate for 1 to 3 min, during which time the reaction rate remained constant, according to the methods of Courtright (1967) for AO and Triplett *et al.* (1982) for XDH.

2.8.1.3 Cytokinin oxidase spectrophotometric assay

Cytokinin oxidase activity was assayed using the method of Liberos-Minotta and Tipton (1995). The reaction mixture in 0.2 M imidazole-HCl buffer (pH 7.5), contained 80 µM iP and 1 mM $CuCl_2$, and was initiated by the addition of enzyme and incubated at 37 °C for 30 min. The reaction was terminated using 40 % (w/v) trichloroacetic acid. Following the addition of *p*-aminophenol reagent (see section 2.3.2 for preparation) and development of colour for 10 min at room temperature, the absorbance at 352 nm was determined in an Anthelie Advanced Spectrophotometer. Cytokinin oxidase activity is expressed as µmol 3-methyl-2-butenal produced mg^{-1} protein, interpolated from a standard curve prepared by reacting 3-methyl-2-butenal with *p*-aminophenol reagent.

2.8.2 Radioactive enzyme assays

2.8.2.1 Cytokinin oxidase assay

Alternatively, CKOX activity was assayed by monitoring the conversion of [2-^{3}H]iP to [^{3}H]adenine according to the method of Motyka and Kamínek (1994). The assay mixture (50 µL final volume) contained 50 mM Tris-HCl buffer (pH 8), 10 µM substrate ([2-^{3}H]iP, 1.25 TBq mmol^{-1}) and 25 µL of enzyme preparation. After incubating the reaction mixture for 4 h at 37 °C, the reaction was terminated by the addition of 10 µL 200 mM Na_2EDTA and 120 µL of cold 95 % (v/v) ethanol, containing 0.75 mM unlabelled iP and adenine. Residual substrate and labelled product were subsequently separated on thin layers of silica gel (GF_{254}), developed once to 10 cm in chloroform/methanol/ammonium hydroxide (25 %, w/v) (9:2:0.1, v/v/v) (Redig *et al.* 1997). Zones corresponding to authentic iP (R_f 0.7-0.8) and Ade (R_f 0.3-0.4) standards were eluted with 2 mL 80 % ethanol, to which was added 5 mL Pico-Fluor™ 40 (Universal LSC cocktail). Radioactivity was determined by liquid scintillation spectrometry using a Packard Tri-Carb® 1500 Scintillation Counter (Packard, Downers Grove, ILL, USA) programmed for automatic quench correction. Cytokinin oxidase activity is expressed as µmol [^{3}H]adenine produced mg^{-1} protein h^{-1}. For substrate specificity analysis *trans*-[2-^{3}H]zeatin was used as a substrate.

2.8.3 Native-PAGE assays

2.8.3.1 Polyacrylamide gel electrophoresis

Native-PAGE was performed with a 10 % acrylamide gel in a Laemmli buffer system (Laemmli 1970), in the absence of sodium dodecyl sulfate (SDS) at 4 °C. A CBS Scientific Vertical Mini-Gel System (Model MGV-202; CBS Scientific Company Inc., Del Mar, CA, USA) was assembled as described in the manufacturer's manual. Before use the glass plates, spacers, combs and Gel Wrap™ gaskets were washed with soap water and cleaned with alcohol. The two glass plates (inner plate 8.5 cm x 11 cm, outer plate 9.5 cm x 11 cm) were positioned in the clamp assembly and separated by 1.0 mm polyethylene spacers. Removable silicone Gel Wrap™ gaskets ensure that the acrylamide solution does not leak from the sandwich assembly.

The separating gel solution (Table 2.1) was run into the space between the two glass plates, to a depth of 2.0 cm from the top of the inner glass plate and was overlayered with distilled H_2O, to exclude atmospheric oxygen, which inhibits polymerisation. Polymerisation involves the production of an acrylamide monomer chain which is cross-linked by the bifunctional compound N, N'-methylenebisacrylamide. N, N, N', N'-tetramethylethylenediamine catalyses the formation of free radicals from ammonium persulfate, which in turn initiates polymerisation (Hames and Rickwood 1981). Once the gel had set (approximately 1 h, evidenced by the

re-appearance of an interface between the gel solution and the over-layered distilled H_2O) the water was removed. Stacking gel solution (Table 2.1) was poured into the sandwich to the top of the inner glass plate and a 10-well comb inserted to form the sample application wells. Once the stacking gel had set, the comb was removed and the wells were washed with distilled H_2O. The gel sandwiches were then transferred and clamped onto the electrophoresis unit.

Table 2.1 **Reagents for two Laemmli gels (Laemmli 1970) for the CBS Scientific Vertical Mini-Gel System. (For reagent preparation see appendix I)**

Reagent	Separating Gel (10 %)	Stacking Gel (3.5 %)
Gel Buffer (mL)	3.75	2.25
Monomer (mL)	5.0	1.05
Ammonium persulfate (µL)	75	50
TEMED (µL)	15	7.5
Dist. H_20 (mL)	6.25	5.7

Tank buffer was poured into the upper and lower chamber of the electrophoresis unit. The voltage was set at 150 V for 30 min, to remove free radicals formed during the polymerisation process. Sample was mixed with non-reducing treatment buffer and bromophenol blue marker dye (10-12 µL), which migrates with the buffer front and monitors the progress of electrophoresis. The samples were centrifuged at 5,500 g for 5 min, in a Hitachi Himac Miniature Centrifuge (model SCT15B; Hitachi Koki Co. Ltd, Tokyo, Japan), to remove any insoluble material. The sample was loaded onto the gel and the voltage was set at 100 V (maximum current) until the bromophenol blue front had migrated through the stacking gel. The voltage was then increased to 150 V for the remainder of the electrophoretic run. Equal amounts of protein were loaded per sample onto the gel, varying from 20 to 30 µg for activity staining and 10 to 15 µg for silver staining of mesocarp samples and 2 to 3 µg for silver staining of seed and seed coat samples. The temperature of the unit was maintained at 4 °C, by attaching the unit to a Lauda Compact Low-Temperature Thermostat (Messgeräte-Werk Lauda, Lauda-Königshofen, Germany). Electrophoresis was continued until the bromophenol

blue marker dye was 0.5 cm from the bottom of the separating gel. The gels were removed and prepared for activity staining or placed in the appropriate staining solution for visualisation.

2.8.3.2 Activity staining for aldehyde oxidase and xanthine dehydrogenase

Enzyme activity staining is a technique used to locate the relative position of multiple forms of enzymes with common catalytic activity, after they have been resolved by PAGE (Tanksley and Orton 1983). This is usually achieved through the production of a non-diffusable chromogenic precipitate at the site of enzyme activity, such as the conversion of a soluble tetrazolium salt to a coloured insoluble formazan. The quantity of formazan formed is directly proportional to enzyme activity during a given incubation time and in the presence of excess substrate and tetrazolium salt (Rothe 1974).

Following electrophoresis, gels were removed, washed with distilled H_2O and immersed in 0.2 M potassium phosphate buffer (pH 8.0) for 5 min. Following this the gels were washed again with distilled H_2O, placed in the reaction mixture containing 1 mM substrate, 0.2 mM PMS and 1 mM MTT in 0.1 M Tris-HCl buffer (pH 8.0) and incubated for 45 min at 30 °C on an orbital shaker, in the dark. Following staining the gels were fixed in 5 % (v/v) acetic acid to facilitate diffusion of non-reacted substrate out of the gel, to remove background staining and to keep the bands sharp. The gels were removed from the fixing solution, washed with distilled H_2O and scanned immediately using a Hewlett-Packard Scanjet 5300C with HP PrecisionScan Software, Version 3.01 at 300 dpi resolution.

For the assessment of AO activity the following substrates were used I-ald, IA-ald, heptaldehyde, benzaldehyde, citral, acetaldehyde and 3-methyl-2-butenal at a concentration of 1 mM. Aldehyde oxidase activity was also assessed using XAN as a substrate at a concentration of 17 µM. For assessment of XDH activity hypoxanthine and xanthine were used as substrates at a concentration of 1 mM.

2.8.3.3 Silver staining of proteins in polyacrylamide gels

This method provides an attractive alternative to the traditionally used Coomassie Brilliant Blue R-250 protein stain, in that proteins may be detected in the nanogram range. The sensitivity of this technique, which is based on the reduction of silver ions to metallic silver, is only surpassed by radioactive labelling. Blum *et al.* (1987) modified the original silver staining procedure to avoid non-specific background staining without the loss of sensitivity or contrast.

All steps were carried out on an orbital shaker at room temperature and in scrupulously clean glass containers. Gloves were worn at all times to prevent contamination of the gels. After electrophoresis, the gels were soaked in fixing solution overnight and washed in washing solution 1 (3 x 20 min) to remove any acetic acid. Gels were placed in pretreatment solution (1 min), washed in deionised H_2O (3 x 20 sec) and placed in the impregnation solution (25 min). After washing (2 x 20 sec in deionised H_2O), the developer was added. As soon as bands were visible, the gels were washed in deionised H_2O until the bands were fully developed. The stopping solution was added (> 20 min), the gels washed in washing solution 2 (10 min), and scanned immediately as previously described (see section 2.8.3.2). For reagent preparation see appendix I.

2.9 EXTRACTION AND ANALYSIS OF PURINES AND PLANT HORMONES

2.9.1 Cytokinin extraction

Freeze-dried tissue (0.5 g), together with PVP (Polyclar AT, 100 % w/w), was homogenised in 100 mL 80 % (v/v) ethanol and extracted overnight in darkness at 4 °C. The homogenates were filtered under vacuum through Whatman® No. 1 filter paper and the resulting residue washed with 50 mL 80 % ethanol. The filtrate was reduced to dryness *in vacuo* at 35 °C and the residue resuspended in 50 mL 80 % ethanol (Smith and van Staden 1978). The pH of the extract was adjusted to 2.5 with 1N HCl and subsequently passed through a Dowex cation exchange resin column (H+ form: 200-400 mesh, column 3 cm x 8 cm) at a flow rate of 15 mL h^{-1}. The column was washed with 100 mL 80 % ethanol and the adsorbed CK were eluted from the column with 100 mL 5 N NH_4OH. The ammonia fraction was collected and reduced to dryness *in vacuo* at 35 °C (van Staden 1976a). The residue was resuspended in 80 % ethanol and applied as a 1 cm strip on Whatman® No. 1 chromatography paper (23 cm x 57 cm). The CKs were separated using descending paper chromatography and developed to 40 cm in iso-propanol/ammonia/water (10:1:1, v/v/v). The chromatograms were dried in a stream of air at 50 °C for 24 h, after which they were divided into ten equal R_f strips and assayed using the soybean callus bioassay (van Staden *et al.* 1972; Smith and van Staden 1978).

2.9.2 Purine extraction

Purines were extracted according to the method of Gilmore and Björkman (1994). Milled, freeze-dried tissue (0.5 g) was extracted on ice, with 10 mL ice-cold 5 % (w/v) perchloric acid, by homogenising for 60 sec. Extracts were chilled on ice for 5 min, homogenised for another 30 sec and the resulting homogenate centrifuged at 10,000 g for 5 min at 2 °C. The supernatant was adjusted to pH 3-3.5 with 5 M KOH and chilled on ice for 5 min to allow the precipitation of $KClO_4$. The mixture was centrifuged at 10,000 g for 2 min at 2 °C, after which PVP (Polyclar AT, 50 % w/w) was added to the supernatant. The PVP mixture was kept on ice

and stirred intermittently for 20 min, followed by centrifugation at 10,000 g for 5 min at 2 °C. The supernatant was titrated to between pH 6.5 and 7 with 0.5 M and 0.05 M KOH and chilled on ice for 5 min to allow $KClO_4$ to precipitate. After centrifuging at 10,000 g for 2 min at 2 °C, the supernatant was dried *in vacuo* at 35 °C and resuspended in HPLC starting buffer (0.02 M $(NH_4)H_2PO_4$ in 2.5 mM N,N-dimethyloctylamine, adjusted to pH 3 with acetic acid) and filtered through a 0.2 µm syringe filter.

2.9.3 ABA and IAA extraction

Tissue (0.5 g DW or 5 g FW) together with PVP (Polyclar AT, 10 % w/w) was homogenised in ice-cold 80 % (v/v) methanol containing BHT and DDC (both 100 mg L^{-1}) as antioxidants and [G-^{3}H]ABA and 3-[5(n)-^{3}H]IAA (both 20 000 dpm/sample), added to correct for losses, and extracted overnight in darkness at 2 °C. All further extraction procedures were carried out in dim light as both IAA and ABA are sensitive to light. Anti-oxidants were included in the extraction solvent as IAA and ABA are oxidised when exposed to air (Yokota *et al.* 1980). Homogenates were filtered under vacuum through Whatman® No. 1 filter paper and the filtrate reduced to dryness *in vacuo* at 35 °C. The residue was resuspended in water (adjusted to pH 3 with 0.1 N HCl) and partitioned three times against ethyl acetate (pK_a for IAA is 4.54 and pK_a for ABA is 4.7). The pooled ethyl acetate fractions were reduced to dryness *in vacuo* at 35 °C, resuspended in water (adjusted to pH 8 with 0.05 N NaOH) and partitioned three times against hexane. The aqueous fraction was loaded onto a DEAE-Sephadex A-25 anion exchange column (5 mL bed volume), pre-equilibrated with 0.5 M sodium formate. The column was washed with two 10 mL aliquots of water (pH 8) and the acids eluted onto a pre-wetted C_{18} (SPE) column with four 5 mL aliquots of 0.2 M formic acid. The C_{18} column was washed with 10 mL 90 % methanol and the eluate reduced to dryness *in vacuo* at 35 °C. Samples were resuspended in methanol and methylated by the addition of excess ethereal diazomethane (see section 2.3.3 for preparation) over 20 min. After removal of the ether phase under a stream of nitrogen, methylated samples were dried, partitioned three times into ethyl acetate and passed through a nitrile (SPE) cartridge column. The ethyl acetate was removed under nitrogen, the sample resuspended in 20 % methanol for analysis by reversed phase HPLC, and filtered through a 0.2 µm syringe filter.

2.9.4 ABA, IAA and CK extraction

The second protocol for ABA and IAA extraction involved using a novel dual mode Waters Oasis® MCX column, containing a copolymer which allows separation of compounds by reversed-phase and cation-exchange modes (Dobrev and Kamínek 2002). This method was employed for two reasons, firstly it is a rapid method that allows easy manipulation of a large

number of samples and secondly, CK can be purified simultaneously. Through the step-wise elution of solvents containing an increasing concentration of methanol and NH_4OH separation of IAA plus ABA, CK nucleotides and CK bases plus CK ribosides plus CK glucosides can be achieved. Other advantages of this method of extraction include high recoveries, high loading capacity (5-10 g FW plant material/column) with reasonably high recoveries and an acceptable degree of purification (80-250 fold reduction in dry mass of the initial extract) (Dobrev and Kamínek 2002).

Milled freeze-dried tissue (0.5 g) together with PVP (Polyclar AT, 10 % w/w) was homogenised in ice-cold 80 % (v/v) methanol containing BHT and DDC (both 100 mg L^{-1}) as antioxidants and [G-^{3}H]ABA, 3-[5(n)-^{3}H]IAA, [2-^{3}H]iP and *trans*-[2-^{3}H]zeatin (all 50 000 dpm/sample), added to correct for losses, and extracted overnight, in darkness, at 2 °C. Homogenates were filtered under vacuum through Whatman® No. 1 filter paper and passed through pre-wetted Sep-Pak® Plus †C_{18} (SPE) cartridges. The eluent was reduced to dryness *in vacuo* at 35 °C and reconstituted in 5 mL 1 M formic acid. This was subsequently passed through an Oasis® MCX (150 mg/6 cc) cartridge, pre-conditioned with 5 mL methanol and 5 mL water. The cartridge was washed with 5 mL 1 M formic acid and 5 mL methanol. The methanol fraction was collected and dried *in vacuo* at 35 °C, as it contained the carboxylic acids, ABA and IAA. It was then prepared for HPLC by resuspending it in 1 mL methanol and filtering it through a 0.2 µm syringe filter. The Oasis® MCX cartridge was subsequently eluted with 1) 5 mL 0.17 M NH_4OH; 2) 5 mL 0.17 M NH_4OH in 60% methanol; and 3) 5 mL 0.34 M NH_4OH in methanol. Cytokinin nucleotides were located in eluate 1 and CK bases plus ribosides plus glucosides were located in eluate 2.

2.9.5 Cytokinin bioassays

Callus from soybean cotyledons (*Glycine max* (L.) Merrill cv. Acme) is CK-dependent and is capable of rapidly metabolising CK (Forsyth and van Staden 1986). The bioassay exhibits a linear relationship between response and concentration over a wide range of CK concentrations from 2 x 10^{-8} M to 5 x 10^{-5} M (Miller 1963; Manos and Goldthwaite 1976; van Staden and Davey 1979). Callus was initiated according to the method described by Miller (1963; 1965) and was maintained on Millller's medium (Miller 1965), supplemented with kinetin (0.5 mg L^{-1}) and NAA (2 mg L^{-1}), for numerous subcultures (see appendix I for media preparation).

Each R_f zone, of the paper chromatogram, was cut into small pieces and placed in 50 mL flasks, to which 1 % (w/v) agar was added. Miller's medium (30 mL) was then added to each flask. The flasks were stoppered with non absorbent cotton wool bungs and covered with

aluminium foil, prior to autoclaving at 121 °C and 1 Pa for 20 min, in a Tomy Autoclave Model SD-30N (Tomy Seiko Co. Ltd, Tokyo, Japan). Transfer instruments were autoclaved at the same time. The flasks were transferred to a sterile cabinet and the agar was allowed to set under UV light. Three small pieces of callus (approximately 10 mg) were transferred to each flask. The flasks were placed in a growth room at a constant temperature (26 °C ± 2 °C) and continuous low light intensity (0.72 µmol m^{-2} s^{-1}), supplied by cool white fluorescent tubes, for 28 d. The combined mass of the three callus pieces was recorded as callus yield (van Staden 1976b). The bioassays were performed in triplicate and the mean values were determined.

2.9.6 HPLC quantification of purines

High performance liquid chromatography of purines was carried out using a 5 µm C_{18} column (250 mm x 4 mm i.d., Nucleosil 100-5) with a 100-5 guard column (8 mm x 4 mm i.d.) and eluted over 40 min with a linear gradient of 100 % - 80 % 0.02 M $(NH_4)H_2PO_4$ in 2.5 mM N,N-dimethyloctylamine (adjusted to pH 3 with acetic acid) (Solvent A) in methanol (Solvent B), at a flow rate of 1 mL min^{-1}. This was followed by a 3 min linear gradient to 100 % methanol to facilitate cleaning of the column, a 2 min linear gradient to 100 % Solvent A and a 3 min re-equilibration time. Compounds of interest were detected at 268 nm using a Spectra System UV/VIS 1000 detector (Thermo Separations Products, Freemont, CA, USA) and quantified after calibration with authentic standards (see appendix I for standard chromatogram).

2.9.7 HPLC quantification of ABA and IAA

High performance liquid chromatography of IAA and ABA was carried out using a 5 µm C_{18} column (250 mm × 10 mm i.d., ODS1) eluted over 55 min with a linear gradient of 20 % - 100 % methanol in either 0.005 % aqueous acetic acid (System A) or water (System B), at a flow rate of 2 mL min^{-1}. System A was used to analyse ABA, IAA, PA and DPA (see appendix I for standard chromatogram), whilst System B was used to analyse methylated samples i.e. ABA- and IAA-methyl esters (see appendix I for standard chromatogram). Compounds of interest were detected at 260 nm using a Spectra System UV/VIS 1000 detector and quantified after calibration with authentic standards of ABA, IAA, DPA, PA, ABA- and IAA-methyl esters.

For estimation of losses incurred during extraction, HPLC eluates were fractionated into 1 mL aliquots, dried down in a Savant SC200 SpeedVac® concentrator, connected to a Refrigerated Condensation Trap RT400 (Savant Instruments Inc., Farmingdale, NY, USA) and resuspended in 0.5 mL methanol to which was added 2 mL Pico-Fluor™ 40 (Universal LSC cocktail). Radioactivity was determined by liquid scintillation spectrometry using a Packard Tri-Carb® 1500 Scintillation Counter, programmed for automatic quench correction.

2.9.8 Gas chromatography-electron impact-mass spectrometric identity of ABA and IAA

High performance liquid chromatography purified ABA and IAA 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 x 0.32 mm i.d.) of OV-1 (Supelco Inc., Bellefonte, USA) programmed from 120 °C at 5 °C min^{-1} with He as a carrier gas (1.5 – 2.0 mL min^{-1}) and EI spectra recorded at 70 eV and compared against authentic ABA (Walker-Simmons *et al.* 2000) and IAA (Prinsen *et al.* 2000) methylated standards.

3 CHARACTERIZATION AND TISSUE DISTRIBUTION OF AO, XDH, AND CKOX IN DEVELOPING AVOCADO FRUIT

3.1 INTRODUCTION

Biochemical characterization of the 'Hass' small fruit variant has shown that carbohydrate, isoprenoid and ABA metabolism are all intimately linked to the appearance of this phenotype (Cowan *et al.* 1997; Moore-Gordon *et al.* 1998; Richings *et al.* 2001). This conclusion is based on reduced HMGR and SuSy activity and enhanced insoluble AI activity in small fruit, which are associated with increased respiration, sucrose depletion, an increase in glucose as a portion of the total soluble sugars and increased ABA metabolism (Richings *et al.* 2001). A reduction in fruit size and the appearance of a small fruit phenotype was also correlated with a decreased CK/ABA ratio (Moore-Gordon *et al.* 1998). It is therefore evident that there exists the possibility of cross-talk between sugar and hormone signalling pathways in fruit development and that one of the potential roles of phytohormones in controlling avocado fruit growth, is to detect or communicate changes in sugar content and composition and as a consequence co-ordinate or re-direct growth (Cowan *et al.* 2001).

Although plant hormones have been detected in avocado fruit the information is rudimentary and no attempt has been made to relate differences in hormone levels to the appearance of a small fruit phenotype (Table 3.1). Furthermore, there is little or no information on hormone metabolism in avocado fruit, aside from studies on ABA biosynthesis. This notwithstanding, determination of plant hormone levels alone is often of limited value. Such data reveals little about the site of hormone metabolism within the tissue under investigation, and very little can be inferred about the contribution of hormone metabolism to changes in net hormone levels. This limitation can be minimized by measuring the activity of key enzymes involved in hormone metabolism, in addition to quantifying endogenous levels of the respective hormones.

The involvement of MoCo-containing AOs in the conversion of XAN to ABA (Walker-Simmons *et al.* 1989; Sindhu *et al.* 1990; Leydecker *et al.* 1995; Cowan 2001; Milborrow 2001) and IA-ald to IAA (Koshiba *et al.* 1996; Tsurusaki *et al.* 1997; Lips *et al.* 1999) is of particular interest as allocation of the MoCo to either NR or other MoCo-requiring enzymes such as XDH and AO can potentially regulate sink strength and fruit growth in avocado (Campbell *et al.* 2000; Cowan *et al.* 2001).

Table 3.1 **Previous findings of hormone content and composition in the tissues of developing avocado fruit. (RIA, Radioimmunoassay).**

Hormone	Method of determination	Seed	Seed coat	Mesocarp	Reference
CK	Bioassay	high initially declines as fruit matures	initially very high declines as fruit matures	moderate "bound" CK-like levels declines as fruit matures	Blumenfeld and Gazit 1970; Gazit and Blumenfeld 1970
	RIA	iP: >30 to 14 ng g^{-1} FW iPA: 5 ng g^{-1} FW	iP: 30 to 0 ng g^{-1} FW iPA: 20 to 0 ng g^{-1} FW	iP: 20 to 5 ng g^{-1} FW iPA: 5 ng g^{-1} FW	Cutting *et al.* 1986
IAA	Bioassay	high initially declines as fruit matures	high initially declines as fruit matures	moderate declines during fruit growth, small peak close to maturity	Gazit and Blumenfeld 1972
	RIA	70 to 15 ng g^{-1} FW at maturity	70 to 0 ng g^{-1} FW at maturity	steady decline from 30 to 10 ng g^{-1} FW	Cutting *et al.* 1986
ABA	RIA	varied between 35 and 65 ng g^{-1} FW	varied between 35 and 65 ng g^{-1} FW declined to 0 at maturity	40 to 100 ng g^{-1} FW from mid growth to maturity	Cutting *et al.* 1986
	Reversed-phase HPLC	14 µg g^{-1} DW during linear phase of growth	-	29.3 to 10.9 µg g^{-1} DW over course linear phase of growth	Cowan *et al.* 1997 Richings *et al.* 2000
GA	Bioassay	no measurable activity	initially very high declines as fruit matures	no measurable activity	Blumenfeld and Gazit 1972

Initial evidence for the involvement of a MoCo-AO in ABA biosynthesis came from studies using MoCo-deficient mutants of barley (Walker-Simmons *et al.* 1989), tobacco (Leydecker *et al.* 1995), tomato (Marin and Marion-Poll 1997) and *Arabidopsis* (Schwartz *et al.* 1997a). These mutants lacked MoCo-containing AO and XDH activities and had impaired ABA production. Lee and Milborrow (1997) presented further evidence of the involvement of a MoCo-AO in ABA biosynthesis by demonstrating XAN accumulation in avocado fruit treated with tungstate. Through the addition of cinchonine, which forms an insoluble complex with tungstate, they were also able to show restoration of ABA production. These findings, taken together, lead to the distinct possibility that XAN oxidase is a MoCo-containing AO. Recently two AO genes have been identified in *Arabidopsis* leaves that are rapidly induced after desiccation (Seo *et al.* 1999) and an AO isoform, designated AOδ, has been found that has high specificity for AB-ald and is expressed mainly in rosette leaves of *Arabidopsis* (Seo *et al.* 2000a;b).

Mutants impaired in MoCo biosynthesis would also be expected to exhibit impaired IAA biosynthesis, if a MoCo-AO is indeed involved in the final step of its biosynthesis. However, MoCo mutants exhibit no obvious IAA deficiency or auxotrophy phenotype (Seo *et al.* 1998). One possible explanation is that the mutants are leaky and small quantities of IAA are sufficient to promote normal growth. Another possibility is that several parallel pathways for IAA biosynthesis exist in plants, operating at different stages of development and/or in different organs or tissues (Normanly *et al.* 1995; Kawaguchi and Syōno 1996; Normanly 1997). Some investigators have shown that an AO, tentatively designated IA-ald oxidase, may be involved in IAA synthesis (Rajagopal 1971; Bower *et al.* 1978; Miyata *et al.* 1981), but the actual function of the enzyme in IAA biosynthesis has not been definitively confirmed. Maize AO has exhibited a high affinity for IA-ald (K_m 3-5 μM), which indicates that even at low concentrations of IA-ald, the aldehyde could still be converted to IAA (Koshiba *et al.* 1996). Subsequently, Seo *et al.* (1998) demonstrated that an AO isoform, designated AOα, in *Arabidopsis* plants had high affinity for I-ald and IA-ald and was overexpressed in the IAA overproducing *sur1* mutant, as compared to the wild type, suggesting the involvement of this enzyme in the final step of IAA biosynthesis.

Xanthine dehydrogenase is another MoCo-requiring enzyme and catalyses the first oxidative step in purine catabolism (Nguyen 1986). It is ubiquitous and ensures that purine compounds, originating from CK and nucleic acid degradation, are irreversibly committed into purine catabolism (Wasternack 1982; Nguyen 1986). Xanthine dehydrogenase catalyses the formation of uric acid from xanthine and hypoxanthine and is thus necessary for the synthesis of ureides in higher plants (Nguyen 1986). This enzyme is inhibited by excess substrate (Bray

1963) and product (Nguyen 1979; Boland 1981), as well as substrate and product analogues (Nguyen 1986), such as adenine and guanine (Woo *et al.* 1981).

It has been suggested that the size of the MoCo pool is not consistent, but varies in response to nutritional and environmental factors (Sagi *et al.* 1997; Sagi and Lips 1998). It is therefore hypothesized that under conditions where nitrate assimilation is reduced and/or XDH inhibited, more MoCo might be expected to be available for AO required for ABA and IAA biosynthesis. One possible means by which XDH might be inhibited is through elevated adenine levels, one source of which is the irreversible degradation of isoprenylated CK. The degradation of CK is catalysed by the enzyme CKOX, which raises the possibility that changes in activity of this enzyme might impact on the activity of XDH, through the production of adenine. The result of CKOX activity is the irreversible loss of CK structure and thus biological activity and in this way CKOX is thought to play an important role in controlling the internal pool of CK in plants. Available evidence suggests that the activity of CKOX and the degradative metabolism of CKs can be mediated by four principle mechanisms: (1) CK supply; (2) phenylurea compounds; (3) auxin levels; and (4) glycosylation and/or isozyme variation (Jones and Schreiber 1997).

There have been very few investigations of the activity of AO, XDH and CKOX in fruit tissues and certainly none considering all three simultaneously. The linkage of these enzymes to hormone homeostasis has been alluded to before (Cowan *et al.* 1999; 2001), but a direct relationship has yet to be established. The plants of choice used to investigate these enzymes remain those with a rapid growth cycle, e.g. *Arabidopsis thaliana*, *Nicotiana sp.*, *Zea mays* and other cereal crops. The tissues extracted to evaluate activity of these enzymes are mainly leaves or roots and in the case of CKOX, maize kernels. The use of the avocado fruit is thus a first, with the 'Hass' small fruit phenotype providing an ideal system in which to probe more detailed aspects of the control of final fruit size. To further characterize the 'Hass' avocado small fruit phenotype, it was therefore necessary to compare and contrast the endogenous hormone profile of seed, seed coat, and mesocarp tissue in normal and small fruit. In addition, certain key enzymes involved either directly or indirectly in hormone metabolism were assayed to determine activity and tissue distribution in normal and small fruit.

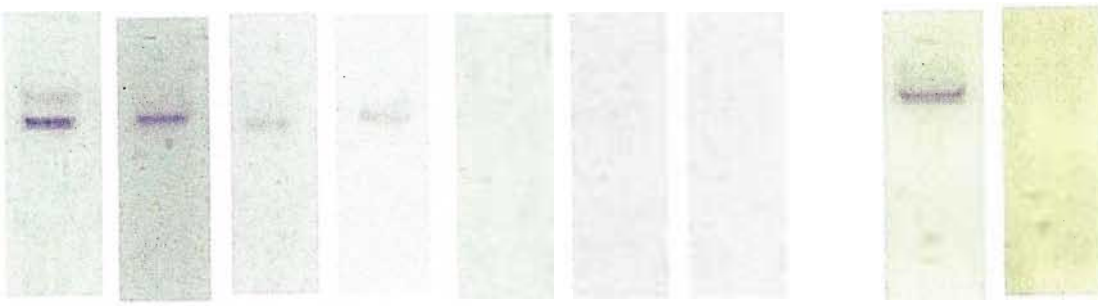
3.2 RESULTS

3.2.1 Characterization of AO, XDH, and CKOX in 'Hass' avocado fruit

3.2.1.1 Aldehyde oxidase

The substrate preference of AO, from mesocarp tissue of 'Hass' avocado fruit, for seven aldehydes was studied by comparing the formazan band intensity after activity staining, following native-PAGE (Fig. 3.1). Of the substrates tested I-ald (1) and citral (2) were used

efficiently by the enzyme. However, the enzyme was less active with benzaldehyde (3) and heptaldehyde (4) and showed no activity in the presence of acetaldehyde (5), 3-methyl-2-butenal (6) and IA-ald (7). Only 17 µM of XAN could be produced after numerous preparations and thus activity with XAN was determined relative to a substrate at a similar concentration. For this purpose I-ald was chosen at a concentration of 20 µM. Although activity was still evident when using I-ald (A), the enzyme exhibited no affinity for XAN (B). This lack of activity may not be attributable to a lack of substrate specificity, but could be due to the presence of minor contaminating compounds in the XAN preparation and/or the concentration of 17 µM may be too low to detect activity when using this substrate.



(1) (2) (3) (4) (5) (6) (7) (A) (B)

(1)	**indole-3-aldehyde**	**(5)**	**acetaldehyde**
(2)	**citral**	**(6)**	**3-methyl-2-butenal**
(3)	**benzaldehyde**	**(7)**	**indole-3-acetaldehyde**
(4)	**heptaldehyde**		
(A)	**indole-3-aldehyde (20 µM)**	**(B)**	**xanthoxal (17 µM)**

Figure 3.1 **Zymogram showing AO activity in extracts from 'Hass' avocado mesocarp tissue (harvested 112 days after full bloom (DAFB)), following activity staining using different substrates. Following native-PAGE, the activity bands were developed separately, with strips from each lane, using seven aldehydes (1-7) at a concentration of 1 mM and two aldehydes (A and B) at a concentration of 20 µM and 17 µm respectively. The number at the bottom of each lane corresponds to the aldehyde used. Each lane was loaded with an equal amount of protein (25 µg/lane).**

Through the use of spectrophotometric assays (Courtright 1967), K_m and V_{max} values were calculated for AO in mesocarp and seed tissue of 'Hass' avocado fruit from Lineweaver-Burk plots, using I-ald, citral, benzaldehyde and heptaldehyde as substrates (Table 3.2). All the aldehydes were used efficiently by AO in both seed and mesocarp tissue, with K_m values ranging from 1.37 mM for heptaldehyde to 6.41 mM for benzaldehyde in mesocarp tissue and 0.46 mM for benzaldehyde to 1.17 mM for citral in seed tissue. Similar values have been reported for AO in maize (Koshiba *et al.* 1996) and *Nicotiana plumbaginifolia* (Akaba *et al.* 1998).

The effect of heat pre-treatment on AO activity in mesocarp tissue is illustrated in Figure 3.2, where enzyme activity is directly proportional to the intensity of the formazan band formed following activity staining. The enzyme lost all activity after being boiled for 2.5 min, but was relatively stable between 50 °C and 60 °C, with some catalytic activity still detected at 70 °C. The loss of formazan stain at high temperatures demonstrates that this is an enzyme catalysed reaction.

Table 3.2 **Kinetic properties of AO in mesocarp and seed tissue of 'Hass' avocado fruit (harvested 112 DAFB) with various substrates. Enzyme activity was determined by monitoring the decrease in absorbance of DCIP (Courtright 1967). The K_m and V_{max} values were calculated based on Lineweaver-Burk plots of the data.**

Substrate	Tissue	K_m	V_{max}
		mM	nmol DCIP mg^{-1} protein min^{-1}
Indole-3-aldehyde	mesocarp	2.81	175.44
	seed	0.8	1000.00
Citral	mesocarp	1.68	322.58
	seed	1.17	1666.67
Heptaldehyde	mesocarp	1.37	175.44
	seed	0.66	1111.11
Benzaldehyde	mesocarp	6.41	43.86
	seed	0.46	909.09

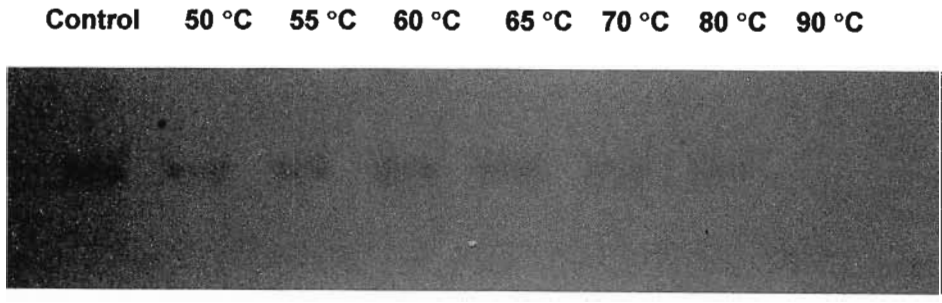


Figure 3.2 **Zymogram showing AO activity in extracts from 'Hass' avocado mesocarp tissue (harvested 84 DAFB), following heat pre-treatment prior to electrophoresis. Extracts were heated in a water bath for 2.5 min at different temperatures, followed by immediate cooling in ice. Activity was determined using 1 mM l-ald as the substrate. Each lane was loaded with 20 µg protein.**

3.2.1.2 Xanthine dehydrogenase

Xanthine dehydrogenase in 'Hass' avocado seed and mesocarp tissues has a pH optimum between 6.0 and 7.0 (Fig. 3.3). The enzyme showed no affinity for xanthine, but exhibited activity when hypoxanthine was used as the substrate (Fig. 3.4). The K_m of XDH for

hypoxanthine in 'Hass' avocado mesocarp tissue was 54 µM and the V_{max} was 270.27 nmol DCIP reduced mg^{-1} protein min^{-1}, as calculated from a Lineweaver-Burk plot of the data. K_m values for hypoxanthine of a similar order have been calculated for XDH in *Chlamydomonas reinhardtii* (160 µM) (Pérez-Vicente *et al.* 1988) and *Nicotiana tabacum* (29 µM) (Nguyen and Nato 1983).

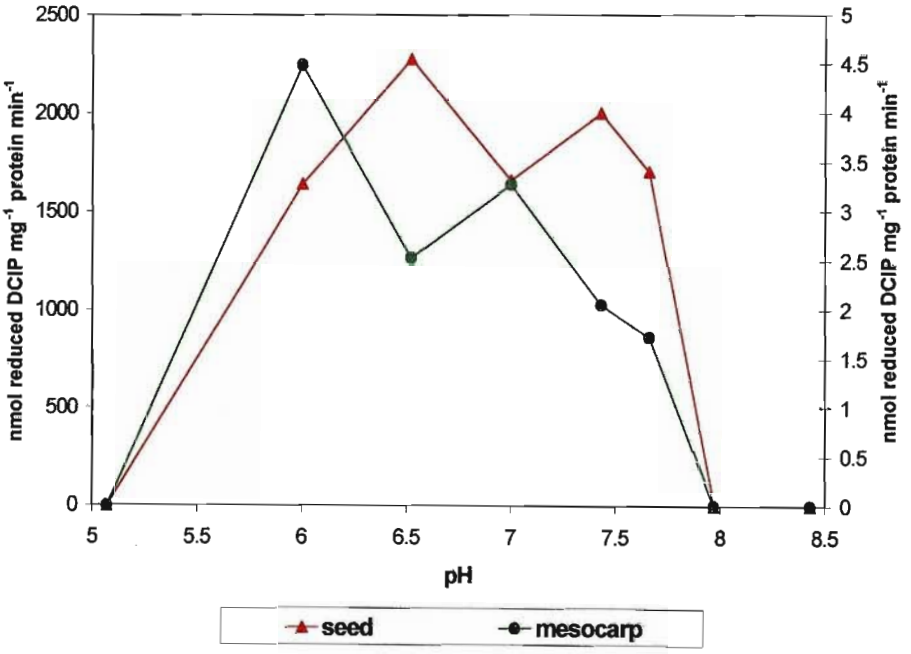
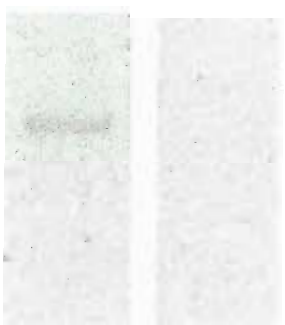


Figure 3.3 **The effect of pH on XDH activity in seed (left axis) and mesocarp (right axis) tissue of normal 'Hass' avocado fruit. Enzyme activity was determined according to the method of Triplett *et al.* (1982).**



(A) **(B)**

Figure 3.4 **Zymogram showing XDH activity in extracts from 'Hass' avocado mesocarp tissue (harvested 112 DAFB), following activity staining with 1 mM hypoxanthine (A) and 1 mM xanthine (B). Following native-PAGE, the activity bands were developed separately, with strips from each lane. Each lane was loaded with 25 µg protein.**

Nicotinamide adenine dinucleotide was a more effective electron acceptor in the enzymatic oxidation of hypoxanthine than DCIP, in both seed and mesocarp tissue of normal 'Hass'

avocado fruit (Table 3.3). This preference of XDH for NAD^+ over DCIP has also been found in *Chlamydomonas reinhardtii* (Pérez-Vicente *et al.* 1988), *Rhodopseudmonas capsulata* (Aretz *et al.* 1981) and *Pisum sativum* leaves (Nguyen and Feierabend 1978).

Table 3.3 **Comparison of two electron acceptors for XDH in seed and mesocarp tissue of normal 'Hass' avocado fruit, harvested 240 DAFB. Enzyme activity was assayed according to the method of Triplett *et al.* (1982). Values are the mean of three replicates ± SE.**

Tissue	Electron acceptor	Concentration	XDH
		mM	µmol cofactor reduced mg^{-1} protein min^{-1}
Seed	NAD^+	2.5	32.62 ± 3.0
	DCIP	0.69	16.6 ± 4.6
Mesocarp	NAD^+	2.5	2.0 ± 0.19
	DCIP	0.69	0.72 ± 0.63

3.2.1.3 Cytokinin oxidase

Cytokinin oxidase in tissues of 'Hass' avocado fruit has a pH optimum between 8.0 and 9.0 (Fig. 3.5). The K_m of CKOX in 'Hass' avocado mesocarp tissue for iP, determined using the spectrophotometric assay of Liberos-Minotta and Tipton (1995), based on a Lineweaver-Burk plot of the data, was 0.706 µM and the V_{max} was 20.24 µmol 3-methyl-2-butenal mg^{-1} protein. On the other hand, a Lineweaver-Burk plot of the data generated from the radioactive CKOX assay (Motyka and Kamínek 1994) yielded a K_m of 7.425 µM and a V_{max} of 1.928 µmol [^{3}H]adenine mg^{-1} protein h^{-1} for iP. Cytokinin oxidase from plant tissues is known to exhibit marked differences in pH optima (6.5 - 8.5) and kinetic constants, with K_m values ranging from 3 µM to 27 µM for iP (McGaw and Horgan 1983; Laloue and Fox 1989; Kamínek and Armstrong 1990; Motyka and Kamínek 1992; Bilyeu *et al.* 2001).

A test for substrate preference of the CKOX enzyme in 'Hass' avocado mesocarp tissue, using the assay of Motyka and Kamínek (1994), revealed that the enzyme was marginally more efficient in oxidizing iP than zeatin (Table 3.4). Similar results have been found in *Zea mays* (Whitty and Hall 1974; McGaw and Horgan 1983; Bilyeu *et al.* 2001), *Nicotiana tabacum* (Pačes and Kamínek 1976), *Phaseolus vulgaris* (Chatfield and Armstrong 1986), *Triticum aestivum* (Laloue and Fox 1989) and *Petunia hybrida* (Auer *et al.* 1999).

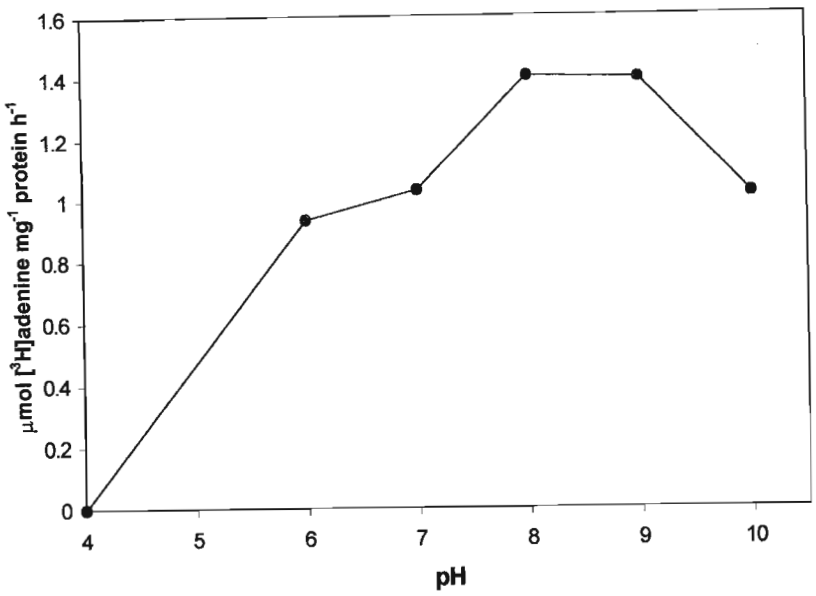


Figure 3.5 **The effect of pH on CKOX activity in mesocarp tissue of normal 'Hass' avocado mesocarp tissue. Enzyme activity was determined according to the method of (Motyka and Kamínek 1994).**

Table 3.4 **Substrate preference of CKOX from mesocarp tissue of normal 'Hass' avocado fruit, harvested 112 DAFB. Enzyme activity was determined according to the method of Motyka and Kamínek (1994). Data are the mean of 4 replicates ± SE.**

Substrate	Concentration	CKOX
	µM	µmol [^{3}H]adenine mg^{-1} protein h^{-1}
Isopentenyl adenine	10	0.7325 ± 0.489
Zeatin	10	0.3789 ± 0.137

3.2.2 Tissue distribution and activity of XDH, IA-ald oxidase, XAN oxidase, and CKOX in normal and small 'Hass' avocado fruit

The activity and distribution of IA-ald oxidase, XAN oxidase, XDH and CKOX in tissues of normal and phenotypically small 'Hass' avocado fruit are presented in Table 3.5. Aldehyde oxidase activity in avocado fruit was determined using two substrates. Activity, using I-ald as a substrate (designated XAN oxidase), was present in all tissues of avocado fruit, where activity was significantly greater in the seed and seed coat of the small fruit variant as compared to seed and seed coat from normal fruit. There was no difference in XAN oxidase activity in

Table 3.5 **Tissue distribution and activity of IA-ald and XAN oxidase, XDH, and CKOX in normal and phenotypically small 'Hass' avocado fruit, harvested 240 DAFB, during the linear phase of fruit growth. Data for IA-ald, XAN oxidase, and XDH are the mean of 3 replicates ± SE, whilst data for CKOX are the mean of 5 replicates ± SE.**

Tissue	Phenotype	IA-ald oxidase	XAN oxidase	XDH	CKOX		
		nmol DCIP mg^{-1} protein min^{-1}			µmol 3-methyl-2-butenal mg^{-1} protein	µmol [^{3}H]adenine mg^{-1} protein h^{-1}	average
Seed	normal	29.78 ± 14.04	6.64 ± 0.30	9.93 ± 5.81	250.86 ± 52.86	48.68 ± 20.23	149.77 ± 36.55
	small	2.51 ± 1.74	19.58 ± 4.44	16.19 ± 1.73	256.77 ± 57.29	276.74 ± 171.58	266.76 ± 114.44
Seed coat	normal	ND[†]	16.76 ± 1.90	ND	32.01 ± 10.79	76.56 ± 11.25	54.29 ± 11.02
	small	ND	43.96 ± 9.22	ND	47.45 ± 18.40	ND	23.73 ± 9.20
Mesocarp	normal	ND	7.48 ± 2.84	3.29 ± 1.45	7.31 ± 3.16	1.02 ± 0.31	4.165 ± 1.74
	small	ND	8.76 ± 2.90	23.44 ± 6.80	9.26 ± 3.81	6.48 ± 1.99	7.87 ± 2.90

[†]**ND = no activity detected**

mesocarp tissue of normal and small fruit. In contrast, AO activity, using IA-ald as a substrate (designated IA-ald oxidase), was only detected in seed tissue where it was found to be ten-fold greater in normal fruit. Xanthine dehydrogenase activity was only detected in seed and mesocarp tissue, where it was significantly greater in tissues from the small fruit variant.

Cytokinin oxidase activity in 'Hass' avocado fruit tissues was determined using two methods. The first method is based on the formation of a highly coloured Schiff base, through the reaction of the *p*-aminophenol reagent with 3-methyl-2-butenal, a product of CK degradation (Liberos-Minotta and Tipton 1995). This reaction is measured spectrophotometrically and involves the use of a crude enzyme extract. As such this is a simple and rapid method of assaying for CKOX activity and a good starting point for establishing the existence of CKOX activity in normal and small 'Hass' avocado fruit. The second method demands the use of a radioactive cytokinin substrate, which is labelled on the adenine ring. Activity is determined by monitoring the amount of radioactive adenine formed as a result of the incubation of the radioactive substrate with a more highly purified enzyme extract (Motyka and Kamínek 1994). This method serves to verify the results obtained with the first assay.

The results in Table 3.5 demonstrate that CKOX activity is present in the tissues of 'Hass' avocado fruit. Although there were no significant differences in CKOX activity between normal and small fruit tissues, when employing the method of Liberos-Minotta and Tipton (1995), small fruit tissues did consistently possess marginally higher activity than normal fruit tissues. In contrast, the method of Motyka and Kamínek (1994) revealed significant differences in CKOX activity between normal and small fruit tissues. Activity was higher in seed and mesocarp tissue of small fruit, but was greater in seed coat tissue of normal fruit, and no activity was detected in the senesced seed coat tissue of small fruit. The difference in the results of these two methods is most probably attributable to the increased sensitivity of the radioactive CKOX assay and the use of a more highly purified extract in this procedure.

3.2.3 Tissue distribution of IAA, ABA, CK, and purines in normal and small 'Hass' avocado fruit

Abscisic acid plays an important role in the determination of final fruit size in 'Hass' avocado, as application of ABA during the linear phase of fruit growth results in seed coat senescence and the development of the small fruit phenotype (Moore-Gordon *et al.* 1998). The endogenous ABA levels in tissues of normal and small fruit, harvested during the linear period of growth, were analysed by HPLC and the results are presented in Table 3.6. Abscisic acid

levels were significantly higher in small fruit seed tissue compared to seed tissue of normal fruit. Although not significant, ABA levels were also marginally higher in the mesocarp of small fruit. These results agree with the findings of Moore-Gordon *et al.* (1998) and Richings *et al.* (2000) and serve to highlight that differences in ABA levels are associated with differences in final fruit size.

In light of the fact that an AO catalyses the final step in IAA biosynthesis (Koshiba *et al.* 1996; Lips *et al.* 1999) and that differences in activity of this enzyme (IA-ald oxidase) were found between normal and small fruit (Table 3.5), it could be expected that IAA levels may also differ between these two phenotypes. Indole-3-acetic acid levels were therefore analysed by HPLC in normal and small fruit tissues and the results are presented in Table 3.6. In contrast to IA-ald oxidase activity, where greater activity was found in seed tissue of normal fruit (Table 3.5), IAA levels were three-fold higher in seed tissue of small fruit as compared to seed tissue of normal fruit. Indole-3-acetic acid was also marginally higher in the mesocarp of small fruit, but this difference was not significant. One possible explanation for this apparent anomaly is that early seed coat senescence in small fruit (Moore-Gordon *et al.* 1998) prevents basipetal movement of seed-derived IAA resulting in apparent accumulation, assuming that IAA is derived from IA-ald in avocado fruit.

Cytokinin-like activity, determined by bioassay (Miller 1963), has been tentatively classed into two groups, viz. glucosides (R_f 1 to 5) and bases plus ribosides (R_f 6 to 10), based on co-chromatography with authentic standards (see appendix II). There was very little difference in the levels of CK glucosides in normal and small avocado fruit (Table 3.6). The highest levels of these compounds were detected in seed tissue, suggesting a storage role for this tissue. Although not significant, higher levels of CK bases and ribosides were found in mesocarp tissue of small fruit, whilst in seed coat tissue higher levels were found in normal fruit. There was no difference in the level of CK bases and ribosides in seed tissue of normal and small fruit.

The differences in activity of CKOX and XDH between normal and small fruit (Table 3.5) were expected to reflect altered purine levels, as both the product and substrate of these enzymes are purines. Purine levels were therefore analysed by HPLC in normal and small 'Hass' avocado fruit tissues and the results are presented in Table 3.6. Purine content and composition was markedly affected by the expression of the small fruit phenotype. The adenine content of seed and mesocarp tissue of small fruit was reduced by 90%, as compared to seed and mesocarp tissue of normal fruit. Whilst hypoxanthine levels were substantially

Table 3.6 **Levels of IAA, ABA, CK-like activity, and purines in tissues of normal and small 'Hass' avocado fruit harvested 256 DAFB, during the linear phase of fruit growth. Data are the mean of 3 replicates.**

Tissue	Phenotype	IAA	ABA	CK-like activity		Purines		
				glucosides	bases and ribosides	adenine	hypoxanthine	xanthine
		nmol g^{-1} FW		callus yield (g)		µmol g^{-1} DW		
Seed	normal	342.45[a†]	0.85[a]	0. 114[b]	0.311[b]	241.20[b]	51.39[a]	13.78[a]
	small	996.98[b]	14.29[b]	0.093[b]	0.322[b]	24.19[a]	35.16[a]	0.83[a]
Seed coat	normal	ND[‡]	ND	0.005[a]	0.404[b]	ND	ND	ND
	small	ND	ND	0.026[a]	0.331[b]	ND	ND	ND
Mesocarp	normal	44.16[a]	0.67[a]	0.011[a]	0.062[a]	320.70[b]	215.53[a]	79.94[b]
	small	77.746[a]	1.11[a]	0.009[a]	0.147[a]	19.66[a]	576.33[b]	nd[★]

[†]Values followed by different letters are significantly different (for ABA $LSD_{(0.05)}$ = 6.57; for IAA $LSD_{(0.05)}$ = 9.36; for CK-like glucosides $LSD_{(0.05)}$ = 0.0383; for CK-like bases and ribosides $LSD_{(0.05)}$ = 0.1038; for adenine $LSD_{(0.05)}$ = 149.98; for hypoxanthine $LSD_{(0.05)}$ = 246.35 and for xanthine $LSD_{(0.05)}$ = 37.30)

[‡] ND = not determined

[★]nd = not detected

elevated in mesocarp tissue of small fruit, the reverse was true for seed tissue of small fruit, where marginally higher levels were detected in seed tissue of normal fruit. Xanthine levels were elevated in both seed and mesocarp tissue of normal fruit.

3.2.4 Cytokinin- and auxin-induced CKOX activity

The rapid stimulation of CKOX activity by external application of CKs was first demonstrated in cultured tobacco cells (Terrine and Laloue 1980). Subsequently, increases in endogenous CK levels were also shown to induce CKOX activity (Jones *et al.* 1992; Dietrich *et al.* 1995). This increase in CKOX activity is relatively rapid, but transient and is stimulated in the presence of both substrate and non-substrate CKs (Kamínek *et al.* 1997). Data to support the universality of this phenomenon has not been forthcoming and it has been suggested that this effect may be tissue-specific (Jones and Schreiber 1997). The determination of the impact of exogenously applied CK on CKOX activity in 'Hass' avocado fruit is therefore essential in cementing the hypothesis that CK-induced CKOX activity could result in increased adenine levels.

Results of *in vivo* treatment with CK on CKOX activity in normal avocado mesocarp tissue are presented in Figure 3.6. After incubation for 6 h, CKOX activity was higher in both iP- and zeatin-treated tissue as compared to the control. After 12 h CKOX activity was greatly elevated in iPA-treated tissue, but returned to a level similar to the control after 24 h, indicating the previously reported transient nature of CKOX stimulation by CKs (Kamínek *et al.* 1997). The elevated level of CKOX activity in iP-treated tissue, relative to the control, after 6 h was maintained at this level until 24 h. Zeatin-treated tissue exhibited activity no different to the control after 12 h but after 24 h activity was once again elevated. As expected adenine had no effect on CKOX activity.

Cytokinin levels can also be modulated by other phytohormones. An example of this occurs in maize kernels where auxin levels increase at the same time as CK levels decline (Lur and Setter 1993), resulting in a sharp decline in the CK to auxin ratio. This increase in kernel auxin level also roughly coincides with the period in which maize CKOX activity increases (Cames and Wright 1988; Lur and Setter 1993). In tobacco pith explants auxin stimulates the oxidative breakdown of CK *in vitro* by activating CKOX (Palni *et al.* 1988). As with CK stimulation of CKOX activity, auxin-induced CKOX activity is possibly tissue-specific, with little known regarding this phenomenon in tissues of developing seed and vegetative tissues. As the CK to auxin ratio plays an important role in the control of cell division (Jacobs 1995; Coenen and Lomax 1997) and as CKOX is thought to play an important role in the mediation of this ratio, the impact of auxins on CKOX activity in 'Hass' avocado fruit tissues was established.

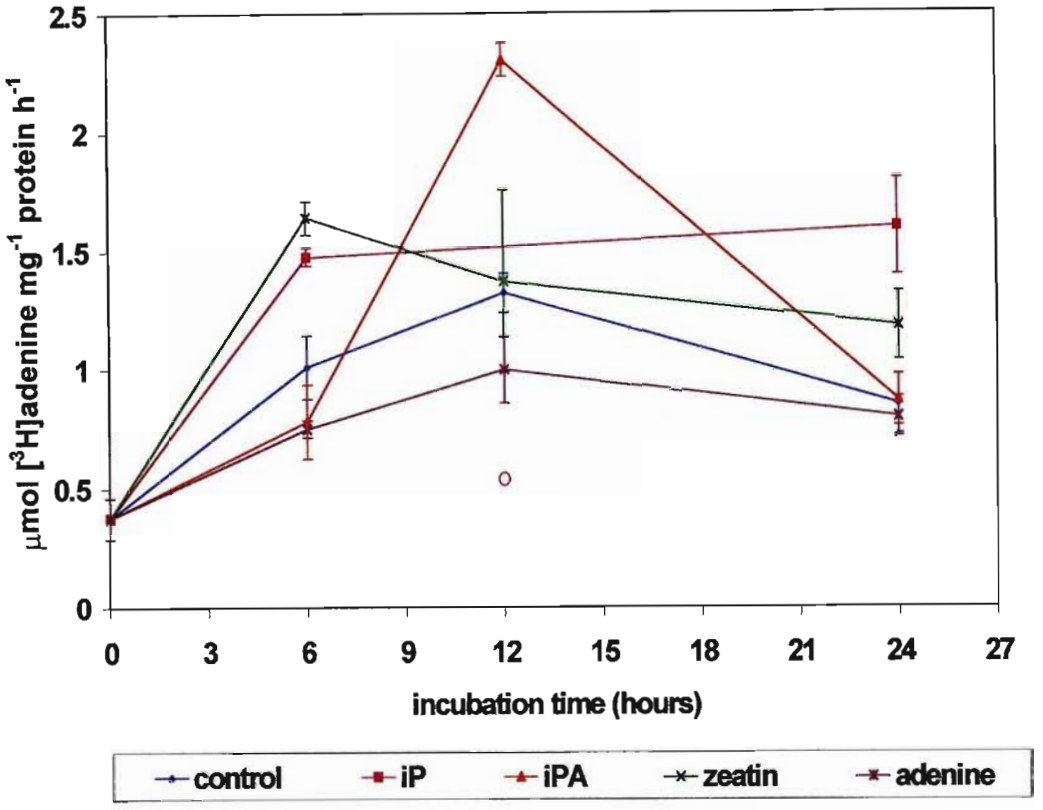


Figure 3.6 **The effect of *in vivo* treatment of CK on CKOX activity in normal 'Hass' avocado mesocarp tissue. Fruit were harvested 171 DAFB and intact fruit supplied with solutions of CKs (all 100 µM), via the pedicel and then incubated for 6, 12 or 24 h at room temperature (see section 2.5.1). Data are the mean of 3 replicates, with vertical bars representing the standard error. (o represents a low leverage point and was excluded)**

Results of *in vivo* treatment with auxins on CKOX activity in normal 'Hass' avocado mesocarp tissue are presented in Figure 3.7. After 6 h IAA-treated tissue exhibited no CKOX activity and was the only treatment that differed from the control. Activity in this tissue increased up until 24 h after treatment, but remained significantly lower than the control. After 12 h tissue treated with indole-butyric acid (IBA) showed considerably higher activity than the control, with activity continuing to increase up until 24 h after treatment. Tissue treated with α-naphthalene acetic acid (NAA) did not exhibit any differences in CKOX activity from the control tissue over the entire 24 h incubation period. The lack of effectiveness of IAA in inducing CKOX activity is most likely attributable to metabolism *in vivo*, which will greatly reduce effectiveness. Indole-3-acetic acid metabolism is most likely in the form of conjugation, as in most plant tissues exogenously applied IAA is rapidly conjugated to form IAA-aspartate (Andreae and van Ysselstein 1956; Sudi 1964; Venis 1964; Sudi 1966; Venis 1972; Slovin and Cohen 1992; Sitbon *et al.* 1993; Sztein *et al.* 1995). Although NAA is resistant to conjugation (Ribnicky *et al.* 1996) its ineffectiveness could possibly be a result of inadequate transport within the avocado fruit.

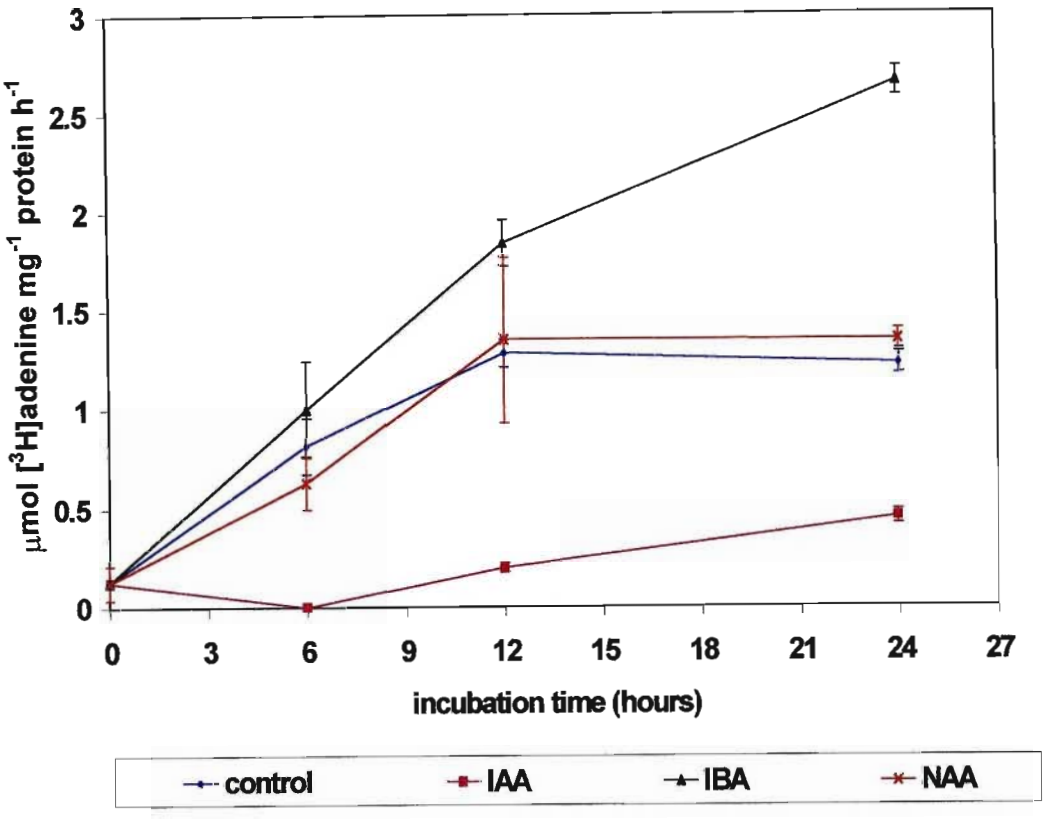


Figure 3.7 **The effect of *in vivo* treatment of auxins on CKOX activity in normal 'Hass' avocado mesocarp tissue. Fruit were harvested 232 DAFB and intact fruit supplied with solutions of auxins (all 100 µM), via the pedicel and then incubated for 6, 12 or 24 h at room temperature (see section 2.5.1). Data are the mean of 3 replicates, with vertical bars representing the standard error.**

3.2.5 Effect of adenine and allopurinol on XDH activity

Xanthine dehydrogenase activity is very sensitive to excess substrate (Bray 1963) and product (Nguyen 1979; Boland 1981). In addition, it is also inhibited by product and substrate analogues (Nguyen 1986), such as adenine and guanine. A particularly potent inhibitor of XDH is the hypoxanthine isomer allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine) (Weir and Fischer 1970; Bray 1975), which is often used to specifically detect XDH activity in plants (Leydecker *et al.* 1995). In humans allopurinol inhibits xanthine oxidase (XO) by binding tightly to the reduced molybdenum component of the enzyme after having first being oxidized to oxypurinol (Massey *et al.* 1970; Spector and Johns 1970; Dollery 1991). Oxypurinol and ribonucleosides of both allopurinol and oxypurinol were identified as the major metabolic conversion products in leaves of allopurinol treated tobacco plants (Montalbini and Della Torre 1995), suggesting a similar mechanism of inhibition in plants.

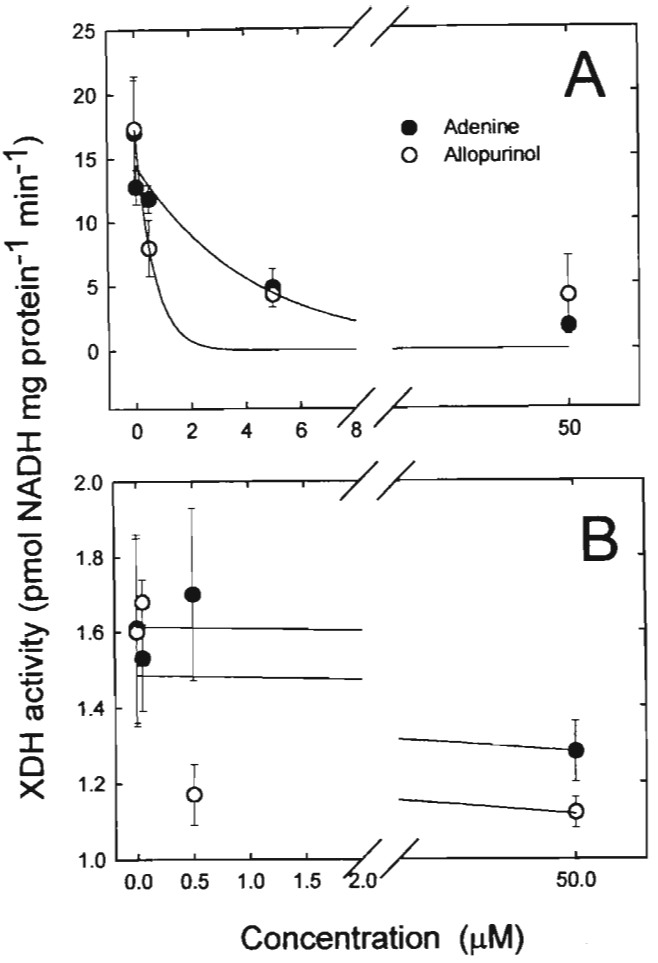


Figure 3.8 **The effect of allopurinol and adenine on *in vitro* activity of XDH in extracts prepared from seed (A) and mesocarp (B) tissue of normal 'Hass' avocado fruit, harvested 256 DAFB. Activity was assayed according to the procedure of Triplett *et al.* (1982), using NAD^+ as an electron acceptor. Treatments were formulated in 2 % (v/v) DMSO and 50 mM Tris-HCl buffer (pH 7.8) and added directly to crude enzyme preparations before reaction initiation. Each data point is the mean of 3 replicates, with vertical bars representing the standard error.**

Figure 3.8 confirms that XDH activity is found in both seed and mesocarp tissue of normal 'Hass' avocado fruit and that *in vitro* activity was reduced by the addition of either adenine or allopurinol. Activity of XDH was ten-fold higher in seed tissue and adenine- and allopurinol-induced inhibition of XDH was more pronounced in these extracts (Fig. 3.8A), than in extracts of mesocarp tissue (Fig. 3.8B). Similar results were found when mesocarp extracts were subjected to activity staining in the presence of adenine (Fig. 3.9A) and allopurinol (Fig. 3.9B), following native-PAGE. Xanthine dehydrogenase activity, indicated by the intensity of the formazan stain, decreased with increasing adenine and allopurinol concentration. At 50 μM adenine a small quantity of formazan stain was still visible, whereas, at the same concentration of allopurinol, there was no stain visible, indicating that allopurinol is a more potent inhibitor of XDH than adenine. This effect is also evident in Figure 3.8. The inhibition of

XDH by allopurinol (Fig. 3.9B) confirms the specificity of the staining technique for XDH (Leydecker *et al.* 1995).

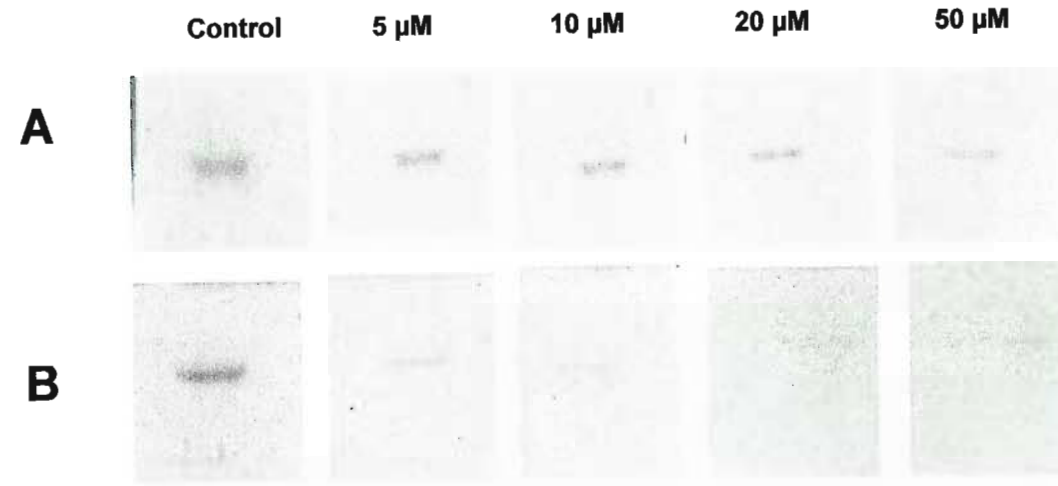


Figure 3.9 **Zymograms showing the effect of adenine (A) and allopurinol (B) on *in vitro* activity of XDH in extracts prepared from mesocarp tissue of normal 'Hass' fruit (harvested 112 DAFB), following native-PAGE. Activity bands were developed separately, with strips from each lane, in staining solution containing 1 mM hypoxanthine, 0.2 mM PMS, 1 mM MTT and the various concentrations of adenine or allopurinol in 0.1 M Tris-HCl buffer (pH 8.0). Lanes were loaded with equal amounts of protein (25 µg/lane).**

3.3 CONCLUSION AND SUMMARY

Results presented in this chapter have demonstrated the presence of AO, XDH, and CKOX in tissues from developing avocado fruit. Two AO activities could be distinguished in terms of hormone metabolism. One showed a preference for IA-ald and the other for I-ald, which was equated with XAN oxidase activity. As expected, iP was the preferred substrate for CKOX and activity of this enzyme was enhanced by auxin and CKs. Adenine, the product of CKOX activity, inhibited XDH activity. Tissue distribution and activity of these enzymes in normal and small fruit was mirrored by a change in the content and composition of IAA, ABA, CK, and purines across the fruit. Thus, high IA-ald oxidase activity was associated with low IAA levels in seed tissue. Low XAN oxidase activity gave low ABA in seed, which occurred concomitantly with low XDH activity and high levels of adenine, xanthine and hypoxanthine. Cytokinin oxidase activity was not markedly changed across the fruit and this was reflected in CK levels that were similar in seed and seed coat tissue but lower in mesocarp tissue. In terms of fruit size, seed of the small fruit variant had low IA-ald oxidase activity but high XAN oxidase and XDH, and contained substantially more IAA and ABA. In summary,

(1) Xanthoxal oxidase activity was detected in all tissues of avocado fruit, where activity was significantly greater in seed and seed coat tissue of the small fruit variant. Indole-3-acetaldehyde oxidase activity was only detected in seed tissue, where it was ten-fold

higher in normal seed. Xanthine dehydrogenase activity was only detected in seed and mesocarp tissues where it was significantly greater in these tissues from the small fruit variant. Cytokinin oxidase activity was significantly higher in seed and mesocarp tissue of small fruit, with no activity detected in senesced seed coat tissue of small fruit.

(2) Expression of the small fruit phenotype resulted in the accumulation of ABA and IAA and the depletion of adenine and xanthine in seed and mesocarp tissues. Although there were no differences between normal and small fruit with respect to CK glucosides, the level of CK bases and ribosides was slightly higher in mesocarp tissue of small fruit and seed coat of normal fruit. Hypoxanthine levels were significantly higher in seed tissue of small fruit and mesocarp tissue of normal fruit.

(3) Cytokinin oxidase activity was stimulated by the exogenous application of CKs (iPA, iP and zeatin) and auxin (IBA). Xanthine dehydrogenase activity was inhibited in the presence of both adenine and allopurinol, in both seed and mesocarp tissue of normal 'Hass' avocado fruit. Allopurinol was a more effective inhibitor of XDH than adenine.

4 EFFECT OF CHANGES IN CKOX, AO, AND XDH ACTIVITY ON TISSUE ABA, CK, AND IAA CONTENT OF DEVELOPING AVOCADO FRUIT

4.1 INTRODUCTION

Antagonism between CK and ABA in the mediation of plant physiological processes is well documented, and includes organ senescence (Beevers 1976; Biswal and Biswal 1988; Noodén 1988; Smart 1994), stomatal closure, leaf and fruit abscission, and seed germination (Salisbury 1994). The importance of CK-ABA interaction in the metabolic control of avocado fruit development has also been demonstrated. Evidence for this is based on the findings that: 1) final fruit size is linearly correlated with the endogenous CK/ABA ratio; 2) 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 iP; and 3) ABA-induced phenotypic variation (including a decline in fruit growth and early seed coat senescence) was negated in the presence of iP (Cowan *et al.* 1997; Moore-Gordon *et al.* 1998). As iP did not fully restore HMGR activity of ABA-treated fruit, but reversed ABA-induced retardation of fruit growth, it was suggested that the interaction between CK and ABA is at a site some distance from HMGR in the ABA biosynthetic pathway.

A detailed study of the impact of CK on ABA metabolism revealed that CK stimulated the oxidative catabolism of ABA (Cowan *et al.* 1999). Further chemical dissection of the response of ABA metabolism to CK, allopurinol (an isomer of hypoxanthine and potent inhibitor of XDH) and tungstate (an inhibitor of AO) indicated the involvement of a MoCo-containing AO. These studies also showed that allopurinol and adenine, a product of CKOX activity and inhibitor of XDH activity (Nguyen 1986), promoted ABA catabolism. Since CKOX is a substrate-inducible enzyme (Kamínek *et al.*1997) it was suggested that CK-induced CKOX activity contributed to the regulation of endogenous ABA during plant organ growth (Cowan *et al.* 1999). A change in CK metabolism is thus postulated to impact on ABA levels through changes in XDH activity and altered MoCo allocation to AO. As the conversion of IA-ald to IAA also requires a MoCo-containing AO (Koshiba *et al.* 1996; Sekimoto *et al.* 1998; Lips *et al.* 1999), it is possible that inhibition of XDH may also impact on auxin levels thereby mediating the balance between CK, ABA and IAA (Taylor and Cowan 2001).

Results in Chapter 3 demonstrated differences in hormone levels between normal and small fruit at a point in the linear phase of fruit growth. This was particularly evident in seed tissue of

small fruit, which contained elevated ABA and IAA. This change in hormone levels was associated with higher CKOX, XDH, and XAN oxidase activity, but low IA-ald oxidase activity. In an attempt to establish a link between the changes in activity of these enzymes and changes in ABA, IAA, and CK, fruit were treated with compounds known to affect CKOX and XDH activity, and the activity of AO assessed in relation to endogenous ABA and IAA.

Cytokinin oxidase activity in 'Hass' avocado fruit is enhanced by applied CK and auxins (see section 3.2.4; Figs 3.6 and 3.7). Furthermore, XDH activity was inhibited by *in vitro* treatment of fruit with adenine and allopurinol (see section 3.2.5; Figs 3.8 and 3.9). The following questions thus require answering: 1) Does the induction of CKOX by CK result in sufficient adenine to inhibit XDH activity? and, 2) Can *in vivo* treatment of fruit with adenine and allopurinol inhibit XDH and alter endogenous ABA and IAA? In addition, the proposal that MoCo is limiting in plants (Sagi *et al.* 1997; Sagi and Lips 1998) can be partially addressed by determining the effect of exogenous molybdate on AO activity and ABA and IAA.

4.2 RESULTS

4.2.1 Cytokinin alteration of purine, ABA, and IAA metabolism

4.2.1.1 Impact of CK on XDH and AO activity in mesocarp tissue

The effect of applied CK on XDH and XAN oxidase activity in avocado mesocarp tissue is shown in Figure 4.1. In general, XDH activity was inhibited by all of the CKs tested over the 24 h incubation period (Fig. 4.1A). In contrast, XAN oxidase activity displayed differential responses to CK treatment (Fig. 4.1B). Zeatin-treatment, and to a lesser extent iPA, caused a rapid but transient increase in activity of XAN oxidase whereas iP and adenine treatment sustained mesocarp XAN oxidase for 12 and 24 h respectively.

4.2.1.2 Impact of CK on IAA levels and ABA metabolism

A previous report indicated that CK stimulates the conversion of XAN to ABA by enhancing the activity of the MoCo-requiring AO that catalyses this step (Cowan *et al.* 1999). In an attempt to verify this and to reconcile changes in XDH and XAN oxidase activity with changes in ABA levels, ripening fruit were treated with CK and the levels of IAA, ABA and DPA quantified by HPLC and the results are presented in Table 4.1. Ripening avocado mesocarp tissue was chosen because of its high metabolic activity with respect to ABA (Adato *et al.* 1976). Results from both experiments indicated that CK impacts on both IAA and ABA metabolism (Table 4.1). Cytokinin treatment reduced IAA, but the rate of change was less than that observed in non-CK-treated tissue. Peroxidases are ubiquitous enzymes that have been shown to decarboxylate IAA *in vitro* (Normanly 1997). These enzymes are present at the

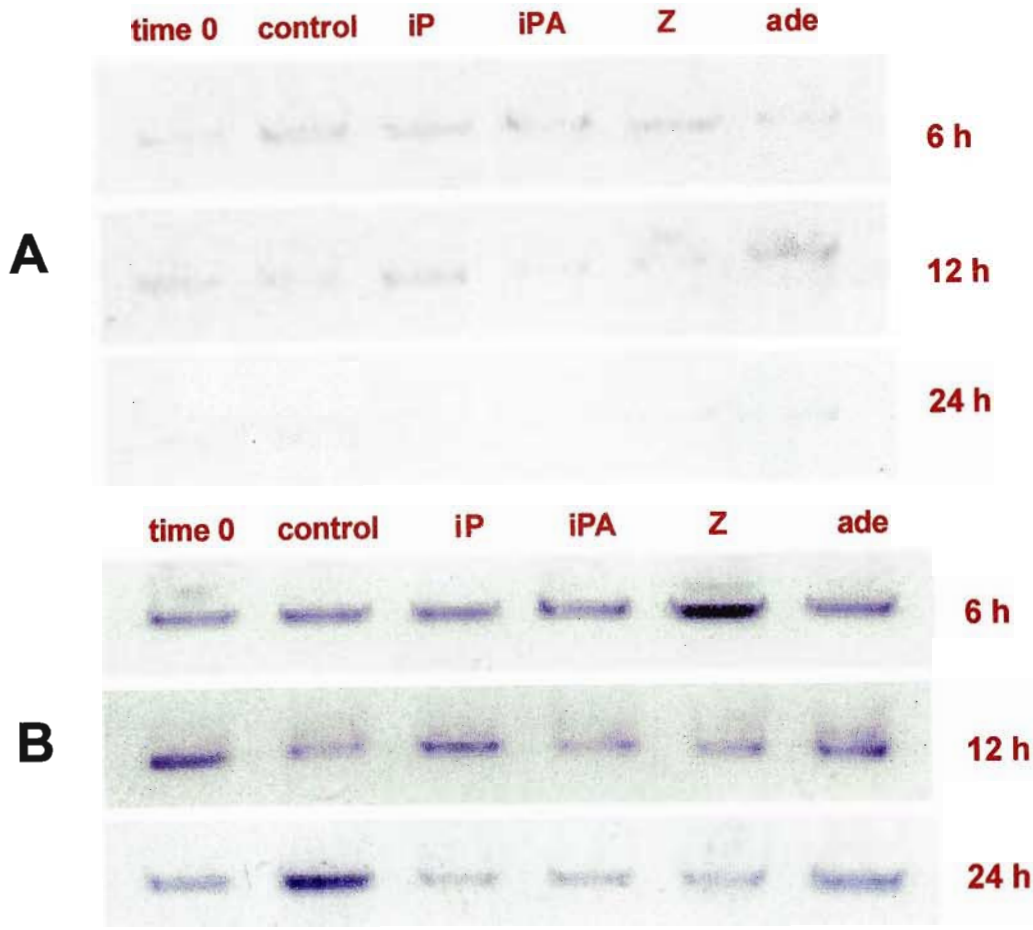


Figure 4.1 **Zymogram showing the effect of applied CK on mesocarp XDH (A) and AO (B) activity. Fruit were harvested 171 DAFB and intact fruit supplied with solutions of CK (all 100 µM), via the pedicel. Fruits were incubated for 6, 12 and 24 h at room temperature (see section 2.5.1). Following native-PAGE, activity was determined using 1 mM hypoxanthine (A) or 1 mM indole-3-aldehyde (B) as the substrate. Each lane was loaded with an equal amount of protein (26 µg/lane).**

cut surfaces of plant tissues (Catalá *et al.* 1994; Bandurski *et al.* 1995; Östin 1995) and their decarboxylase activity could account for the rapid loss of IAA in the control. The relatively higher levels of IAA in CK-treated tissue could be indicative of inhibition of peroxidase activity due to the senescence-delaying effects of CK or as a result of inhibition of IAA conjugation. Cytokinin treatment has also been demonstrated to increase active IAA as a result of the inhibition of IAA conjugation (Coenen and Lomax 1997).

In both experiments CK stimulated ABA metabolism which was manifested by increased accumulation of DPA (Table 4.1). The accumulation of DPA was accompanied by a decline in ABA. However, in the first experiment iP- and adenine-treated tissue exhibited elevated levels of both ABA and DPA, which in the adenine-treated tissue was significantly different to the control. As ABA is known to stimulate its own oxidative catabolism by enhancing the activity of ABA 8'-hydroxylase (Uknes and Ho 1984; Railton and Cowan 1987; Gergs *et al.* 1993; Babiano 1995; Cutler *et al.* 1997) it is likely that the depletion of ABA in CK-treated tissue was a result of sustained catabolism. The linear nature of the early ABA catabolic pathway also

implies that an increase in DPA reflects higher rates of metabolism and rapid flow of carbon through this pathway.

Table 4.1 **Effect of applied CK on IAA, ABA and DPA in mesocarp tissue of ripening 'Hass' avocado fruit. Solutions of Tween 20/acetone/water (1:1:8, v/v/v), containing CK (100 µM), were supplied to fruit via the cut surface and incubated in a water-saturated environment at 25 °C for 24 h (see section 2.5.2). Data are expressed as net change (i.e. $t_{24} - t_0$).**

Exp.	Treatment	IAA	ABA	DPA
		nmol g^{-1} FW (% of control)		
1				
	control	-3095.44 (100)	66.22 (100)	96.53 (100)
	iP	-2543.09*† (122)	88.34 (133)	168.93 (175)
	zeatin	-1904.18* (162)	10.11 (15)	259.92 (269)
	adenine	-2730.81* (113)	542.20* (818)	1823.08* (1888)
2				
	control	-785.10 (100)	128.70 (100)	575.57 (100)
	iP	-744.83 (105)	73.59* (57)	1244.41* (216)
	zeatin	-775.14 (101)	17.05* (13)	393.37 (68)
	adenine	-662.04* (119)	60.59* (47)	832.60 (145)

†**Values followed by * are significantly different ($P \leq 0.05$) from the control**

If purines do indeed impact on MoCo allocation and utilization, then ABA levels in avocado mesocarp tissue should change in response to application of CK analogues. Ripening avocado mesocarp tissue was thus treated with CK analogues and ABA, PA and DPA levels determined by HPLC. Results in Table 4.2 demonstrate that CK analogues increased ABA metabolism, which is in agreement with the data in Table 4.1. This increase in ABA metabolism was manifest as an increase in ABA and its breakdown products, PA and DPA, in both experiments. In the first experiment 2,6-dichloropurine was the most effective CK analogue in stimulating ABA metabolism, whereas in the second experiment both 6-chloropurine and 2-methylthio-6-chloro-9-methylpurine were highly effective.

Cytokinins were also supplied to whole fruit in an attempt to overcome the confounding influence of seed coat senescence and to assess the impact of CKs on ABA and IAA

metabolism in seed tissue. In this way, an attempt was made to simulate the situation in small fruit (see section 3.2.3). Treatment with iP increased IAA levels in both seed and mesocarp (Table 4.3). By comparison, zeatin, iPA and adenine increased IAA content of mesocarp tissue, but decreased IAA in seed tissue. This is probably indicative of transport of IAA from seed to mesocarp tissue. Application of iP, iPA and adenine decreased ABA in mesocarp tissue, but had little impact on ABA in seed tissue. However, zeatin reduced ABA in seed tissue but had little effect on ABA in mesocarp tissue. These results indicate that CKs differ in their ability to alter ABA and IAA metabolism.

Table 4.2 **Effect of CK analogues on ABA metabolism in mesocarp tissue of ripening 'Hass' avocado fruit. Solutions of Tween 20/acetone/water (1:1:8, v/v/v), containing either 6-chloropurine (6.5 µmol), 2,6-dichloropurine (5.3 µmol), 2-methylthio-6-chloropurine (1.8 µmol) or 2-methylthio-6-chloro-9-methylpurine (1.9 µmol), were supplied to fruit via the cut surface and incubated in a water-saturated environment at 25 °C for 48 h (see section 2.5.2).**

Exp.	Treatment	ABA	PA	DPA	Total
		nmol g^{-1} FW (% of control)			
1					
	control	235.62 (100)	55.69 (100)	275.77 (100)	567.07 (100)
	6-chloropurine	241.37 (102)	52.89 (95)	387.893 (141)	682.16 (120)
	2,6-dichloropurine	281.68*† (120)	16.95* (30)	766.53* (278)	1065.16* (188)
	2-methylthio-6-chloropurine	208.903 (89)	26.52 (48)	536.70 (195)	772.11 (136)
2					
	control	274.98 (100)	75.542 (100)	175.41 (100)	525.94 (100)
	6-chloropurine	587.31* (213)	341.05* (451)	247.38 (191)	1175.74* (224)
	2,6-dichloropurine	394.80 (144)	104.71 (139)	176.76 (101)	676.26 (129)
	2-methylthio-6-chloro-9-methylpurine	427.93 (156)	105.31 (139)	382.95* (218)	916.19* (174)

†**Values followed by * are significantly different ($P \leq 0.05$) from the control**

Table 4.3 **Effect of applied CK on IAA and ABA in seed and mesocarp tissue of normal 'Hass' avocado fruit harvested in the linear phase of fruit growth, 171 DAFB. Solutions of CK (100 µM) were supplied to intact fruit via the pedicel and the fruit incubated at 25 °C for 24 h prior to extraction of ABA and IAA (see section 2.5.1). Data are the mean of three replicates.**

Treatment	IAA		ABA	
	nmol g^{-1} DW			
	seed	mesocarp	seed	mesocarp
control	3301.78 (100)	371.34 (100)	95.96 (100)	33.33 (100)
iP	4961.93*† (150)	2362.83* (636)	85.16 (89)	31.18* (94)
iPA	1638.44* (50)	2540.16* (684)	108.03 (113)	29.18* (88)
zeatin	2642.25 (72)	698.26 (188)	162.34* (169)	32.14 (96)
adenine	2160.91* (65)	1085.82 (292)	107.47 (112)	29.30* (88)

†**Values followed by * are significantly different ($P \leq 0.05$) from the control**

4.2.2 Molybdate and allopurinol effects on ABA, IAA, and purine metabolism

To further dissect the response of ABA and IAA metabolism to CK, fruit were treated with molybdate (which is incorporated into the MoCo) and allopurinol (an isomer of hypoxanthine and potent inhibitor of XDH) and the activity of XDH and AO assessed (Fig. 4.2). In addition ABA, IAA, CK-like activity, and levels of selected purines were determined in similarly treated tissue and the data is presented in Table 4.4. As allopurinol is a specific inhibitor of XDH (Weir and Fischer 1970; Bray 1975), the hypothesis that the inhibition of this enzyme will result in the more efficient utilization of the MoCo by AO can be tested. Furthermore, through the provision of excess Mo the reported limiting nature of the MoCo can be assessed.

4.2.2.1 Molybdate effects

Activity of XDH and XAN oxidase was stimulated in mesocarp tissue treated with molybdate (Fig. 4.2). Molybdate increased the ABA content of the seed but had little or no effect on ABA in mesocarp tissue when applied to whole fruit (Table 4.4). The reverse was true for IAA, and in these fruit IAA was decreased by 56 % in mesocarp tissue but remained unchanged in the seed. Furthermore, CK-like activity and xanthine increased in response to molybdate treatment in mesocarp tissue. In ripening mesocarp tissue, ABA was reduced by molybdate treatment, but IAA was elevated (Table 4.5). This indicates that overall ABA metabolism was stimulated by exogenous molybdate, but that IAA turnover was presumably inhibited.

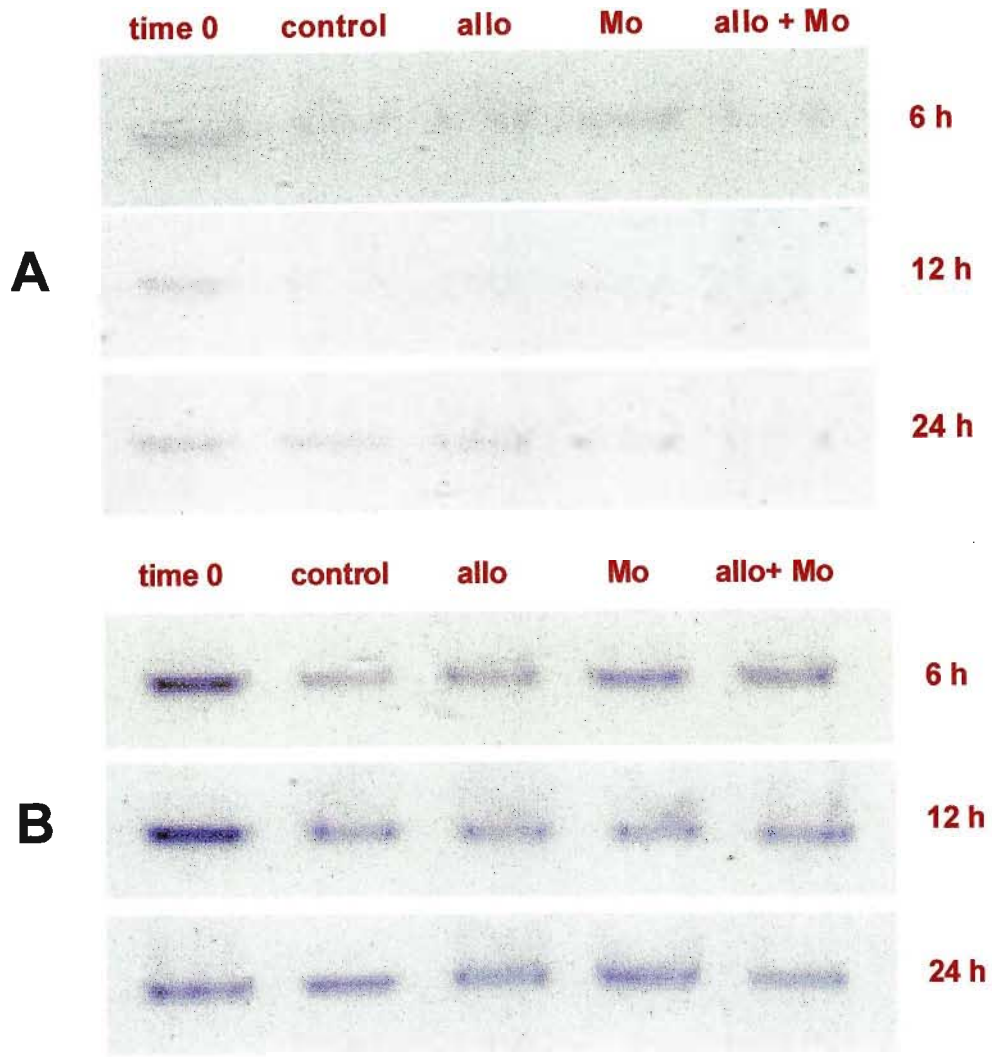


Figure 4.2 **Zymogram showing the effect of allopurinol and molybdate on XDH (A) and AO (B) activity. Fruit were harvested 177 DAFB and intact fruit supplied with solutions of allopurinol (100 µM), molybdate (100 µM) and allopurinol (50 µM) + molybdate (50 µM) via the pedicel. Fruits were incubated for 6, 12 and 24 h at room temperature (see section 2.5.1). Following native-PAGE, activity was determined using 1 mM hypoxanthine (A) or 1 mM indole-3-aldehyde (B) as the substrate. Each lane was loaded with an equal amount of protein (24 µg/lane).**

4.2.2.2 Allopurinol effects

Although XDH activity was not apparently affected by allopurinol treatment of mesocarp (Fig. 4.2A), activity of XAN oxidase was rapidly and transiently increased (Fig. 4.2B) similar to the effect observed in zeatin- and iPA-treated tissue (cf. Fig. 4.1). Allopurinol increased the ABA content of seed tissue but had little or no effect on ABA in mesocarp tissue (Table 4.4). In contrast, IAA in seed tissue was reduced by allopurinol treatment and significantly increased in mesocarp tissue. Whilst CK-like activity was reduced in allopurinol-treated mesocarp from whole fruit, adenine and hypoxanthine increased, a result that supports inhibition of XDH by allopurinol in avocado tissue. In ripening mesocarp tissue allopurinol increased ABA content, but reduced IAA (Table 4.5).

Table 4.4 **Effect of allopurinol (100 µM), molybdate (100 µM) and allopurinol (50 µM) + molybdate (50 µM) on ABA, IAA and CK-like activity in seed and mesocarp tissue and selected purines in mesocarp tissue of normal 'Hass' avocado fruit harvested in the linear phase of fruit growth, 269 DAFB. Solutions of allopurinol and/or molybdate were supplied to intact fruit via the pedicel and then incubated at 25 °C for 24 h prior to ABA, IAA, CK and purine extraction (see section 2.5.1). Total CK-like activity represents the sum of activity at all 10 R_f zones and is expressed as total callus yield in grams.**

Treatment	ABA		IAA		CK-like		Purines		
	nmol g^{-1} FW (% of control)				callus yield (g) (% control)		µmol g^{-1} DW (% control)		
	seed	mesocarp	seed	mesocarp	seed	mesocarp	adenine	hypoxanthine	xanthine
control	5.32 (100)	5.32 (100)	6.45 (100)	5.86 (100)	0.641 (100)	0.845 (100)	151.08 (100)	118.47 (100)	16.20 (100)
molybdate	7.65*† (144)	3.84 (72)	5.32 (82)	2.59* (44)	0.671 (104)	1.322* (156)	192.58 (127)	87.70 (74)	39.59* (244)
allopurinol	9.02* (170)	5.13 (96)	3.42* (53)	27.11* (463)	0.824 (129)	0.425* (50)	371.97* (246)	363.33* (307)	14.34 (88)
allopurinol+ molybdate	3.71 (70)	8.42* (158)	8.37* (130)	28.02* (478)	0.304* (47)	0.783 (93)	ND‡	219.35 (185)	21.52 (133)

† **Values followed by * are significantly different ($P \leq 0.05$) from the control**
‡**ND = not detected**

4.2.2.3 Allo+Mo effects

A combined treatment of allopurinol + molybdate (allo+Mo) reduced XDH activity (Fig. 4.2A) but caused a transient increase in XAN oxidase (Fig. 4.2B). Application of allo+Mo to whole fruit resulted in increased ABA and IAA content of mesocarp tissue, but had no apparent effect on seed ABA (Table 4.4). Indole-3-acetic acid levels were, however, increased in seed tissue in response to allo+Mo treatment. Cytokinin-like activity was reduced in seed tissue by allo+Mo treatment, but in mesocarp tissue CK-like activity remained unchanged. Although adenine was not detected in mesocarp tissue treated with allo+Mo, levels of hypoxanthine and xanthine increased. Both ABA and IAA were increased in mesocarp tissue from ripening fruits in response to allo+Mo treatment (Table 4.5). As mentioned above, the increase in IAA was presumably a result of inhibition of IAA turnover.

Table 4.5 **ABA and IAA in mesocarp tissue of ripening 'Hass' avocado fruit treated with molybdate (100 µM), allopurinol (100 µM) or, allopurinol (50 µM) + molybdate (50 µM), applied in Tween 20/acetone/water (1:1:8, v/v/v). Solutions were supplied to the cut surface of mesocarp tissue and incubated for 48 h in a water-saturated environment at room temperature prior to extraction of ABA and IAA (see section 2.5.2).**

Treatment	ABA	IAA
	nmol g^{-1} FW (% of control)	
control	4.00 (100)	77.77 (100)
molybdate	1.46*† (36)	242.84* (312)
allopurinol	8.89* (222)	44.60 (57)
allopurinol + molybdate	6.09* (152)	239.34* (307)

†Values followed by * are significantly different ($P \leq 0.05$) from the control

The effect of allo+Mo on ABA and IAA metabolism was further assessed by treating ripening avocado fruit with varying ratios of allo+Mo. Results in Figure 4.3 show that allopurinol, at 100 µM, increased the level of ABA, but reduced the level of IAA. Molybdate had the opposite effect in that ABA levels were reduced and IAA levels elevated. When allopurinol and molybdate were applied in an equimolar ratio both ABA and IAA levels increased, but not to the same extent as that observed in tissue treated with allopurinol or molybdate only. Unequal molar ratios of allopurinol and molybdate had little effect on ABA levels relative to the control, but resulted in changed IAA levels. When molybdate was applied in excess of allopurinol IAA decreased, however, when the situation was reversed IAA was increased. This indicates that

differential regulation of XDH and, possibly AO, can operate to alter the ratio of ABA to IAA in plant tissues.

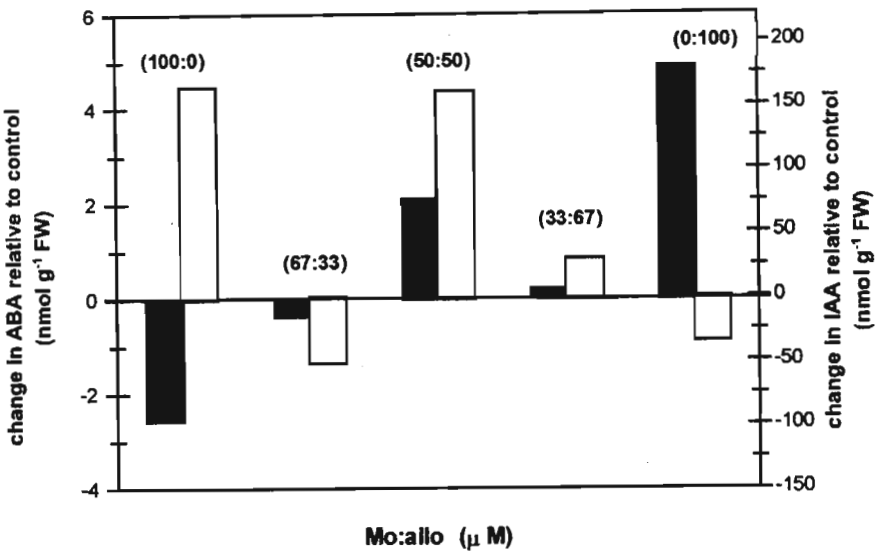


Figure 4.3 **Effect of varying ratios of molybdate and allopurinol on ABA (solid bars) and IAA (open bars) in ripening mesocarp tissue. Solutions of allopurinol and/or molybdate in Tween 20/acetone/water (1:1:8, v/v/v) were supplied to the fruit via the cut surface and incubated at 25 °C for 48 h in a water-saturated environment prior to extraction of ABA and IAA (see section 2.5.1). Values in parentheses represent the ratio of molybdate to allopurinol.**

4.3 CONCLUSION AND SUMMARY

Results presented in this chapter demonstrate that treatment of avocado fruit tissues with CK, molybdate and allopurinol resulted in changes in the activity of both XDH and XAN oxidase, which in turn were associated with altered levels of ABA and IAA. In general, CK treatments decreased XDH activity, enhanced XAN oxidase activity, increased levels of IAA, and increased the overall metabolism of ABA. Manipulation of MoCo-containing enzyme activity using allopurinol, molybdate, and CK changed the ABA/IAA ratio. In summary,

(1) Xanthine dehydrogenase activity was inhibited by adenine, iPA and zeatin, whilst XAN oxidase activity was stimulated in iP-, iPA-, zeatin- and adenine-treated mesocarp tissue. Cytokinin and CK analogues stimulated overall ABA metabolism in whole fruit and in ripening mesocarp tissue. This was manifested in elevated PA and DPA, which was accompanied by either elevated or reduced ABA, depending on the time of incubation and the stage of ripening of the fruit. Treatment with CK generally increased

IAA in mesocarp tissue, but reduced seed IAA content, possibly indicating transport of IAA from the seed to the mesocarp.

(2) Molybdate consistently increased both XDH and XAN oxidase activity in mesocarp tissue throughout the experimental period. Mesocarp tissue treated with molybdate displayed decreased ABA and IAA, but increased CK-like activity and xanthine. Abscisic acid increased in seed tissue treated with molybdate.

(3) Although allopurinol had no effect on XDH activity, elevated XAN oxidase activity was observed in this tissue after 6 h. Allopurinol applied to ripening mesocarp tissue increased ABA, but decreased IAA. When applied to whole fruit, ABA increased in seed tissue, whilst IAA increased in mesocarp tissue and decreased in seed tissue. Mesocarp tissue treated with allopurinol also displayed lower CK-like activity, but increased adenine and hypoxanthine.

(4) Mesocarp tissue treated with allo+Mo displayed lower XDH activity than the control, but higher XAN oxidase activity. This tissue had higher ABA and IAA. Cytokinin-like activity was lowered in seed tissue treated with allo+Mo, whereas hypoxanthine and xanthine increased. When allo+Mo was applied in an equimolar ratio, ABA and IAA increased. However, when unequal molar ratios were applied the treatment had no effect on ABA but altered IAA.

5 RELATIONSHIP BETWEEN ACTIVITY OF CKOX, AO AND XDH AND CHANGES IN PLANT HORMONES DURING THE LINEAR PHASE OF AVOCADO FRUIT GROWTH

5.1 INTRODUCTION

Cell number and cell size influence the capacity of developing fruits to import assimilate, and therefore contribute directly to fruit growth (Bohner and Bangerth 1988a; 1988b). The control of fruit size requires maximization of cell division and expansion during development (Valmayor 1967; Coombe 1976) with any reduction in the availability of required resources impacting on fruit growth. The bulk of carbon needed by developing fruits is derived from source tissue (Ho 1998), which in avocado include photosynthetically active leaves and fruitlets (Coombe 1976; Thorne 1985; Blanke and Lenz 1989; Blanke and Whiley 1995). Development of these structures is additionally sustained by mobilization of stored tree reserves that occur during times of organ growth (Kozlowski 1992). Phloem is the most likely path of solute movement in dicotyledonous species, and in developing fruit phloem unloading occurs in the testa (Thorne 1985). In avocado fruit it is the developing pachychalaza (i.e. seed coat) that supplies photoassimilates, minerals and water to the growing fruit (Moore-Gordon *et al.* 1998). The continuous rate of cell division in avocado fruit, from fruit set to maturity, demands maintenance of symplastic continuity throughout fruit growth and development. Termination of symplastic continuity in small fruit, through early senescence of the seed coat, severs the supply of necessary nutrients to the developing fruit, thereby causing alterations in the expression of genetic factors controlling fruit growth.

Fruit development is governed by three genetic factors which determine: (1) the number of carpel cells; (2) the number of cell divisions during the cell proliferation phase; and (3) the duration and enlargement rate of individual cells in the cell enlargement phase (Higashi *et al.* 1999). It is currently unknown which genetic factor plays the most important role in controlling final fruit size in higher plants, but environmental factors and phytohormones are known to modify the expression of these genes. Appreciation of the pleiotropic effects of plant hormones suggests that no single growth regulator can be responsible for a complex process such as fruit morphogenesis (Trewavas 1980; 1983) and it is generally accepted that phytohormones exert multiple control on organ development by alterations in concentration and as a result of changes in sensitivity of the affected tissues (Trewavas 1982; Firn 1986; Trewavas 1991; Bradford and Trewavas 1994).

Previous reports indicate that CK, IAA, GA, and ABA levels changed over the course of avocado fruit growth (Blumenfeld and Gazit 1970; Gazit and Blumenfeld 1970; Blumenfeld and Gazit 1972; Gazit and Blumenfeld 1972; Cowan *et al.* 1997). The highest levels of IAA, GA and CK were found in endosperm tissue, where levels remained high provided the tissue was still present in the fruit, i.e. until 3 months after fruit set (Blumenfeld and Gazit 1970; Blumenfeld and Gazit 1972; Gazit and Blumenfeld 1972). Cytokinin and IAA levels were higher in seed and seed coat than in the mesocarp (Blumenfeld and Gazit 1970; Gazit and Blumenfeld 1970; Gazit and Blumenfeld 1972), whereas levels of ABA were similar in the seed and mesocarp (Richings *et al.* 2000; Taylor and Cowan 2001). No GA activity was detected in seed or mesocarp tissue (Blumenfeld and Gazit 1972).

The previous chapters established that differences in hormone levels exist between normal and small fruit, which probably occur as a result of changes in the activity of enzymes considered to play an important role in controlling hormone levels. To further evaluate the role of these enzymes in the control of hormone homeostasis and final fruit size of the 'Hass' avocado, enzyme activity and hormone levels were determined in the major tissues of developing fruit during the linear phase of growth.

5.2 RESULTS

5.2.1 Fruit growth

The linear phase of fruit growth was established by determining the percentage increase in fruit length, diameter, and fresh mass of both normal and small fruit and the results are presented in Figures 5.1 and 5.2. At the first sampling interval, 84 DAFB, there was no evidence of early seed coat senescence. However, 112 DAFB fruit with senesced seed coats were readily distinguishable. As a consequence, growth of the two populations of fruit could be monitored with relative ease, particularly as the expected difference in fruit size between the normal and small fruit phenotypes was clearly discernible (Figs 5.1 and 5.2). As illustrated in Figure 5.1, the increase in fruit length occurred more rapidly than increased growth in diameter in normal fruit and terminated sooner. By comparison, expansion growth in small fruit was greater than elongation growth, which also terminated earlier. Since the increase in fruit size (measured as a change in fresh mass) was linear over the course of the experiment (Fig. 5.2), radial growth must compensate for elongation growth during the later stages of the linear phase of avocado fruit growth. The data further suggests that longitudinal growth is more sensitive than radial growth and that factors contributing to this process during early fruit development might in fact be those that are limiting in the small fruit. In an effort to determine whether these 'limiting factors' were tissue-specific, the change in proportion of the major tissues comprising the whole fruit was monitored. Results in Figure 5.3 show that growth

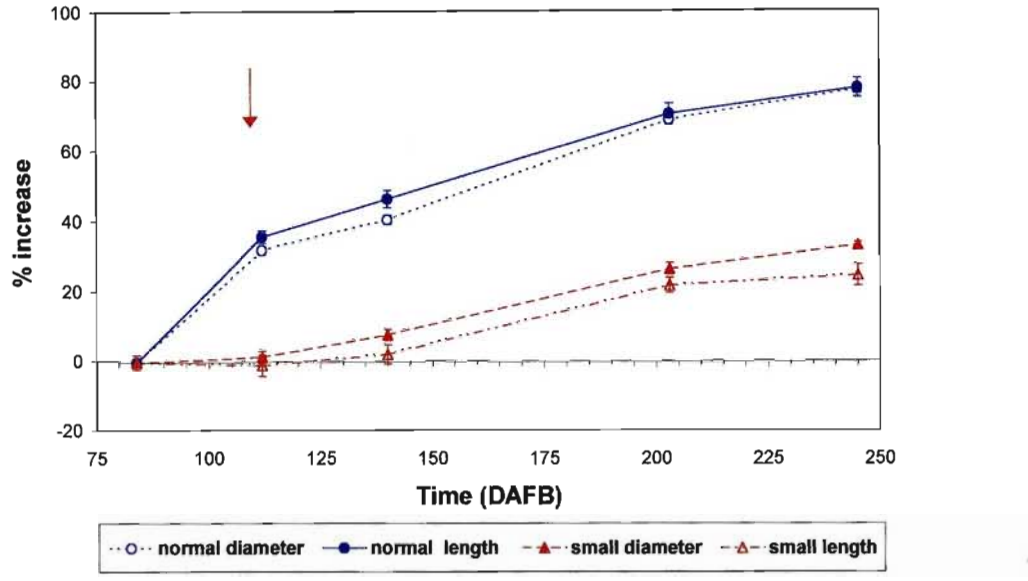


Figure 5.1 **Percentage increase in fruit length and diameter of normal (—) and small (···) 'Hass' avocado fruit over the linear phase of growth. The appearance of the first small fruit is indicated by (↑). (Growth curves were constructed from a total of 103 normal fruits and 79 small fruit).**

of these tissues differed between normal and small 'Hass' avocado fruit. Seed tissue displayed the greatest increase in growth (Fig. 5.3A), with seed coat and mesocarp displaying similar growth rates (Fig. 5.3B and C), implying that changes in fruit size occur largely as a result of seed growth.

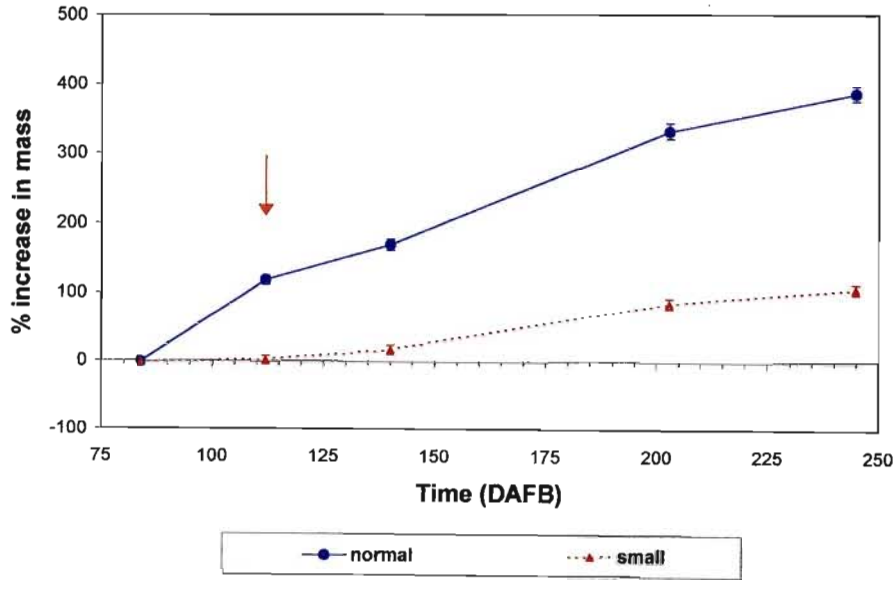


Figure 5.2 **Percentage increase in fresh mass over the linear phase of growth for normal (—) and small (···) 'Hass' avocado fruit. The appearance of the first small fruit is indicated by (↑). (Growth curves were constructed from a total of 103 normal fruits and 79 small fruit).**

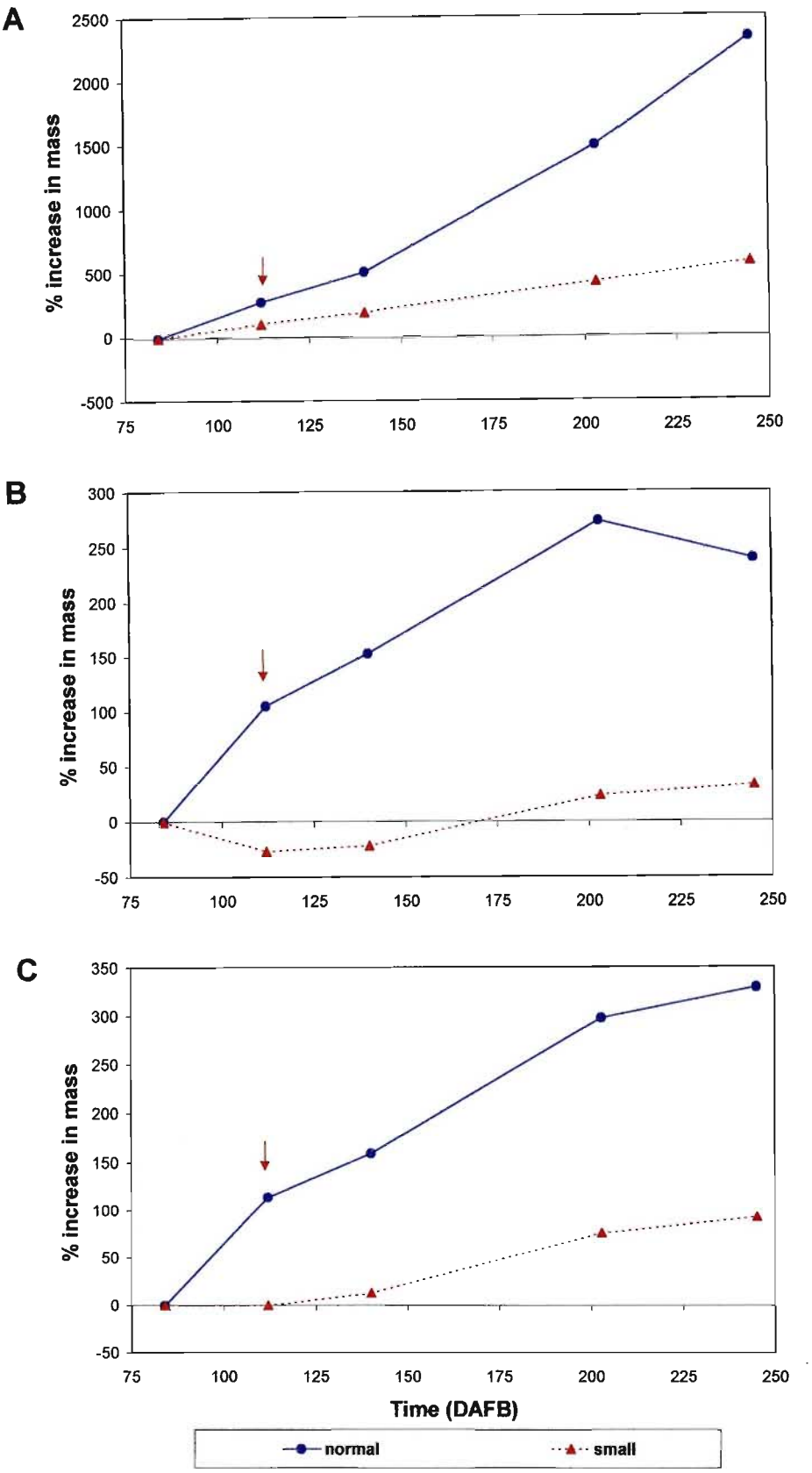


Figure 5.3 **Percentage increase in seed (A), seed coat (B) and mesocarp (C) tissue in normal (—) and small (···) 'Hass' avocado fruit over the linear phase of growth. The appearance of the first small fruit is indicated by (↑). (Graphs were constructed from a total of 103 normal fruit and 79 small fruit).**

Throughout the linear phase of growth, normal and small fruit were comprised chiefly of mesocarp tissue. Even so, the proportion of mesocarp declined from 95 % (84 DAFB) to 84 % and 89 % (245 DAFB) in normal and small fruit respectively. A decline in the relative amount of mesocarp tissue was mirrored by an increase in the relative amount of seed tissue and, in normal fruit, seed tissue increased from 3 % at 84 DAFB to 15 %, 245 DAFB. In small fruit, however, seed tissue only increased to 10 % at 245 DAFB from the initial 3 %. The amount of seed coat tissue was 2 % and 1.4 % of the whole in normal and small fruit respectively, and remained constant throughout the linear phase of growth. At 245 DAFB these values had decreased to 1.5 % and 1 % respectively presumably indicative of seed maturation and the onset of seed coat senescence.

5.2.2 Endogenous CK-like activity and CKOX

Despite the rapid improvement of techniques used in the isolation, purification and identification of CK, bioassays remain an integral part of the identification process. Bioassays assist in the establishment of the existence of active endogenous plant growth regulators and provide primary qualitative information on the nature of endogenous hormones in an extract (Horgan 1981). The simplicity of bioassays is an advantage and in most cases the detection limits and linear range are adequate, but they are not always very selective. Thus, to be strictly accurate, only compounds that have been chemically identified should be named CK. Other compounds should be referred to as cell-division-inducing compounds, or CK-like compounds and should be qualified by reference to the bioassay used (see appendix II for standards) (van Staden and Davey 1979).

Levels of CK-like activity shown in Figure 5.4A, B and C are presented as total callus yield from all 10 R_f zones of the soybean callus bioassay described in section 2.9.5. This was done for ease of presentation in order to show trends in CK-like activity between normal and small fruit during the linear phase of growth. In all tissues of 'Hass' avocado fruit total CK-like activity increased initially and then declined rapidly as the fruit approached maturity. There was no significant difference between CK-like activity in seed, seed coat, and mesocarp tissue of normal and small fruit at any time in the linear phase of fruit growth. The highest levels of CK-like activity were detected in seed coat tissue, followed by seed and mesocarp tissue. In seed coat and mesocarp tissue CK-like activity reached a maximum 112 DAFB in both normal and small fruit (Figs 5.4B and C). In seed tissue CK-like activity reached a maximum 140 DAFB and then declined (Fig. 5.4A).

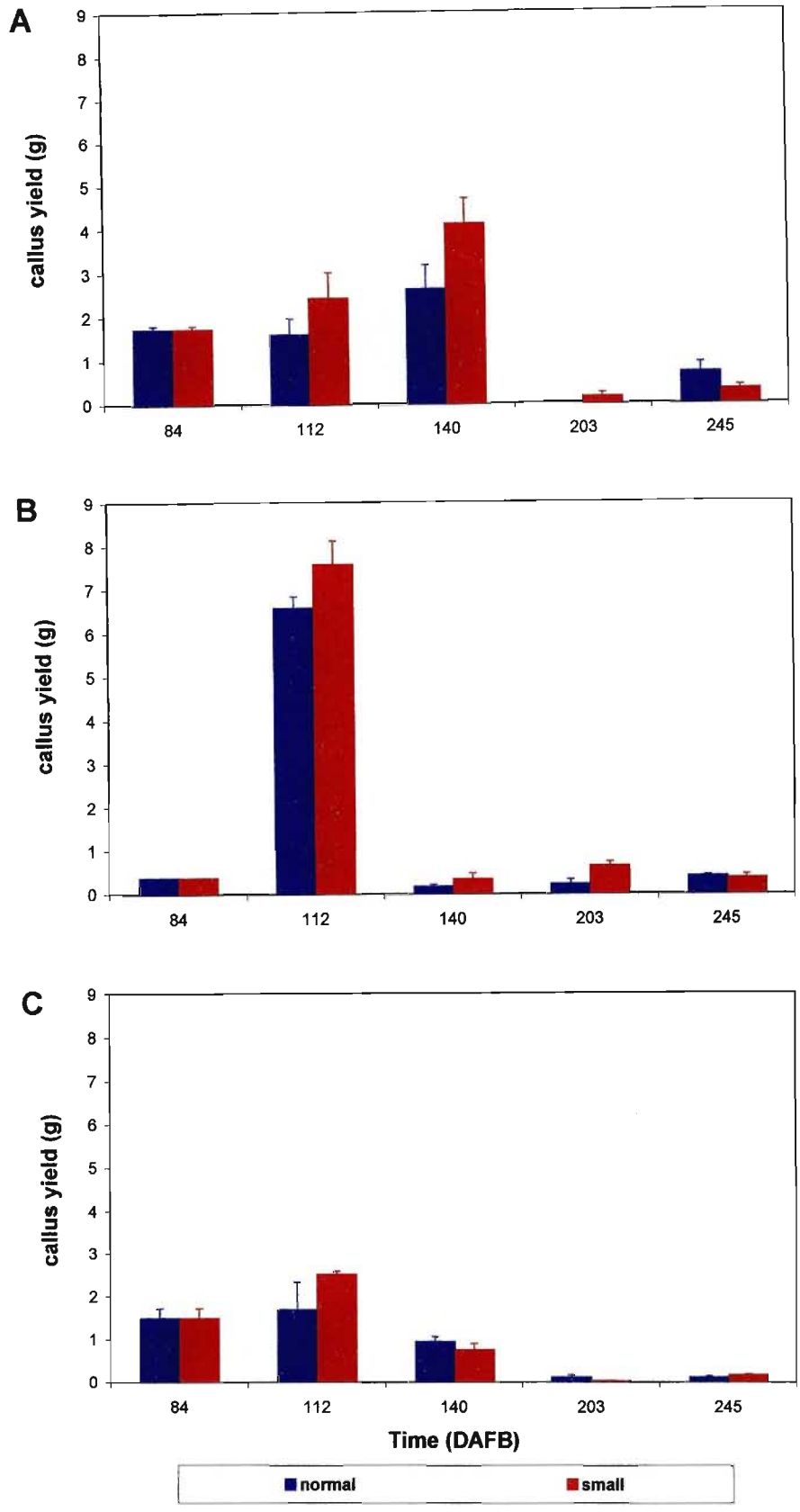


Figure 5.4 **Total CK-like activity in normal and phenotypically small 'Hass' avocado seed (A), seed coat (B) and mesocarp (C) tissue over the course of linear fruit growth. Total CK-like activity represents the sum of activity at all 10 R_f zones and is expressed as callus yield in grams. Data are the mean of three replicates with vertical bars representing the standard error.**

'Hass' avocado fruit CKOX is a substrate-inducible enzyme with a preference for either iP or zeatin (see section 3.2.4). Thus, to evaluate the relationship between endogenous CK-like activity and CKOX, only those zones from the chromatogram corresponding to iP and zeatin (R_f zones 6-9; see appendix II for CK standards) were evaluated in relation to changes in CKOX activity. The results shown in Figure 5.5 indicate that over the linear phase of fruit growth CKOX activity declined in seed and mesocarp tissue (Fig. 5.5A and C). By comparison, CKOX activity increased transiently in seed coat tissue and reached a maximum 140 DAFB (Fig. 5.5B). The increase in iP- and zeatin-like activity in seed coat tissue preceded maximum CKOX activity suggesting induction of the enzyme by its substrate. It is therefore tempting to speculate that the decline in CK-like activity in seed and mesocarp tissue (Fig. 5.4A and C) which occurred after induction of seed coat CKOX was as a result of this activity, and that induction of seed coat CKOX establishes a gradient for removal of CK from seed and mesocarp tissue that coincides with the cessation or dramatic slowing of cell division cycle activity in the fruit.

5.2.3 Changes in MoCo-containing enzyme activity during the linear phase of avocado fruit growth

5.2.3.1 Activity of XDH and XAN oxidase in mesocarp tissue

Xanthine dehydrogenase activity was lower in mesocarp tissue of small fruit throughout the linear phase of fruit growth (Figs 5.6A and B). In mesocarp of normal fruit XDH increased and reached a maximum 140 DAFB. The decline in activity after 140 DAFB coincided with cessation of longitudinal growth of the fruit (cf. Fig. 5.1). Likewise, in small fruit, maximum XDH activity was associated with maximum longitudinal growth, and arrest of elongation growth in this phenotype was followed by a decline in XDH activity. Xanthoxal oxidase showed two peaks of activity in mesocarp of normal fruit, one 84 DAFB and the other, 203 DAFB (Fig. 5.7A). In mesocarp from the small fruit phenotype, however, maximum XAN oxidase activity was sustained until 140 DAFB and, thereafter, appeared to decline (Fig. 5.7B). This observation seems to indicate that activity of mesocarp XAN oxidase is intimately related to appearance of the small fruit phenotype in 'Hass' avocado.

5.2.3.2 Changes in activity of XDH, XAN oxidase and IA-ald oxidase in extracts of seed and seed coat tissue

The activity of MoCo enzymes in seed and seed coat tissue was determined spectrophotometrically as activity was too low to be detected by activity staining in polyacrylamide gels. The nature of the extracts also prevented attempts at concentration as the concentrated extract became gelatinous and failed to enter the gel uniformly, thereby preventing the detection of specific bands of AO and XDH activity.

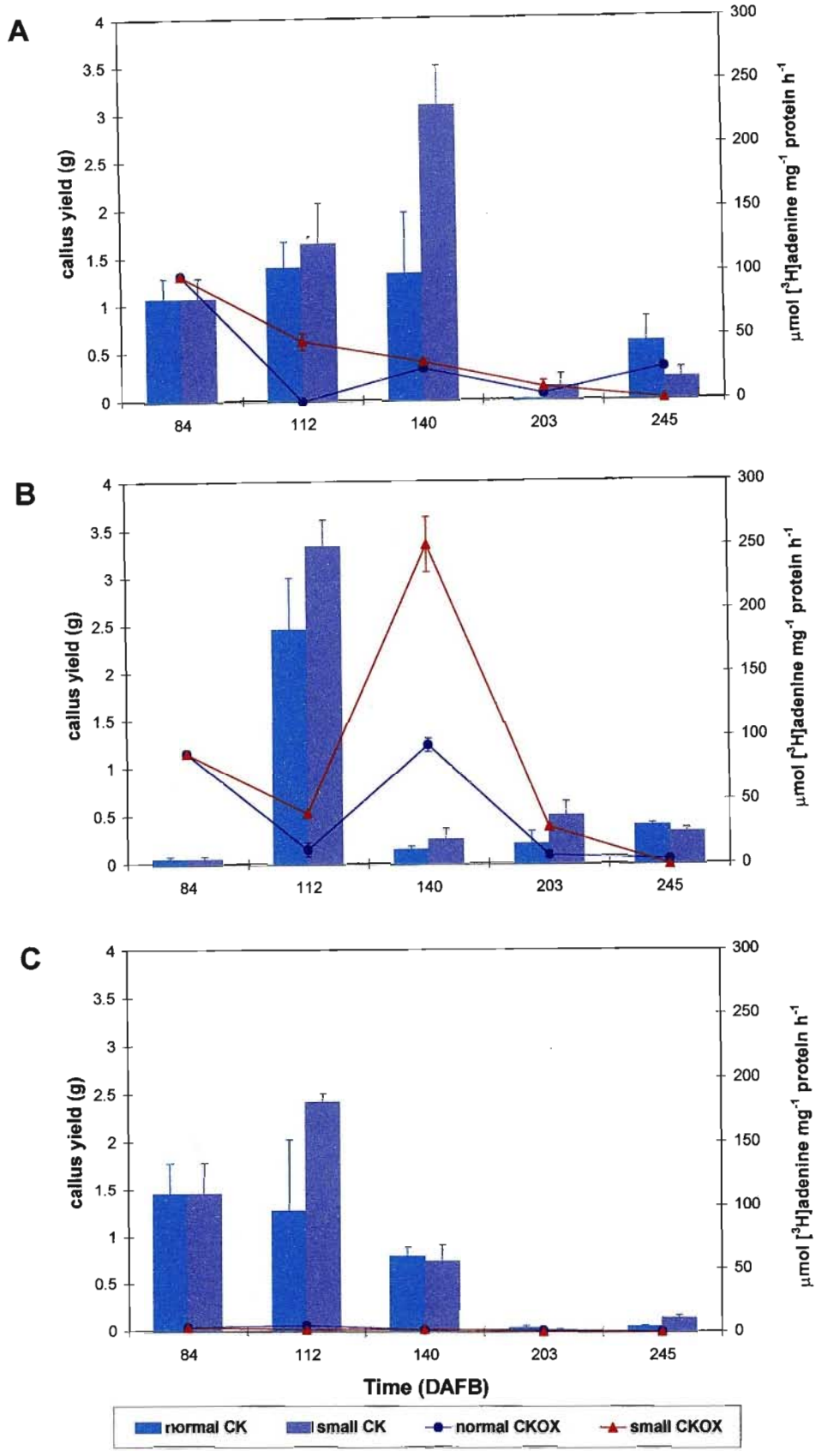


Figure 5.5 **Cytokinin oxidase and CK-like activity (in R_f zones corresponding to iP and zeatin) in seed (A), seed coat (B) and mesocarp (C) tissue of normal and small 'Hass' avocado fruit over the linear phase of growth. Data are the mean of three replicates with vertical bars representing the standard error.**

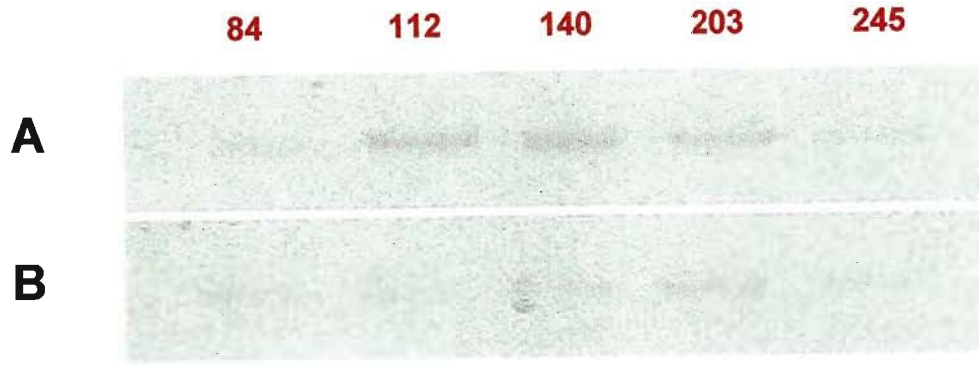


Figure 5.6 **Zymogram showing XDH activity in mesocarp tissue from normal (A) and small (B) 'Hass' avocado fruit over the course of linear fruit growth. Numbers above the zymogram indicate the number of days after full bloom. Following native-PAGE, activity was determined using 1 mM hypoxanthine as the substrate. Each lane was loaded with an equal amount of protein (25 µg/lane).**

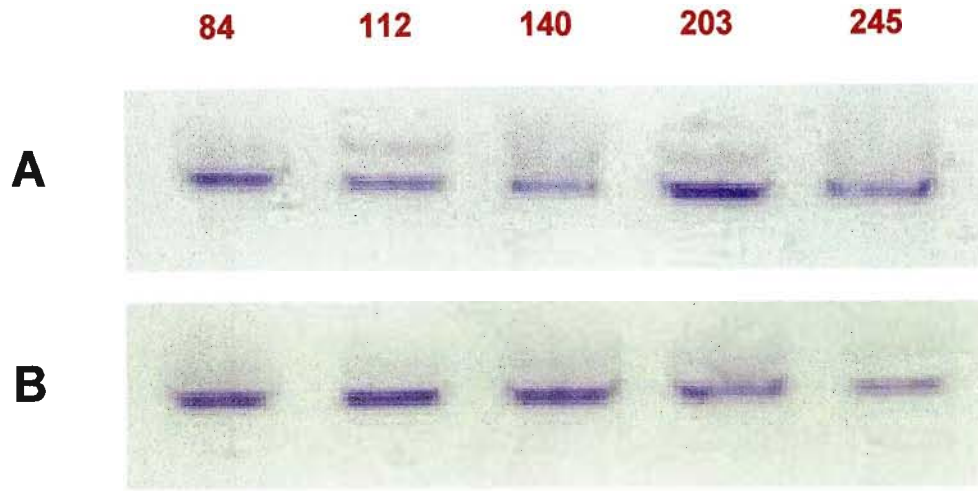


Figure 5.7 **Zymogram showing XAN oxidase activity in normal (A) and small (B) mesocarp tissue from 'Hass' avocado fruit over the course of linear fruit growth. Numbers above the zymogram indicate the number of days after full bloom. Following native-PAGE, activity was determined using 1 mM indole-3-aldehyde as the substrate. Each lane was loaded with an equal amount of protein (25 µg/lane).**

Spectrophotometric analysis of changes in XDH and AO activity in extracts prepared from seed tissue of normal and small fruit revealed the trends shown in Figure 5.8A and B. There was no significant change in activity of seed XDH throughout the course of growth of the small fruit phenotype whereas, in seed of normal fruit, activity of XDH tended to increase (Fig 5.8A) and was coincident with the increase in seed size. Aldehyde oxidase was assayed in extracts of seed tissue from both phenotypes using IA-ald (for estimation of IA-ald oxidase activity) and I-ald (for estimation of XAN oxidase activity). The results in Figure 5.8B show that IA-ald oxidase activity declined rapidly in seed of both normal and small fruit whereas XAN oxidase activity displayed two peaks of activity. The first peak in activity, 112 DAFB, was similar for

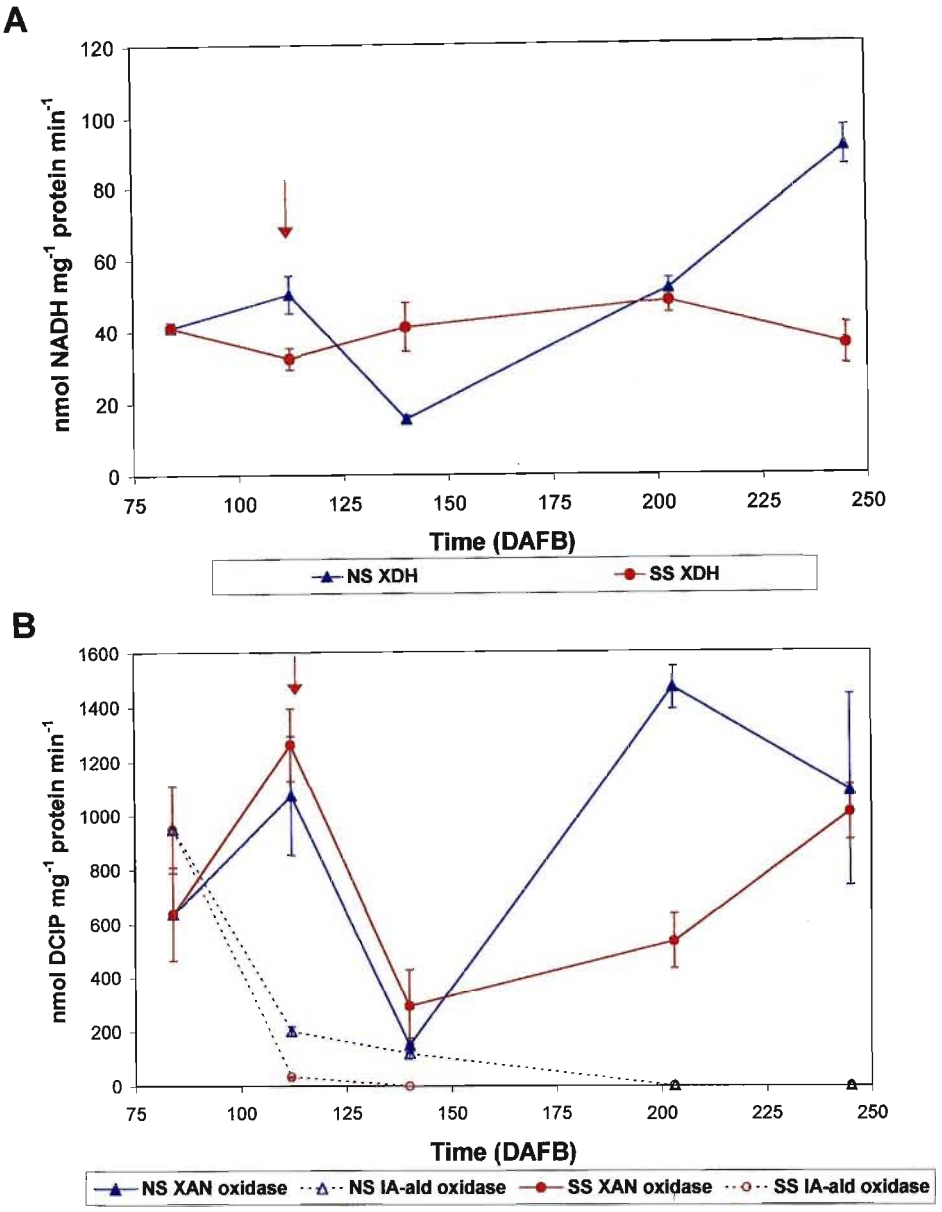


Figure 5.8 **XDH (A) and AO (B) activity in extracts prepared from seed tissue of normal (—) and phenotypically small (—) 'Hass' avocado fruit over the course of linear fruit growth. The appearance of the first small fruit is indicated by (↑). Data are the mean of three replicates, with vertical bars representing the standard error. NS, seed of normal fruit; SS, seed of small fruit.**

seed from small and normal fruit whereas the second peak was significantly higher and occurred earlier in seed of normal fruit. Together, these observations indicate that XDH activity is high in actively growing avocado seeds. Furthermore, these results suggest that both IA-ald oxidase and XAN oxidase are required early in avocado seed growth whereas only XAN oxidase activity is required later in seed development. Thus, a reduction in activity of XAN oxidase in the latter stage of seed growth and development appears to correlate with the appearance of a small fruit phenotype in 'Hass' avocado. Caution should, however, be exercised when interpreting these results as they are from a single growth season and may therefore not be consistent across growth seasons.

In extracts prepared from seed coat tissue derived from normal fruit, XDH activity increased to a maximum 203 DAFB (Fig. 5.9). Thereafter, activity declined. Not surprisingly, the trend with regard to the change in seed coat XDH activity corresponded to the increase in mass of this tissue in normal fruit. In light of these findings, it was perhaps not unexpected that no XDH activity was detected in seed coat tissue of small fruit. There was little difference in XAN oxidase activity in extracts prepared from seed coat tissue of normal and small fruit, although activity was not sustained in the small fruit (Fig. 5.9).

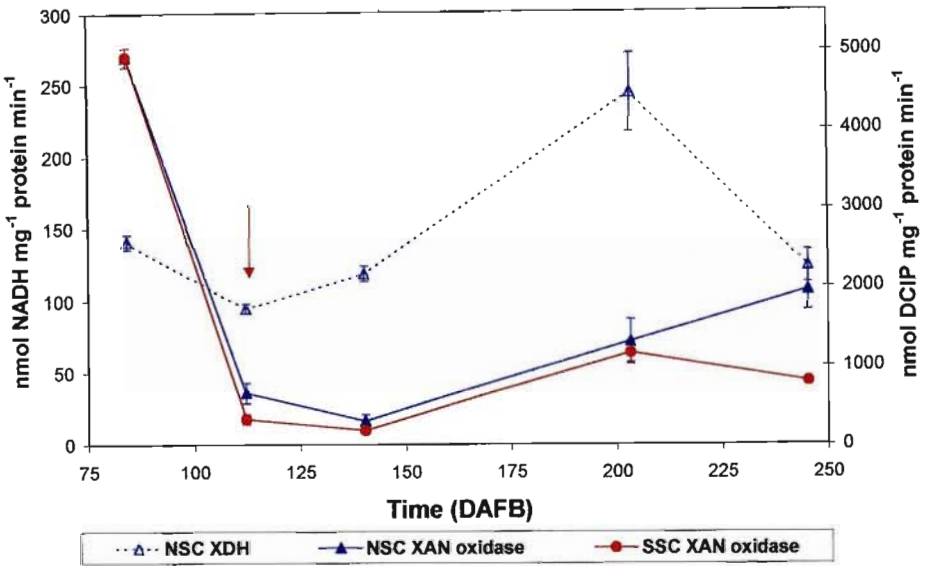


Figure 5.9 **Changes in activity of XDH and XAN oxidase in extracts prepared from seed coat tissue of normal (—) and phenotypically small (--) 'Hass' avocado fruit over the course of linear fruit growth. The appearance of the first small fruit is indicated by (↑). Data are the mean of three replicates, with vertical bars representing the standard error. NSC, seed coat of normal fruit; SSC, seed coat of small fruit.**

5.2.4 Changes in ABA and IAA during the linear phase of avocado fruit growth

Changes in endogenous ABA and IAA in the major tissues of developing normal and small 'Hass' avocado fruit during the linear phase of growth are illustrated in Figure 5.10. The IAA content of seeds of normal fruit was initially high, reaching a maximum 112 DAFB, and then declined gradually with approaching seed and fruit maturity (Fig. 5.10A). The decline in seed IAA content was mirrored by a transient increase in IAA content of the seed coat (Fig. 5.10B) but sustained accumulation of this hormone in mesocarp tissue (Fig. 5.10C). These results seem to indicate that seed-derived IAA is preferentially partitioned to the mesocarp via a functional seed coat in normal fruit. Support for this conclusion is evident from the situation for IAA in the small fruit phenotype. In this variant the decline in seed IAA (Fig. 5.10A) was followed by substantial IAA accumulation in seed coat tissue (Fig. 5.10B). An increase in IAA in the mesocarp occurred early and was transient (Fig. 5.10C) probably reflecting the loss of

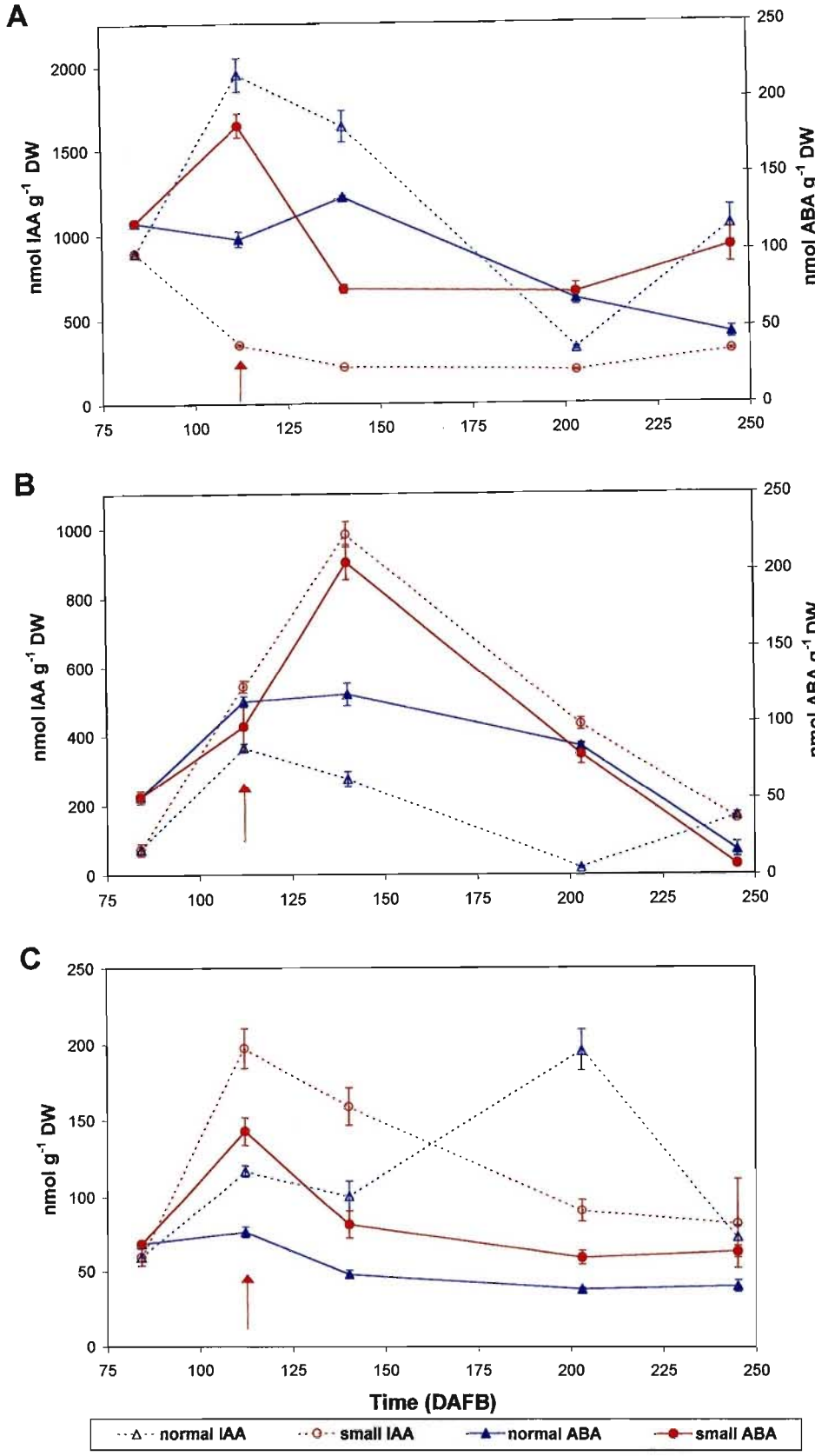


Figure 5.10 **Changes in endogenous ABA and IAA in normal (—) and phenotypically small (—) 'Hass' avocado seed (A), seed coat (B) and mesocarp (C) tissue over the course of linear fruit growth. The appearance of the first small fruit is indicated by (↑). Data are the mean of three replicates with vertical bars representing the standard error.**

structural integrity between seed coat and mesocarp in phenotypically small fruit due to early senescence of the seed coat.

The situation as regards the change in endogenous ABA is somewhat more complex as each of the major fruit tissues appears to be competent for ABA biosynthesis. However, some of the seed-produced ABA may be localized to the seed coat because the decline in seed and mesocarp ABA of normal fruit 112 DAFB (Fig. 5.10A and C) was mirrored by a protracted accumulation of ABA in the seed coat (Fig. 5.10B). Also, seed coat tissue from the small fruit accumulated substantial amounts of ABA (Fig. 5.10B) although this may be indicative of senescence-induced ABA synthesis. Equally plausible is ABA-induced ABA catabolism in both seed and mesocarp tissue of normal fruit. Nevertheless, the early increase in seed and seed coat ABA occurred coincident with the first peak in XAN oxidase activity (cf. Fig. 5.8B) indicative of a positive relationship between activity of this enzyme and changes in ABA during avocado fruit growth and development.

5.3 CONCLUSION AND SUMMARY

The first small fruit, with readily distinguishable senesced seed coats, were observed 112 DAFB. These fruit differed from normal fruit in both longitudinal and radial growth, with the former exhibiting a greater sensitivity to factors contributing to a decline in growth. Although the growth of all three major tissues differed between normal and small fruit, the disparity in seed growth between the two phenotypes is likely to have the greatest impact on final fruit size. The slowing down of longitudinal growth and cell division cycle activity in normal fruit was associated with an increase in CKOX activity in seed coat tissue and a general decline in CK-like and XDH activity. In fact high XDH activity occurs in actively growing seeds and seed coats when CK-like activity is high. Furthermore, whilst IA-ald oxidase and XAN oxidase activity is required early in seed growth, only XAN oxidase is required in the latter stages of growth where a decline in activity correlates with small fruit. In normal fruit IAA is preferentially partitioned to the mesocarp from the seed via the seed coat. However, the senescence of the seed coat in small fruit results in the loss of structural integrity and therefore a transient peak of IAA in mesocarp tissue and accumulation in seed coat. Abscisic acid accumulation in seed and seed coat tissue occurred concomitant with the early peak in XAN oxidase activity and was more pronounced in tissues of the small fruit variant. In summary,

(1) Small fruit were first distinguishable 112 DAFB. In contrast to normal fruit, the increase in expansion growth was greater than the increase in elongation growth in small fruit. There was a significant difference in the percentage increase in growth of all of the major tissues of normal and small 'Hass' avocado fruit over the course of linear fruit

growth, which was manifest in a significant divergence of the percentage change in fruit fresh mass between normal and small fruit.

(2) Cytokinin-like activity increased initially and then declined as fruit approached maturity. The highest levels of CK-like activity were detected in seed coat tissue, followed by seed and mesocarp and there was no significant difference between normal and small fruit at any point in the linear phase of growth. Cytokinin oxidase activity in seed and mesocarp tissue declined over the linear phase of fruit growth, whilst CKOX activity in seed coat tissue increased transiently. This increase in CKOX activity followed the peak in iP- and zeatin-like activity, thereby suggesting the induction of the enzyme by its substrate.

(3) A decline in XDH activity in mesocarp tissue corresponded to a decline in longitudinal growth of both normal and small fruit. A similar situation prevailed in seed tissue where high XDH activity was correlated with actively growing avocado seeds. Similarly, XDH activity was not detected in senesced seed coat tissue of small fruit. Both mesocarp and seed tissue displayed two peaks in XAN oxidase activity, the second of which occurred in the latter stages of fruit growth and was not evident in small fruit. However, the sustained XAN oxidase activity early in development of the small fruit variant suggests that activity of this enzyme is intimately related to the appearance of this phenotype.

(4) The IAA content of seed was initially high, declining as fruit approached maturity. Levels were consistently lower in seed tissue of small fruit. The decline in seed IAA content in normal fruit was matched by a transient increase in IAA of the seed coat, but sustained accumulation in the mesocarp. In small fruit, however, substantial IAA accumulated in seed coat tissue which was significantly higher than that in normal fruit, whilst IAA accumulation in mesocarp was only transient. Although all the major fruit tissues appear to be competent for ABA biosynthesis, it is possible that some of the seed-produced ABA may be localized to the seed coat. Seed coat tissue of small fruit also accumulated substantial amounts of ABA. In general, larger amounts of ABA accumulated in small fruit tissues at key physiological events in fruit growth.

6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION

There are two major reasons for using 'Hass' avocado as a model system to study the metabolic control of fruit growth with emphasis on the regulation of final fruit size (Cowan *et al.* 2001). Firstly, 'Hass' avocado is a major export crop in South Africa and reducing the number of "small fruit" produced in any season will ensure substantial economic benefit to the region. Secondly, in the absence of evergreen tree-crop mutants with which to dissect the mechanisms contributing to the control of final fruit size, 'Hass' avocado and its small fruit variant present an ideal system to investigate the physiology, biochemistry and molecular biology of fruit growth in a subtropical species. A recent review on the molecular biology of fruit maturation and ripening has highlighted the dearth of information on the molecular development of fleshy fruit (Giovannoni 2001). In fact, this author concludes the review by stating, "insights into early regulation of fruit development ... represent avenues through which future research activities will follow for the dissection of common regulatory control systems..." Nonetheless, the early events in the development of fleshy fruits have been likened to those of leaves (Gillaspy *et al.* 1993), and it is distinctly possible that the molecular mechanisms responsible for governing leaf size apply equally to fleshy fruits. The size of plant cells, and thus plant organs, is determined by genetic, structural, and physical factors that are influenced by internal and external signals. While our knowledge of the molecular mechanisms involved remains rudimentary, it is apparent that a major limitation to cell and organ growth is the entry and subsequent metabolism of nutrients. In addition to genetic factors that determine pattern formation (e.g. *ROTUNDIFOLIA3*), organ identity (e.g. *UNUSUAL FLORAL ORGAN*), and meristematic competence (e.g. *AINTEGUMENTATA*) all of which contribute to plant organ size, mutants defective in hormone synthesis and signalling demonstrate that a major consequence of modified hormone signalling is an alteration in final organ size. For example, the *Arabidopsis ethylene-overproduction1* (*eto1*) and *constitutive triple response1* (*ctr1*) mutations result in smaller than normal organs due to reductions in cell size and cell number. Ethylene and CK increase organ expansion along the transverse axes whereas GA and auxin regulate expansion along the longitudinal axes and influence stature and size (Shibaoka and Nagai 1994). The role of auxin signalling in final cell size seems not to be in doubt as overexpression of the *AUXIN-BINDING PROTEIN1* (*ABP1*) in tobacco increased the size of leaf cells dramatically (Jones *et al.* 1998). But *ABP1* does not appear to function in controlling organ size as an increase in cell size was accompanied by a reduction in cell number resulting in no major effect on leaf size. In contrast, studies using the *REVOLUTA (REV)/*

INTERFASCICULAR FIBERLESS1 (*IFL1*) gene indicate that the polar flow of auxin may be the cue for control of organ size. In *rev/ifl1* mutants, growth and cell proliferation persisted longer than in the wild type leading to larger organ size (Talbert *et al.* 1995; Zhong and Ye 1999). *REV/IFL1* function is required for the activity of auxin polar transport and for normal development of interfascicular cells (Zhong and Ye 2001). Thus, *REV/IFL1* links polar auxin transport to fiber cell differentiation, possibly to the regulation of secondary meristem formation, and determination of the extent of organ growth.

Interfascicular cells form between vascular bundles and join up with the fascicles to form a continuous ring of meristematic tissue. In avocado fruit, the vascular strands in the mesocarp are numerous and arranged in two concentric rings. They are surrounded by oil-containing parenchyma cells and these cells are in stages of active division as long as the fruit is attached to the tree. Thus cell division in the mesocarp parenchyma results in continuous enlargement throughout the developmental life of the fruit from fruit set to maturity. It is presumed that seed-derived hormones are translocated via the chalaza into the vasculature from where they exert their effects on cell division in the mesocarp. In fact, non-development of the pachychalaza in seedless fruit is believed to inhibit meristematic activity in the chalazal region, reduce sink strength, and retard fruit growth (Steyn *et al.* 1993). Although ethylene stimulates senescence of the pachychalaza, production of ethylene in senescing pachychalazal tissue is considered a result rather than a cause of seed coat degeneration (Davenport and Manners 1982). Thus other hormones and presumably developmental factors would appear to trigger degeneration (or arrested development) of the pachychalaza and chalazal tissue to cause appearance of the small fruit variant in 'Hass' avocado.

The present programme of research has shown that the increase in size of 'Hass' avocado fruit occurs largely as a result of seed growth and that longitudinal growth in small fruit is a more sensitive process, implying that either auxin or GA, or both, are limiting during the early development of this variant. However, a three-fold increase in IAA content of seed of small fruit was evident 256 DAFB and kinetic studies showed that accumulation of IAA in seed coat tissue of this variant during the linear phase of growth probably occurred as a consequence of the loss of structural integrity between seed coat (pachychalaza) and mesocarp tissue due to early seed coat senescence. Once produced, IAA normally diffuses into surrounding tissues and eventually away from the developing fruit. Thus, low extractable auxin correlates with the rapid growth phase of avocado fruit (Gazit and Blumenfeld 1972). Since application of CK to normal fruit increased IAA in mesocarp but reduced the IAA content of seed tissue, polar transport of IAA during avocado fruit growth may be coupled to CK signalling and/or action. Cytokinin treatment of normal fruit did not influence seed ABA but, and as expected, reduced

the ABA content of mesocarp tissue. Together, this information indicates that changes in plant hormone homeostasis may indeed be responsible for the high incidence of a small fruit variant in 'Hass' avocado. Using a model to describe the control of plant organ size, Mizukami (2001) assigns a major role to growth promoters that activate growth signal mediators such as *AINTEGUMENATA,* which stimulate growth co-ordinators (e.g. D-type cyclins) to activate and couple growth and cell division to maintain meristematic competence. In all probability the growth promoters are hormones (CK, IAA and GA) and control of hormonal balance likely impacts on the signal mediators to either suppress or maintain meristematic competence in affected organs. Whilst the importance of carbohydrates, other metabolites, mineral nutrients and water in fruit growth is acknowledged, this investigation focused on the importance of plant hormones to fruit growth, which act either directly or indirectly to alter gene expression. The role of hormone homeostasis in the control of final fruit size of avocado was established by comparing and contrasting hormone levels and activity of key enzymes involved in hormone metabolism, between normal 'Hass' avocado fruit and its small fruit phenotype. In addition this system was used to test the hypothesis that changes in hormone homeostasis occur as a result of differences in the activity of the MoCo-containing enzymes XDH and AO.

Results from this study indicate that CKOX, XDH and AO activity are correlated with final fruit size. Whilst high XDH and IA-ald oxidase activity was correlated with active tissue growth, high CKOX and XAN oxidase activity during early fruit development coincided with a decline in fruit growth. Furthermore, inhibition of XDH and a corresponding increase in AO activity in response to CK occurred coincident with increased levels of IAA and increased overall ABA metabolism. Cytokinin oxidase plays a crucial role in this process, as it is the induction of this enzyme by CK that is likely to set in motion events leading to changes in IAA and ABA. Thus these enzymes have an important role to play in the control of hormonal balance in fruit tissues. The importance of hormone homeostasis in the determination of final fruit size is demonstrated by the finding that fruit tissue growth was negatively correlated with an imbalance in the CK/IAA/ABA ratio in favour of IAA or ABA or both; an effect that is most likely mediated through their impact on sink strength and cell division cycle activity.

6.1.1 The biochemical basis of plant hormone homeostasis in avocado fruit

Moore-Gordon (1997) suggested that a change in the CK/ABA ratio was the trigger that elicited reduced growth and the appearance of the small fruit phenotype in 'Hass' avocado. This ratio is likely to be mediated via hormone interaction as Cowan *et al.* (1999) demonstrated CK stimulation of the oxidative catabolism of ABA. These authors proposed that a number of enzymes play a key role in mediating this interaction, including CKOX and the Mo-hydroxylases, AO and XDH. An initial probe into the validity of this proposal confirmed that

changes in the activity and tissue distribution of these enzymes between normal and small avocado fruit were mirrored by a change in the content and composition of IAA, ABA, CK and purines. At this time a low CK/ABA and CK/IAA ratio in seed tissue of small fruit was associated with low IA-ald oxidase activity, but high XAN oxidase, XDH and CKOX activity. Due to these differences between normal and small fruit a role for CKOX and Mo-hydroxylase activity in the control of hormone homeostasis and final fruit size was partly established.

No XDH mutants have been described for higher plants and in MoCo deficient mutants, characterized by a pleiotropic loss of all MoCo-enzyme activities, there are no symptoms that can be traced back specifically to a lack of XDH (Mendel and Schwarz 1999). It therefore seems that XDH does not play a vital role in plant development. It is, however, involved in a number of important physiological processes. These include purine catabolism, pathogen responses and cell death and senescence (Mendel and Schwarz 1999). This study presents another possible role for XDH, that involves mediation and interaction between the CK, ABA and IAA biosynthetic pathways. The demonstration in this study of XDH inhibition by adenine and allopurinol agrees with previous findings (Weir and Fischer 1970; Bray 1975; Boland 1981; Woo *et al.* 1981) and lends support to the theory that adenine-induced alterations in XDH activity might indeed impact on plant hormone homeostasis by facilitating more efficient utilization of the desulfo-MoCo by the AOs involved in ABA and IAA biosynthesis.

Cowan *et al.* (1999) proposed that low XDH activity, typical of that observed in seed of normal fruit 240 DAFB, might be associated with either increased CKOX activity or increased adenine, or both. Results indicate that, whilst lower CKOX activity was present in seed and mesocarp of normal fruit, elevated levels of adenine and xanthine were found in these tissues. Together, these confirm the postulated relationship between adenine content of fruit tissues and activity of XDH alluded to above. Cytokinin-induction of CKOX activity has been demonstrated in this (see section 3.2.4) and previous studies (Whitty and Hall 1973; Terrine and Laloue 1980; Jones *et al.* 1992; Dietrich *et al.* 1995), but it is unlikely to account for high CKOX activity in small fruit as CK content did not differ significantly between the two phenotypes. This disparity in CKOX activity is therefore most likely attributable to elevated IAA in small fruit tissues as auxin has been demonstrated to stimulate CKOX activity in avocado mesocarp tissue (see section 3.2.4), with similar observations reported using maize kernels (Cames and Wright 1988; Lur and Setter 1993) and tobacco pith explants (Palni *et al.* 1988). The stimulation of CKOX by auxin has important implications for final fruit size as it is likely to lead to a decline in the CK/IAA ratio that is of importance in the control of cell division cycle activity. In addition, auxin induction of CKOX activity might also impact on ABA metabolism as Cowan *et al.* (1999) suggested that CKOX activity contributed to the regulation of ABA

metabolism during plant organ growth by modulating the activity of XDH. Thus IAA may also regulate ABA metabolism, thereby maintaining plant hormone homeostasis during organ growth.

Although the physiological role of AO in plants remains unclear there is a growing body of evidence to support a function for this enzyme in both ABA (Walker-Simmons *et al.* 1989; Leydecker *et al.* 1995; Marin and Marion-Poll 1997; Schwartz *et al.* 1997a; Seo *et al.* 2000a;b) and IAA biosynthesis (Rajagopal 1971; Bower *et al.* 1978; Miyata *et al.* 1981; Koshiba and Matsuyama 1993; Tsurusaki *et al.* 1997; Seo *et al.* 1998). It is likely there exist several isoforms of the AO enzyme in plants, each with a different physiological function, which are expressed in a developmental- and/or tissue-dependent manner (Akaba *et al.* 1999). A single activity band of AO was detected in mesocarp tissue of 'Hass' avocado fruit following native-PAGE, which exhibited affinity for I-ald, citral, benzaldehyde and heptaldehyde (see section 3.2.1.1). This suggests that only one isoform of AO is present in avocado mesocarp tissue. However, the existence of additional isoforms that have identical mobilities in native-PAGE cannot be excluded. Although no activity was detected using XAN, it cannot be excluded as a substrate as activity may have been too low to be detected by activity staining and/or the presence of minor contaminating substances could have interfered with activity. The use of XAN by some of the *Arabidopsis* AO isoforms in activity gel staining, following native-PAGE, has been alluded to by Seo and Koshiba (2002), suggesting that an AO might indeed be involved in the conversion of XAN to XAN-acid. The higher activity of AO in the presence of I-ald, designated XAN oxidase in this study, in tissues of small fruit occurred co-incident with elevated ABA supporting the involvement of this AO in ABA biosynthesis and the appearance of the small fruit phenotype.

In contrast to mesocarp and seed coat tissue, AO in seed tissue exhibited activity in the presence of IA-ald and I-ald, citral, benzaldehyde and heptaldehyde (see section 3.2.1.1 and 3.2.2). This leads to the possibility that two AO isoforms are present in seed tissue, or at least that mesocarp and seed tissue possess different AO isoforms. This is supported by the finding of an altered ABA/IAA ratio in response to allopurinol and molybdate treatments. Results indicate that these isoforms have different affinity for molybdenum, catalyse AO-mediated reactions in ABA and IAA biosynthesis respectively, and that activity is development- and/or tissue-dependent. Similar findings have been reported for maize and *Arabidopsis* (Koshiba and Matsuyama 1993; Koshiba *et al.* 1996; Sekimoto *et al.* 1998). However, without conclusive native-PAGE evidence for seed tissue this possibility cannot be confirmed. Although in the latter stages of linear fruit growth AO activity in the presence of IA-ald, designated IA-ald oxidase in this study, was not correlated with IAA in normal and small seed

tissues, it is possible that the early senescence of the seed coat in small fruit (Moore-Gordon *et al.* 1998) prevents basipetal movement of seed-derived IAA resulting in an apparent accumulation in this tissue. Indole-3-acetic acid biosynthesis is also complicated by the presence of several possible parallel biosynthetic pathways in plants (Normanly *et al.* 1995; Kawaguchi and Syōno 1996; Normanly 1997), with three indole compounds known to be direct precursors of IAA (Tsurusaki *et al.* 1997). As auxin metabolism has not been studied in avocado fruit, the predominant pathway for IAA biosynthesis in this tissue is unknown and thus a parallel pathway, not involving a MoCo-AO, could be responsible for the observed increase in IAA in small fruit.

From this data it is evident that the presence of a small fruit phenotype in 'Hass' avocado is related to alterations in the activity of CKOX and Mo-hydroxylases which contribute to an imbalance in the CK/ABA/IAA ratio in favour of ABA and IAA. There thus seems to be evidence to support the proposal that the impact of CK on ABA and IAA metabolism is mediated through alterations in XDH activity that lead to the redistribution of MoCo to AO isoforms involved in ABA and IAA biosynthesis.

6.1.2 Implications of the modulation of CKOX, AO, and XDH activity on hormone homeostasis

6.1.2.1 Purine modulation of hormone homeostasis

In agreement with the findings of Cowan *et al.* (1999), CK was found to stimulate the oxidative catabolism of ABA to PA and DPA in both whole and ripe fruit, mesocarp and seed tissue. Similar results were obtained using CK analogues, thereby confirming that purines do indeed impact on ABA metabolism. The effect of CKs on ABA metabolism has been proposed to be two-fold firstly, the oxidation of XAN is stimulated and, secondly, the conversion of ABA to PA and DPA is enhanced (Cowan *et al.* 1999). This latter effect is due to the fact that ABA enhances its own catabolism by inducing ABA 8'-hydroxylase activity (Uknes and Ho 1984; Cutler *et al.* 1997). Since CKs were shown to enhance the oxidation of XAN, an overall stimulation of ABA metabolism in response to CK treatment is projected (Cowan *et al.* 1999). Support for this proposal that CK-ABA antagonism occurs at the level of MoCo biosynthesis is derived from the *aba1* mutant of *Nicotiana plumbaginifolia,* which is both CK resistant and ABA deficient. This mutant lacks MHS activity, which catalyses the sulfuration of the desulfo form of MoCo required by AO and XDH (Leydecker *et al.* 1995; Akaba *et al.* 1998), and is thus both ABA deficient and wilty (Blonstein *et al.* 1991) due to impairment in the conversion of XAN to ABA (Parry *et al.* 1991).

The increase in oxidation of XAN to ABA in response to CK (Cowan *et al.* 1999) seems to involve an increase in the activity of the AO, XAN oxidase. As an AO is also proposed to act at the final step in IAA biosynthesis, the question was posed in this study as to whether CK impacts on IA-ald oxidase activity in a similar manner to XAN oxidase. As predicted, treatment of fruit with CK did indeed change IAA levels. Whilst IAA decreased in seed in response to CK, levels in mesocarp tissue increased. Since no IA-ald oxidase activity was detected in mesocarp tissue an increase in IAA content in this tissue implies transport of seed-derived IAA into this tissue. Polar transport of IAA during avocado fruit development may therefore be coupled to CK signalling and/or action. In addition, as the increase of IAA in mesocarp tissue was greater than the reduction of IAA in seed tissue, IAA biosynthesis must have been stimulated in the presence of CK, indicating that increased availability of MoCo will also impact on AO isoforms involved in IAA biosynthesis.

The mediation of hormone homeostasis through alterations in XDH activity was investigated in more detail using allopurinol and molybdate. In agreement with the studies of Montalbini and Della Torre (1995) treatment of avocado fruit with allopurinol resulted in the accumulation of hypoxanthine and adenine, confirming the inhibitory action of allopurinol on XDH activity in avocado fruit tissues. Furthermore, allopurinol-treated seed tissue exhibited a decline in the CK/ABA ratio as a result of unchanged CK but elevated ABA. Hormone homeostasis was further perturbed in this tissue due to reduced IAA, confirming the importance of XDH in hormone homeostasis. Molybdate, on the other hand, stimulated overall hormone metabolism, confirming that MoCo-requiring enzymes mediate hormone levels and that MoCo availability can be limiting. Similar results have been reported in barley seeds where ABA increased in response to application of exogenous molybdate (Omarov *et al.* 1999).

6.1.2.2 Purines and MoCo enzyme activity

Although Cowan *et al.* (1999) proposed that CK-induced ABA metabolism involved XDH inhibition, these authors did not quantify XDH or AO activity. Their conclusions were based on analysis of ABA metabolism and incorporation of [^{14}C] from 3*R*-[2-^{14}C]mevalonolactone (MVL) into XAN, ABA and DPA in response to CK, allopurinol and molybdate. The present study showed that XDH and XAN oxidase activity was indeed altered in mesocarp tissue in response to CK. Whilst XDH activity was inhibited by CK, XAN oxidase activity was elevated, lending support to the proposal that inhibition of XDH results in more efficient utilization of MoCo by AO. This effect was confirmed in allopurinol- and allo+Mo-treated tissue.

The supposition that supply of MoCo is limiting in plants was supported by evidence of the induction of XDH and XAN oxidase activity in mesocarp tissue of avocado fruit treated with

excess molybdate. Similar "superinduction" of AO and XDH was noted in wild type tomato plants treated with Na_2S (Sagi *et al.* 1999). Furthermore, recent studies have shown that sulfuration of the dioxo-MoCo can be limiting for ABA biosynthesis (Bittner *et al.* 2001; Xiong *et al.* 2001). This conclusion was based on increased expression of the *Arabidopsis* ABA3 gene in response to dehydration treatment. As *ABA3* encodes a MoCo sulfurase it is likely that MoCo sulfuration limits XAN oxidase activity, thereby regulating the last step in ABA biosynthesis. It is also expected that if induction of sulfuration occurs under conditions where XDH is inhibited, the increase in AO activity will be exacerbated. In contrast, IAA biosynthesis appears to be differentially regulated by MoCo availability in response to stress, as MoCo biosynthesis mutants exhibit no obvious IAA deficiency or auxotrophy phenotype (Seo *et al.* 1998). As previously mentioned the existence of multiple parallel pathways for IAA biosynthesis in plants (Normanly *et al.* 1995; Kawaguchi and Syōno 1996; Normanly 1997) implies that a limitation in MoCo availability/biosynthesis will not impact on IAA in the same manner as it does on ABA.

6.1.3 Stress induction of Mo-hydroxylases

Moore-Gordon (1997) found that there is a physiological window 60-90 DAFB when 'Hass' avocado fruit first become susceptible and express the small fruit phenotype. This period was associated with a period of tree stress when there was a rapid rise in canopy temperatures and increased incidence of fruit drop (Moore-Gordon 1997). The incidence of small fruit also increased in trees bearing heavily (Lahav and Kalmer 1977) and in stressed, older trees (Köhne 1992; Whiley *et al.* 1996; Cowan 1997) grown under warm conditions (Cutting 1993), confirming that early seed coat senescence is exacerbated by tree stress. In agreement with these findings, small fruit possessing senesced seed coats were first distinguishable 84-112 DAFB in this study.

An important mechanism whereby plants respond, and adapt, to stress is believed to be through changes in the activity of MoCo-requiring enzymes. This is proposed to occur through changes in the MoCo pool size, which varies in response to nutritional and environmental factors (Sagi *et al.* 1997; Sagi and Lips 1998). An increase in the activity of the Mo-hydroxylases (i.e. AO and XDH) in response to salt stress and ammonium treatment in barley (Omarov *et al.* 1998) and ryegrass (Sagi *et al.* 1998) is thus considered to be part of the mechanism for stress adaptation in plants, which includes elevated ABA synthesis and increased ureide production (Sagi *et al.* 1998). As these two enzymes require a desulfo-MoCo it is further postulated that MHS may play a regulatory role in plants, particularly considering that expression of the *ABA3* gene, encoding this enzyme in *Arabidopsis*, is induced by dehydration treatment (Xiong *et al.* 2001). In addition, the sulfuration step has been shown to

be reversible *in vitro* (Wahl and Rajagopalan 1982; Schwartz *et al.* 1997a) which leads to the possibility that the size of the desulfo-MoCo pool is finely regulated in plants in response to stress.

Elevated XAN oxidase activity in mesocarp and seed tissue of avocado at first appearance of the small fruit could likely be associated with adaptation to imposed stress. However, in contrast to findings in barley (Omarov *et al.* 1998) and ryegrass (Sagi *et al.* 1998), increased AO activity in avocado small fruit tissues was not associated with increased XDH activity, but rather a decline in the activity of this enzyme. This has three possible implications for the ability of the fruit to adapt to stress. Firstly, it has been suggested that elevated XDH is necessary for efficient use of available C to synthesise organic N compounds with a low C/N ratio for transport from roots to shoots (Sagi *et al.* 1999). Secondly, the product of XDH activity, uric acid, is an effective scavenger of active oxygen species (Becker *et al.* 1989; Radi *et al.* 1990) and reduces free radicals produced in response to stress. Finally, as discussed above, a decline in XDH activity might result in further MoCo allocation to XAN oxidase, thereby increasing activity further. The first possibility has little relevance for fruit growth and is likely to apply only in roots where nitrogen fixation occurs. However, the second and third possibilities have severe implications for fruit growth in that reduced XDH activity is likely to exacerbate the effect of stress leading to a dramatic reduction in growth. The decline in XDH activity in small fruit is most probably attributable to adenine-induced inhibition of XDH which occurs as a result of increased CKOX activity in these fruit. This increase in CKOX activity is likely to be ascribed to two factors. Firstly, the slightly higher levels of CK-like activity and, secondly, the significantly higher IAA levels found in small fruit tissues at this stage, both of which have been demonstrated to stimulate CKOX activity in this study (see section 3.2.4).

As mentioned above it is possible that, under stress, activity of AO isoforms involved in ABA biosynthesis are preferentially activated over those involved in IAA biosynthesis. This effect may reside at the level of abundance of AO isoform apoproteins, whose expression may be differentially regulated in response to stress. In *Arabidopsis* leaves subjected to dehydration the expression of the gene encoding the AO isoform involved in IAA biosynthesis remained constant or was slightly reduced (Seo *et al.* 2000a). However, the expression of the gene encoding the AO isoform involved in ABA biosynthesis was rapidly induced following dehydration (Seo *et al.* 2000a). When coupled with increased MoCo sulfuration in response to drought stress (Xiong *et al.* 2001) this suggests that ABA biosynthesis will increase relative to IAA biosynthesis leading to an increase in the ABA/IAA ratio. Further experiments are, however, required to elucidate the exact physiological role of protein regulation in ABA biosynthesis. An increased ABA/IAA ratio in seed tissue of small fruit, which occurs co-

incident with enhanced XAN oxidase activity, but significantly reduced IA-ald oxidase activity, suggests that this may be an attempt to deal with imposed stress and thereby reduce growth. This is in agreement with the proposal that changes in ABA and IAA interact to signal the need for changes in plant functions required for adaptation to stress conditions (Dunlap and Robacker 1990).

6.1.4 Hormone homeostasis in avocado fruit in relation to CKOX, AO, and XDH activity

In general, hormone levels in both normal and small fruit tissues declined as fruit approached maturity, except for auxin levels in mesocarp tissue of normal fruit that peaked close to maturity. This is in agreement with previous studies on hormone levels in avocado fruit (Blumenfeld and Gazit 1970; Gazit and Blumenfeld 1970; Gazit and Blumenfeld 1972; Cutting *et al.* 1986; Cowan *et al.* 1997). Most of the CK and IAA was found in the developing seed, with ABA remaining constant across the fruit, which lends credence to the generally accepted proposal that the developing seed regulates cell division and expansion in surrounding fruit tissues (Gillaspy *et al.* 1993). Seed-derived hormones are partitioned to surrounding fruit tissues via a functional seed coat and thus the loss of structural integrity between seed coat and mesocarp tissue in small avocado fruit will sever the supply of these important growth-promoting substances. In addition to seed coat abortion, small fruit also possess smaller seeds, which make up a smaller percentage of the whole fruit. The aberrant seed growth and seed coat senescence of small fruit therefore implies an inability to sustain cell division in surrounding tissues.

Whilst there was very little difference in CK between normal and small fruit, ABA and IAA were significantly different, resulting in a shift in the hormonal balance in small fruit tissues. In keeping with the findings of Moore-Gordon *et al.* (1998) the CK/ABA ratio of mesocarp tissue was correlated with fruit size throughout the period of measurement in this study. However, had these authors extended their study to seed and seed coat tissue, they would have found that a similar correlation was not evident in these tissues. Although a slightly lower CK/ABA ratio was found in seed tissue of small fruit 112 DAFB, this situation did not persist and a higher CK to ABA ratio was found in seed of small fruit at the next two harvest intervals. Seed coat tissue from small fruit exhibited a similar trend, but there was no difference between the two phenotypes 112 DAFB. Interestingly, the CK/IAA and ABA/IAA ratios were also altered in small fruit tissues, with a decrease in IAA relative to CK and ABA evident in seed tissue of small fruit. However, the reverse was true for both seed coat and mesocarp tissue. Once produced, IAA diffuses into the surrounding tissue and eventually away from the developing organ via the pedicel. Studies using the *REVIIFL1* gene in *Arabidopsis* led to the proposal that polar auxin transport is a cue for the control of organ size (Zhong and Ye 2001). This is

attributed to the finding that polar auxin transport is linked to fiber cell differentiation, regulation of secondary meristem formation and the determination of the extent of organ growth (Talbert *et al.* 1995; Zhong and Ye 1999; 2001). It is thus not surprising that low extractable levels of auxin in mesocarp tissue were correlated with rapid growth of avocado fruit (Gazit and Blumenfeld 1972). The high levels of IAA relative to CK and ABA in both seed coat and mesocarp tissue of small fruit are thus expected to contribute to the decline in growth observed in these tissues and more specifically to the early senescence of the seed coat in these fruit. In small fruit longitudinal growth is a more sensitive process than radial growth and as GA and auxin are proposed to regulate expansion along the longitudinal axis (Shibaoka and Nagai 1994) it follows that one or both of these hormones are limiting in the early development of this variant. As the increase in size of 'Hass' avocado fruit occurs largely as a result of seed growth, the reduced IAA content in seed tissue during the early development of small fruit is likely to account for the reduction in longitudinal growth in these fruit.

The high IAA in seed coat and mesocarp of small fruit during the early stages of linear fruit growth is most probably attributable to a combination of factors. However, it is currently unknown which factor plays the most important role in elevating IAA in these fruit. Firstly, the early senescence of the seed coat in small fruit prevents partitioning of IAA to the mesocarp from the seed, which could explain why IAA accumulation is only transient in mesocarp tissue and why there is substantial accumulation in the seed coat. Secondly, alternate pathways for IAA biosynthesis, not involving a MoCo-AO, may be operational in seed coat and mesocarp, which are elevated in small fruit in response to stress. This second possibility is supported by the belief that different pathways of IAA biosynthesis are operational at different developmental stages, in different tissues or under different environmental conditions (Michalczuk *et al.* 1992; Celenza *et al.* 1995; Koshiba *et al.* 1995; Normanly *et al.* 1995). Finally, the elevated IAA in these tissues may be a consequence of impaired conjugation or increased hydrolysis of conjugates. Most of the IAA in plants exists as conjugates (Cohen and Bandurski 1982) and any factor impacting on conjugate formation or hydrolysis will result in large changes in free IAA. Whatever the cause of substantially elevated IAA in seed coat and mesocarp of small fruit, the consequences are likely to be deleterious for fruit growth.

6.1.5 Direct hormone interaction in avocado fruit

Hormone interaction may either reside at the level of hormone ratio, through changes in the effective concentration or tissue sensitivity of one hormone by another, or by sequential action of different hormones. Direct hormone-hormone interactions are likely to play an important role in the control of avocado fruit size. Aside from the impact of CK on ABA metabolism (Cowan *et al.* 1999) it is also likely that IAA and ABA interact, with high IAA stimulating ABA

biosynthesis (Grossmann *et al.* 1996; Grossmann 2000). Auxin is reported to have a biphasic effect on plant growth and development, with low concentrations of auxin reducing ethylene formation and promoting growth by cell division and elongation, in contrast to high concentrations, which induce phenomena such as epinasty, premature leaf abscission and inhibition of root and shoot elongation, followed by plant senescence (Grossmann 2000). It is clear from these plant responses that ethylene formation is stimulated in response to high IAA and it is these effects that provide the basis for the use of synthetic auxin analogues as herbicides in agriculture (Abeles *et al.* 1992; Cobb 1992; Sterling and Hall 1997; Grossmann 1998).

Auxin has been shown to induce *de novo* synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC), which is the result of increased expression of specific ACC synthase genes or post-transcriptional regulation (Kende and Zeevaart 1997; Grossmann 1998; Taiz and Zeiger 1998; Wei *et al.* 2000). As ACC synthase catalyses the rate-limiting step in ethylene biosynthesis (i.e. the conversion of *S*-adenosylmethione into ACC) (Abeles *et al.* 1992; Kende and Zeevaart 1997), an increase in the activity of this enzyme will have a direct impact on ethylene levels. Furthermore, the induction of ethylene synthesis by auxin is also thought to be related to ABA accumulation and growth inhibition in sensitive dicot species (Grossmann *et al.* 1996; Grossmann 2000). However, whether avocado is one of these sensitive species remains to be demonstrated. Support for ethylene induction of ABA biosynthesis was obtained through the use of the tomato *never ripe* mutant, which is impaired in its ability to bind ethylene efficiently (Wilkinson *et al.* 1995). In contrast to wild type plants, XAN failed to accumulate in mutant plants in response to auxin treatment (Hansen and Grossmann 2000). It was therefore concluded that these plants were unable to increase production of XAN following auxin treatment due to a block in response to ethylene. Further biochemical characterization of the response of ABA biosynthesis to ethylene revealed that NCED is the likely target site of auxin-induced ethylene (Hansen and Grossmann 2000). This is the key regulatory step in ABA biosynthesis and has been shown to be upregulated during stress (Kende and Zeevaart 1997; Neill *et al.* 1998; Cutler and Krochko 1999; Qin and Zeevaart 1999). The inhibitory effect of auxins at high concentrations is therefore mediated through hormonal interaction between ethylene and ABA.

Corroborative evidence to suggest that such an event occurs in avocado fruit is found in seed coat and mesocarp tissue of small fruit where the peak in IAA occurs co-incident with increased ABA. Furthermore, although Davenport and Manners (1982) reported that ethylene stimulates senescence of the pachychalaza, they concluded that ethylene produced in senescing pachychalazal tissue is a result rather than a cause of seed coat degeneration. It is

thus tempting to suggest that elevated IAA in seed coat tissue of small fruit is the cause of ethylene production in this tissue, which possibly combines with increased XAN oxidase activity to elevate ABA to non-physiological levels resulting in the early degeneration of the seed coat in these fruit.

6.1.6 Hormonal regulation of fruit size

Growth promoters have been assigned a major role in activating growth signals mediators, such as *AINTEGUMENATA*, which stimulate growth co-ordinators (e.g. D-type cyclins) to activate and couple growth and cell division to maintain meristematic competence. These growth promoters are in all likelihood hormones and thus changes in hormonal balance during fruit growth have important implications for control of fruit development, as they are likely to affect cell division cycle activity and sink strength.

6.1.6.1 Interaction of hormones with carbohydrates

Developing fruits are terminal sinks and require carbohydrates, other metabolites, mineral nutrients and water to sustain cell division and cell expansion. In tomato, under conditions of limited photoassimilate supply, the main limiting factor for fruit size is cell number (Bertin *et al.* 2002), indicating the importance of carbohydrates in the maintenance of cell division. The importation of photoassimilates into fruit must be monitored continuously and it is predicted that hormones may fulfil this role. Hormones are therefore proposed to co-ordinate fruit growth through detection of changes in sugar content and composition (Cowan *et al.* 2001). Alternatively, carbohydrate status of developing organs impacts hormone metabolism to alter flux through metabolic pathways and signal changes in development. Implicit in this is cross-talk between sugar and hormone signalling, which is supported by recent observations that sugars and hormones interact in the control of plant growth. For example, sucrose overrides auxin-induced *vsp* gene expression in soybean (De Wald *et al.* 1994) and regulates tuber formation in potato by influencing GA (Xu *et al.* 1998), and sucrose negatively regulates the signalling pathway in which transcriptional activation of wheat *WPK4* gene (which encodes a protein kinase capable of phosphorylating HMGR *in vitro*) is mediated by CK (Ikeda *et al.* 1999). In addition, the sugar insensitive (*sis*) mutants of *Arabidopsis*, *sis4* and *sis5*, are allelic to the ABA biosynthetic mutant *aba2* and the ABA insensitive mutant *abi4*, respectively (Laby *et al.* 2000).

In avocado the CK/ABA ratio is regarded as being important with respect to post-phloem solute transport, growth rate and final fruit size (Cowan *et al.* 1997; Moore-Gordon *et al.* 1998). In maize kernels ABA accumulation seems to serve as a biochemical signal to restore source/sink balance when photosynthesis is reduced due to water stress (Ober *et al.* 1991). In

this way, ABA is projected to play an important role in decreasing average kernel size or causing kernel abortion in the apical region of the maize ear in response to stress. Furthermore, Arenas-Huertero *et al.* (2000) showed that a relationship exists between ABA synthesis and plant sugar responses by demonstrating that ABA increased in *Arabidopsis* seedlings after sugar treatment and that treatment with ABA increased sugar sensitivity. While the extent to which the connection between ABA and sugar response is direct or indirect is still unresolved, results indicate that this connection exhibits a substantial degree of specificity as mutations in only specific components of the ABA response pathway(s) confer a *sis* phenotype (Laby *et al.* 2000). Thus, whilst a certain level of ABA is required in the early stages of fruit development to establish sink strength (Dewdney and McWha 1978; Tietz *et al.* 1981), it is evident that abnormally high levels of ABA, such as those found in small fruit, will have deleterious effects on fruit growth. This is thought to occur through alterations in symplastic continuity/solute transport and in particular the loss of plasmodesmata structure/function and cell-to-cell chemical communication (Moore-Gordon *et al.* 1998). This study presents corroborative evidence for a decreased CK/ABA ratio in seed and mesocarp tissue of small fruit, when small fruit were first distinguishable. In addition it reveals that IAA is reduced in seed tissue of the small fruit phenotype. This could contribute to a decline in sink strength in small fruit as auxin has been prescribed a role in directing sink strength through its impact on cell division, elongation and differentiation (Gillaspy *et al.* 1993).

In addition to affecting changes in sugar transport in avocado fruit, hormones are also likely to impact on sugar content and composition and the levels/or activities of sugar-metabolizing enzymes and vice versa. The high ABA content of seed of the small fruit variant was coupled with increased acid invertase activity, an effect that could be mimicked by ABA application to normal fruit (Richings *et al.* 2000). This has been demonstrated previously where both auxin and ABA were reported to increase invertase activity (Ackerson 1985; Poovaiah and Veluthambi 1985; Schaffer *et al.* 1987). This has particular relevance for avocado fruit growth as an over-expression of invertase has been shown to lead to morphological changes (Tymowska-Lalanne and Kreis 1998), stunted growth (Dickinson *et al.* 1991) and arrested development of secondary plasmodesmata (Ding *et al.* 1993), traits which are evident in ABA-treated normal fruit (Moore-Gordon *et al.* 1998; Richings *et al.* 2000). The result of increased invertase and reduced SuSy activity in seed tissue of small fruit, and in ABA treated normal fruit, is sucrose depletion and an increase in glucose as a proportion of total soluble sugar (Richings *et al.* 2000). The sucrose/hexose ratio is proposed to constitute a homeostat in which the relative amount of each component serves to modulate SNF1-related protein kinase 1 (SnRK1)/hexokinase (HXK) activity on one hand and plant hormone metabolism on the other (Campbell *et al.* 2000; Cowan *et al.* 2001).

Sucrose non-fermenting 1 (SNF1) related kinases have the potential to regulate several biosynthetic pathways including isoprenoid synthesis, sucrose synthesis and nitrogen assimilation (Sugden *et al.* 1999) through the phosphorylation and inactivation of key enzymes such as HMGR (Halford *et al.* 1999) and has therefore been implicated in the control of plant cell cycling (Dickenson *et al.* 1999). In addition to HMGR, both SPS and NR are also SnRK1-regulated enzymes. Nitrate reductase is a MoCo-requiring enzyme and is phosphorylated by SnRK1 (Sugden *et al.* 1999) and inactivated following the binding of a 14-3-3 protein to the phosphorylation site (Bachmann *et al.* 1996; Moorhead *et al.* 1996). This enzyme is of particular interest as AO and ABA levels are reduced in plants in which NR has been induced by its substrate (Omarov *et al.* 1998). This implies that, under conditions where NR is under the control of SnRK1, there will be a change in ABA and IAA metabolism and hence plant hormone homeostasis via the differential utilization of MoCo by MoCo-requiring enzymes (Campbell *et al.* 2001). If such conditions persist when sulfuration of the MoCo is increased, AO activity is likely to be greatly enhanced leading to large perturbations in ABA and possibly IAA, which will result in reduced fruit growth.

The cross-talk between sugars and hormones is further emphasised by the finding that plant SNF1-kinase (or SnRK1) is apparently regulated by the *pleiotropic regulatory locus 1* (*PRL1*), an evolutionary conserved α-importin-binding nuclear WD-protein (Salchert *et al.* 1998; Bhalerao *et al.* 1999). The PRL1 appears to function in plant sugar-related gene expression by acting as a negative regulator of SNF1 homologues (Gibson and Graham 1999). This is demonstrated by the finding that the *prl1* mutation results in hypersensitivity to glucose and sucrose. It augments sensitivity of plants to CK, ethylene, auxin and ABA and de-represses genes that are positively or negatively regulated by glucose or CK, or both (Salchert *et al.* 1998; Bhalerao *et al.* 1999). Furthermore, the *prl1* mutation is exacerbated by *amp1* (CK overproducing), a mutation which regulates CK production and cell division (Deikman 1997). SnRK1 is therefore likely to play an integral role in the control of fruit size by mediating hormone levels in response to carbohydrate status and composition and vice versa, which in turn will co-ordinate growth through the regulation of cell division.

6.1.6.2 Hormonal regulation of cell division

'Hass' avocado fruit size is dependent on cell number and not cell size (Cowan *et al.* 1997). The regulation of cell division cycle activity is therefore an important determinant of final fruit size. Cyclin-dependent protein kinases (CDKs) guard the checkpoints of the eukaryotic cell cycle and regulate mitotic cell division. These enzymes are regulated primarily at the post-translational level by phosphorylation and consist of a catalytic CDK subunit (member of the CDC2-like family) and a regulatory cyclin subunit (Pines 1993). Levels of the plant kinase *cdc2*

(also known as $p34^{cdc2}$) are positively correlated with physiological competence to divide (Colasanti *et al.* 1991; Bergounioux *et al.* 1992; Hashimoto *et al.* 1992; Hemerly *et al.* 1992; Miao *et al.* 1993; Ferreira *et al.* 1994; Devitt and Stafstrom 1995; Kvarnheden *et al.* 1995), with the spatial specificity of *cdc2* expression contributing to spatial regulation of cell division in plants (Hemerly *et al.* 1993). In intact *Arabidopsis* plants *cdc2* can be induced or inhibited, depending on the phytohormone applied, with auxin and CK assigned an important role in the control of cell division in plants as they increase transcripts of *cdc2* (Hemerly *et al.* 1993). Mader and Hanke (1996) demonstrated that the withdrawal of CK in cultured soybean cells acted as a switch from mitotic to amitotic cycles with fractions gathering in the G_2/M transition. Subsequently, Zhang *et al.* (1996) showed that CK acts specifically at late G_2 through stimulation of tyrosine dephosphorylation and sequential activation of $p34^{cdc2}$-like H1 histone kinase. Thus, while auxin alone can induce $p34^{cdc2}$ synthesis, cell division requires the activation by CK. Auxin has, however, also been implicated in the control of the duration of G_1 and G_2 (Bayliss 1985; John *et al.* 1993a;b).

In addition to positive regulation of the cell cycle by activation of CDK complexes, negative regulators, termed cyclin-dependent kinase inhibitors (CKIs), can block the activity of the kinase (Nakayama and Nakayama 1998). Controlled proteolysis has recently been shown to be important in the control of cell cycle transition and two genes implicated in auxin responses have been found to be essential for the degradation of cell cycle proteins such as CKIs (Peters 1998). This leads to the attractive possibility that auxins may promote cell division by triggering the degradation of CKIs (Mironov *et al.* 1999), but this has yet to be proven unequivocally. In addition a CKI designated *ICK1* whose expression is induced by ABA, has been found in plants, suggesting that ICK1 may mediate the cytostatic effect of ABA in plants (Wang *et al.* 1998).

The potential role of ABA in blocking or slowing down the cell cycle has been indicated in several studies (Nougaréde *et al.* 1987; Myers *et al.* 1990; Robertson *et al.* 1990; Müller *et al.* 1994; Bracale *et al.* 1997; Liu *et al.* 1997). It is proposed that ABA regulates cell division cycle activity through the inhibition of nucleic acid and protein synthesis (Owen and Napier 1988; Jacqmard *et al.* 1995). The inhibition of HMGR activity by ABA is another possible manner in which ABA might impact on cell division cycle activity (Russell and Davidson 1982; Moore and Oishi 1994). Inhibition of HMGR activity has been shown to reduce cell division cycle activity in cultured tobacco cells (Crowell and Salaz 1992) which is proposed to be a consequence of reduced CK biosynthesis. Chemical inhibition of HMGR has also been demonstrated to arrest avocado fruit growth (Cowan *et al.* 1997; Richings *et al.* 2000) and HMGR activity and

isoprenoid compounds are essential in the determination of final tomato fruit size (Narita and Gruissem 1989; Gillaspy *et al.* 1993; Jelesko *et al.* 1999).

As CK, IAA and ABA have all been found to regulate cell division cycle activity either synergistically or antagonistically, the balance between them is likely to be of the utmost importance in the regulation of this process. Jacqmard *et al.* (1995) speculate that the balance between ABA and CK levels are one of the major factors controlling DNA replication and ultimately the duration of the cell cycle in shoot apices of *Sinapsis*. This ratio has also been implicated in the control of cell division in root-nodule initiation in legumes (Phillips 1971). In addition IAA may serve to degrade a CKI, designated *ICK1*, whose expression is induced by ABA (Wang *et al.* 1998; Mironov *et al.* 1999). The change in the CK/IAA/ABA ratio, in favour of IAA or ABA or both, in small fruit could thus account for reduced cell division cycle activity in these fruit, due to elevated levels of CKIs, inhibition of DNA synthesis and loss of CDK activity.

6.1.7 An integrated model for the control of avocado fruit size through hormone homeostasis

Fruit development is considered to be a result of intricate spatial and temporal interactions between resources required for growth and hormonal mediation through the regulation of gene expression. Based on the findings in this study a model presented in Figure 6.1 is proposed to explain the manner in which hormone homeostasis is mediated in avocado fruit and how this impacts on final fruit size. For this purpose the model links hormone homeostasis with sugar/carbohydrate and adenylate status of the tissue in the control of cell cycle activity and thus final fruit size.

The size of the MoCo pool is regulated by environmental conditions and nutritional status (Sagi *et al.* 1997; Sagi and Lips 1998). Under stressful conditions sulfuration of the dioxo-MoCo is stimulated (Xiong *et al.* 2001) leading to an increase in the activity of the Mo-hydroxylases (AO and XDH) which require a desulfo-MoCo. Under conditions where CKOX is elevated, the resulting rise in adenine will inhibit XDH, which is predicted to result in the more efficient utilization of the MoCo by AO, thereby exacerbating the effect of stress. Two potential avenues exist for regulation of CKOX activity in avocado fruit: firstly, CK levels (Kamínek *et al.* 1997) and, secondly, IAA levels (Coenen and Lomax 1997), both of which serve to increase CKOX activity. The increased activity of AO in response to stress, and possibly decreased XDH activity, will impact on ABA and IAA, as isoforms of AO catalyse the final steps in ABA (Walker-Simmons *et al.* 1989; Sindhu *et al.* 1990; Leydecker *et al.* 1995; Cowan 2001; Milborrow 2001) and IAA biosynthesis (Koshiba *et al.* 1996; Lips *et al.* 1999). Elevated ABA and IAA relative to endogenous CK content will combine to reduce cell division cycle activity

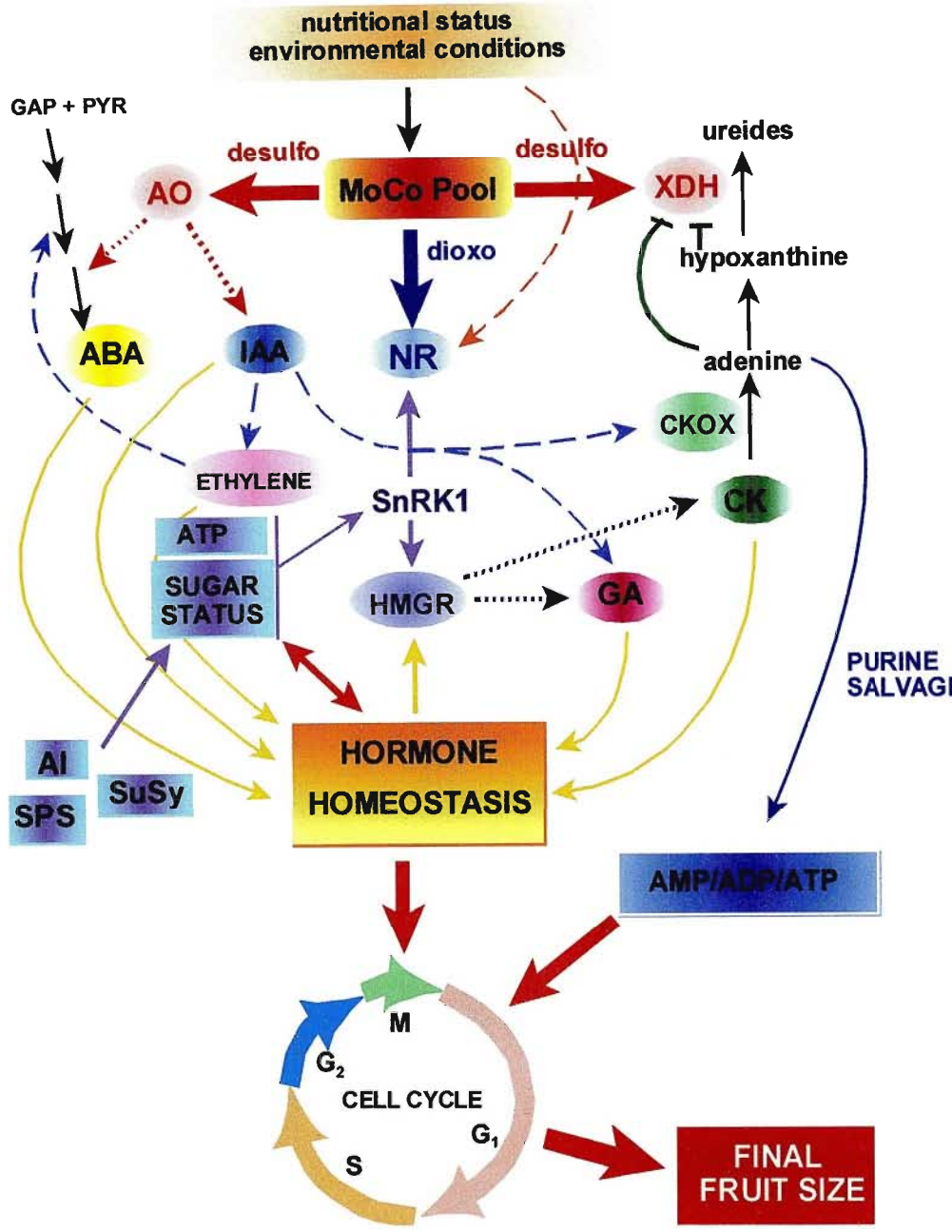


Figure 6.1 **Model depicting the proposed manner in which hormone homeostasis is controlled in 'Hass' avocado fruit and how this impacts on cell cycle activity and final fruit size. ABA, abscisic acid; ADP, adenosine diphosphate; Al, acid invertase; AMP, adenosine monophosphate; AO, aldehyde oxidase; ATP, adenosine triphosphate; CK, cytokinin; CKOX, cytokinin oxidase; IAA, indole-3-acetic acid; G_1, gap 1; G_2, gap 2; GA, gibberellin; GAP, glyceraldehyde phosphate; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; M, mitosis; NR, nitrate reductase; PYR, pyruvate; SnRK1, SNF1-related protein kinase 1; S, DNA synthesis; SPS, sucrose phosphate synthase; SuSy, sucrose synthase; XDH, xanthine dehydrogenase.**

as it is maintenance of the correct hormonal balance in fruit that ensures continuation of cell division cycle activity (Nagl 1971; Nagl 1972; Nagl 1976; Barlow 1976).

Free purines are in a large part salvaged and reused to synthesise nucleotides, and adenine salvage is an important mechanism for rapid adenylate synthesis (Shimazaki *et al.* 1982). Incubation of growing potato tuber discs with adenine resulted in increased levels of adenine nucleotides, decreased levels of glycolytic intermediates and organic acids, increased starch synthesis, increased respiration, an increased ATP/ADP ratio and decreased sucrose synthesis (Loef *et al.* 2001). This effect was, however, dependent on the hexose/sucrose ratio and under conditions where glucose levels are elevated increased adenine has the greatest impact, as ATP levels are low. In fact, Halford *et al.* (1999) suggests that changes in ATP levels might be the signal that indicates the availability of sugar and it therefore possibly links respiration to carbohydrate availability and growth.

High IAA is also reported to stimulate ABA biosynthesis (Grossmann *et al.* 1996; Grossmann 2000). Auxin enhances *de novo* synthesis of ACC (Kende and Zeevaart 1997; Grossmann 1998; Taiz and Zeiger 1998; Wei *et al.* 2000) which leads to an increase in ethylene. Induction of ethylene synthesis by auxin is thought to be related to ABA accumulation and growth inhibition in sensitive dicot species (Grossmann *et al.* 1996; Grossmann 2000). The target site of auxin-induced ethylene in ABA biosynthesis is reported to be the NCED enzyme (Hansen and Grossmann 2000) which catalyses the rate-limiting step in ABA biosynthesis i.e. the conversion of 9-*Z*-xanthophylls to XAN (Schwartz *et al.* 1997b; Tan *et al.* 1997; Cutler and Krochko 1999). Elevated IAA will therefore impact negatively on cell division cycle activity and fruit growth, partly through the stimulation of both ethylene and ABA biosynthesis.

On the other hand, low auxin is expected to have a positive impact on fruit growth. This effect could be partly mediated through the induction of GA biosynthesis, which has been demonstrated in pea fruit (van Huizen *et al.* 1997). 4-chlorindole-3-acetic acid is proposed to originate in seed tissue from where it is transported to the pericarp where it in turn regulates the conversion of GA_{19} to GA_{20} by increasing the level and/or stability of GA 20-oxidase mRNA (van Huizen *et al.* 1997). Normal levels of IAA are also required to maintain normal levels of bioactive GA (GA_1) in pea stems (Ross *et al.* 2000). In addition, the expression of the pea gene *LE* (GA_{20} to GA_1) in stem internodes requires IAA from the shoot apex and expression of *PsGA2ox1* (deactivation of GA_{20} to GA_{29} and GA_1 to GA_8) is reduced by IAA (Ross *et al.* 2000). The existence of such a mechanism in avocado fruit is as yet unproven, but it is possible that seed derived IAA stimulates GA biosynthesis in the surrounding tissues, especially the seed coat, which in turn contributes to the regulation of fruit growth. Although

the role of GA in fruit development is not well understood, it is generally assumed that it is necessary for the stimulation of cell division and the maintenance of cell expansion (Gillaspy *et al.* 1993). These authors also propose that auxin-stimulated GA synthesis and accumulation during maximal fruit growth is required for subsequent expansion and/or sink activity of the fruit cells.

Sugar content and composition coupled with isoprenoid metabolism and hormone homeostasis seem inextricably linked to the fruit developmental programme via sugar-metabolizing enzymes, HMGR and AO. Activity of HMGR is central in fruit growth as it determines the availability of regulatory isoprenoid compounds required for cell division cycle activity, sink strength and fruit growth (Narita and Gruissem 1989; Gillaspy *et al.* 1993; Jelesko *et al.* 1999; Richings *et al.* 2000). As CK and GA have their biosynthetic origins in the isoprenoid pathway HMGR activity will also impact on hormone homeostasis. The activity of this enzyme is apparently modulated by SnRK1 protein kinase in concert with changes in sugar content and composition. A decrease in the sucrose/hexose ratio coupled with changes in adenylate status (i.e. AMP/ATP ratio) impacts on activity of either SnRK1 or HXK to redirect the sugar-induced signalling cascade. In avocado, exogenous sucrose, glucose and ABA effect changes in HMGR activity and fruit size (Cowan *et al.* 1997; Richings *et al.* 2000). Nitrate reductase and SPS are also SnRK1 or sugar-regulated enzymes. Nitrate reductase is phosphorylated by SnRK1 (Sugden *et al.* 1999) and inactivated following the binding of a 14-3-3 protein to the phosphorylation site (Bachman *et al.* 1996; Moorhead *et al.* 1996). Nitrate reductase is a MoCo-requiring enzyme and the endogenous MoCo pool varies in response to nutritional status which is in agreement with findings that NR is particularly sensitive to stress and is influenced by a number of environmental factors (Huffaker *et al.* 1970; Hueur *et al.* 1979; Crawford 1995). According to the model proposed in Figure 6.1, the result of inactive NR is the preferential allocation of MoCo to the remaining two MoCo-requiring enzymes, AO and XDH, and as a consequence increased ABA and IAA biosynthesis. Hormone homeostasis is thus finely controlled in avocado fruit, such that cell division and fruit growth can be regulated in relation to available resources required for growth and prevailing environmental conditions.

6.2 CONCLUSIONS AND FUTURE PROSPECTS

This study has demonstrated that final size of 'Hass' avocado fruit is intimately linked with hormone homeostasis, with an imbalance in the CK/IAA/ABA ratio occurring co-incident with the appearance of the small fruit phenotype and with the associated reduction in fruit growth. It is proposed that in response to a period of tree stress the activity of Mo-hydroxylase enzymes is adjusted. The result of this is an elevation in ABA, but a reduction in IAA relative to

CK in seed tissue of small fruit. It is further proposed that, as the seed is purported to control growth in the surrounding tissue, the change in ABA and IAA in this tissue signals the surrounding tissues to abort development, and hence the elevated levels of both ABA and IAA in these tissues which are associated with a reduction in growth and ultimately senescence (Gazit and Blumenfeld 1972; Zeevaart and Creelman 1988; Grossmann 2000). Whether this change in hormone homeostasis is a cause of seed coat senescence or a result thereof, however, remains to be determined. This study has also demonstrated that, in seed tissue from small fruit, elevated CKOX activity, as a result of CK and/or IAA stimulation, results in the inhibition of XDH through the increased production of adenine. This reduction in XDH activity in seed tissue of small fruit was in turn associated with elevated XAN oxidase, but reduced IAald oxidase activity. Cytokinin oxidase activity thus contributes to the modulation of hormone homeostasis by influencing the allocation of MoCo to the AO isoforms involved in ABA and IAA biosynthesis through the inhibition of XDH.

Hormone-hormone interaction appears to play an important role in the determination of final avocado fruit size. Whilst the interaction between CK and ABA has been demonstrated in this and previous studies (Moore-Gordon *et al.* 1998; Cowan *et al.* 1999), other interaction awaits verification. Firstly, there is a need to determine if avocado fruit are sensitive to high IAA, which results in elevated ethylene and enhanced ABA biosynthesis (Grossmann *et al.* 1996; Grossmann 2000), as this could be part of the mechanism leading to seed coat senescence and overall reduction of growth in small fruit. Included in this is the need to determine the predominant pathway of IAA biosynthesis in avocado fruit tissues. Secondly, the possibility that seed-derived IAA impacts on GA biosynthesis in surrounding fruit tissues needs to be examined, as this could represent an important part of the growth promoting mechanism of auxin in fruit. The complete picture of hormones in avocado fruit also awaits determination. The rudimentary bioassay data available needs to be confirmed, with particular reference to the spectrum of CKs and GAs found in these fruit. As both these hormones have been implicated in the control of cell division in fruit, their detailed analysis in normal and small 'Hass' avocado fruit could provide some understanding of their role in fruit growth. Finally, the hypothesis that alterations in hormone homeostasis in fruit occur as a result of differences in the allocation of MoCo to MoCo-requiring enzymes needs to be tested in another plant system.

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APPENDIX I

1 DETAILS OF PROCEDURES OMITTED FROM MATERIALS AND METHODS

Reagent preparation for polyacrylamide gels and silver staining and Miller's media preparation for CK bioassays (see Table (i)).

2 PURINE STANDARDS (HPLC)

The standards (adenine, hypoxanthine, xanthine, adenosine, adenosine monophosphate, guanosine monophosphate and inosine monophosphate) were separated with a linear gradient of 100 % - 80 % 0.02 M $(NH_4)H_2PO_4$ in 2.5 mM N,N-dimethyloctylamine (adjusted to pH 3) in methanol over 40 minutes at 268 nm on a 5µm C_{18} column (250 x 4 mm i.d., Nucleosil 100-5) with a 100-5 guard column (8 x 4 mm i.d.), at a flow rate of 1 mL min^{-1} (see Figure (i)).

3 ABA, IAA, PA AND DPA STANDARDS (HPLC)

The standards (ABA, IAA, PA and PA) were separated with a linear gradient of 20 % - 100 % methanol, in 0.005 % acetic acid (System A) over 55 minutes at 260 nm on a 5 µm C_{18} column (250 x 10 mm i.d., ODS1) at a flow rate of 2 mL min^{-1} (see Figure (ii)).

4 ABAMe AND IAAMe STANDARDS (HPLC)

The standards (ABA methyl ester and IAA methyl ester) were separated with a linear gradient of 20 % - 100 % methanol in water (System B) over 55 minutes at 260 nm on a 5 µm C_{18} column (250 x 10 mm i.d., ODS1) at a flow rate of 2 mL min^{-1} (see Figure (iii)).

1 DETAILS OF PROCEDURES OMITTED FROM MATERIALS AND METHODS

1.1 Polyacrylamide gel electrophoresis

1.1.1 Reagents for polyacrylamide gels

Monomer solution [30 % (w/v) acrylamide, 0.8 % (w/v) N,N'-methylenebisacrylamide]. Acrylamide (30 g) and N, N'-methylenebisacrylamide (0.8 g) were dissolved in 100 mL of deionised H_2O. The solution was filtered through Whatman® No. 1 filter paper and stored at room temperature, in an amber-coloured bottle.

Tank buffer [25 mM Tris-HCl, 192 mM glycine, pH 8.8]. Tris (6.057 g) and glycine (28.8 g) were dissolved in 1.5 L deionised H_2O, the pH was adjusted to 8.8 with HCl and the buffer made up to 2 L with deionised H_2O. The buffer was stored at 4° C.

Stacking gel buffer [500 mM Tris-HCl, pH 8.8]. Tris (3.0285 g) was dissolved in 40 mL deionised H_2O, the pH was adjusted to pH 8.8 with HCl and the buffer made up to 50 mL with deionised H_2O. The buffer was filtered through Whatman® No. 1 filter paper and stored at 4 °C.

Running gel buffer [1 M Tris-HCl, pH 8.8]. Tris (12.114 g) was dissolved in 75 mL deionised H_2O, the pH was adjusted to pH 8.8 with HCl and the buffer made up to 100 mL with deionised H_2O. The buffer was filtered through Whatman® No. 1 filter paper and stored at 4 °C.

10 % (w/v) Ammonium persulfate. Ammonium persulfate (0.1 g) was dissolved in deionised H_2O (1 mL) just before use.

Non-reducing treatment buffer [50 mM Tris-HCl, 20 % (v/v) glycerol, pH 8.8]. Stacking buffer (1 mL) and glycerol (2 mL) were made up to 10 mL with deionised H_2O.

Molecular mass markers. The standard used for molecular weight estimation was urease from Jack bean (272 kDa (trimer) and 545 kDa (hexamer)). The marker (1 mg) was reconstituted in 750 µL non-reducing treatment buffer to give a final concentration of 1.3 mg mL^{-1}.

1.1.2 Reagents for silver staining procedure

30 % (v/v) Nitric acid. Nitric acid [545 mL of a 55 % (v/v) solution] was diluted to 1 L with deionised H_2O.

Fixing solution [50 % (v/v) methanol, 12 % (v/v) acetic acid, 0.05 % (v/v) formaldehyde]. Methanol (100 mL), acetic acid (24 mL) and formaldehyde (100 µL of a 37 % (v/v) solution) were diluted to 200 mL with deionised H_2O.

Washing solution 1 [50 % (v/v) ethanol]. Ethanol (100 mL) was diluted to 200 mL with deionised H_2O.

Pretreatment solution [0.02 % (w/v) sodium thiosulfate]. $Na_2S_2O_3.5H_2O$ (40 mg) was dissolved in deionised H_2O (200 mL).

Impregnation solution [0.2 % (w/v) silver nitrate, 0.075 % (v/v) formaldehyde]. $AgNO_3$ (400 mg) was dissolved in deionised H_2O (199.85 mL) and formaldehyde (150 µL of a 37 % (v/v) solution) was added just before use.

Developing solution [6 % (w/v) sodium carbonate, 0.0004 % (w/v) sodium thiosulfate, 0.05 % (v/v) formaldehyde]. Na_2CO_3 (12 g) and $Na_2S_2O_3.5H_2O$ (4 mL of pretreatment solution above) were thoroughly mixed in 195 mL of deionised H_2O. Formaldehyde (100 µL of a 37 % (v/v) solution) was added just before use.

Stopping solution [50 % (v/v) methanol, 12 % (v/v) acetic acid]. Methanol (100 mL) and acetic acid (24 mL) were diluted to 200 mL with deionised H_2O.

Washing solution 2 [50 % (w/v) methanol]. Methanol (100 mL) was diluted to 200 mL with deionised H_2O.

1.2 Media preparation for cytokinin bioassays

Table (i) **Basal medium for soybean callus bioassay (Adapted from Miller 1963; 1965).**

STOCK	CHEMICALS	MASS IN STOCK ($g\ L^{-1}$)	VOLUME STOCK ADDED ($mL\ L^{-1}$)
1	KH_2PO_4	3.0	100
	KNO_3	10.0	
	NH_4NO_3	10.0	
	$Ca(NO_3)_2.4H_2O$	5.0	
	$MgSO_4.7H_2O$	0.715	
	KCl	0.65	
	$MnSO_4.4H_2O$	0.14	
2	NaFeEDTA	1.32	10
	$ZnSO_4.7H_2O$	0.38	
	H_3BO_3	0.16	
	KI	0.08	
	$Cu(NO_3)_2.3H_2O$	0.035	
	$(NH_4)Mo_7O_{24}.4H_2O$	0.01	
3	MYO-INOSITOL	10.0	10
	NICOTINIC ACID	0.2	
	PYRIDOXINE HCl	0.08	
	THIAMINE HCl	0.08	
4	NAA	0.2	10

2 PURINE STANDARDS

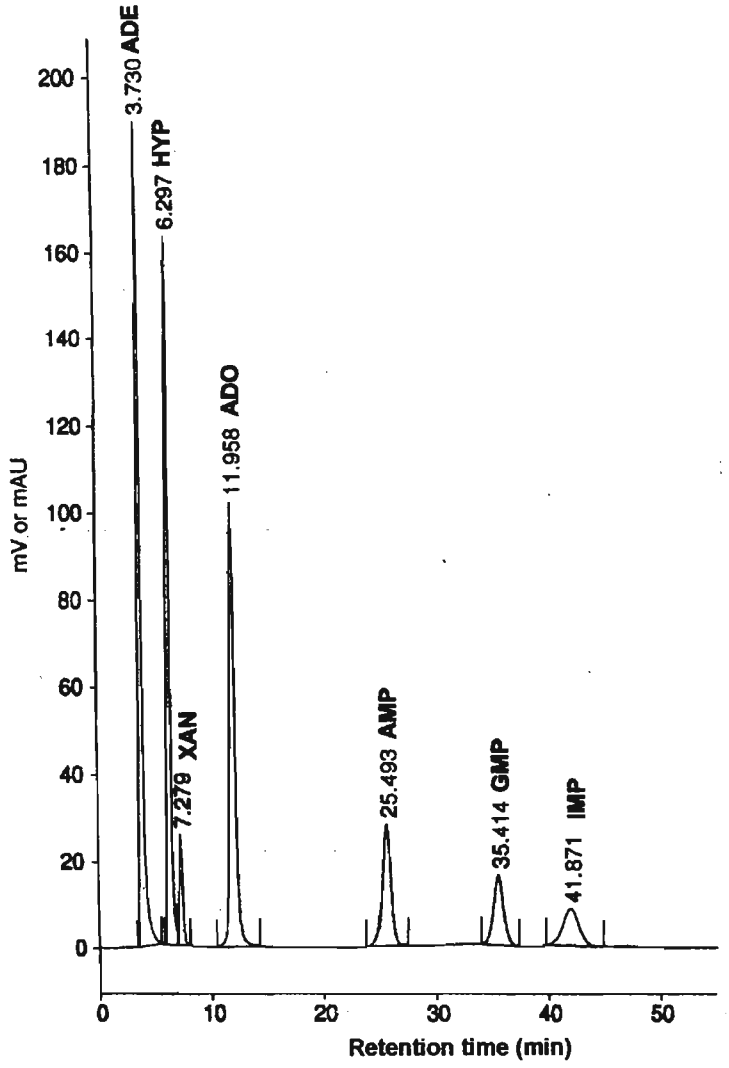

Figure (i) **Chromatogram of standard purine solution. The standards were separated with a linear gradient of 100 % - 80 % 0.02 M $(NH_4)H_2PO_4$ in 2.5 mM N,N-dimethyloctylamine (adjusted to pH 3) in methanol over 40 minutes at 268 nm on a 5μm C_{18} column (250 x 4 mm i.d., Nucleosil 100-5) with a 100-5 guard column (8 x 4 mm i.d.), at a flow rate of 1 mL min^{-1}. ADE, adenine; HYP, hypoxanthine; XAN, xanthine; ADO, adenosine; AMP, adenosine monophosphate; GMP, guanosine monophosphate; and IMP, inosine monophosphate.**

3 ABA, IAA, PA and DPA STANDARDS

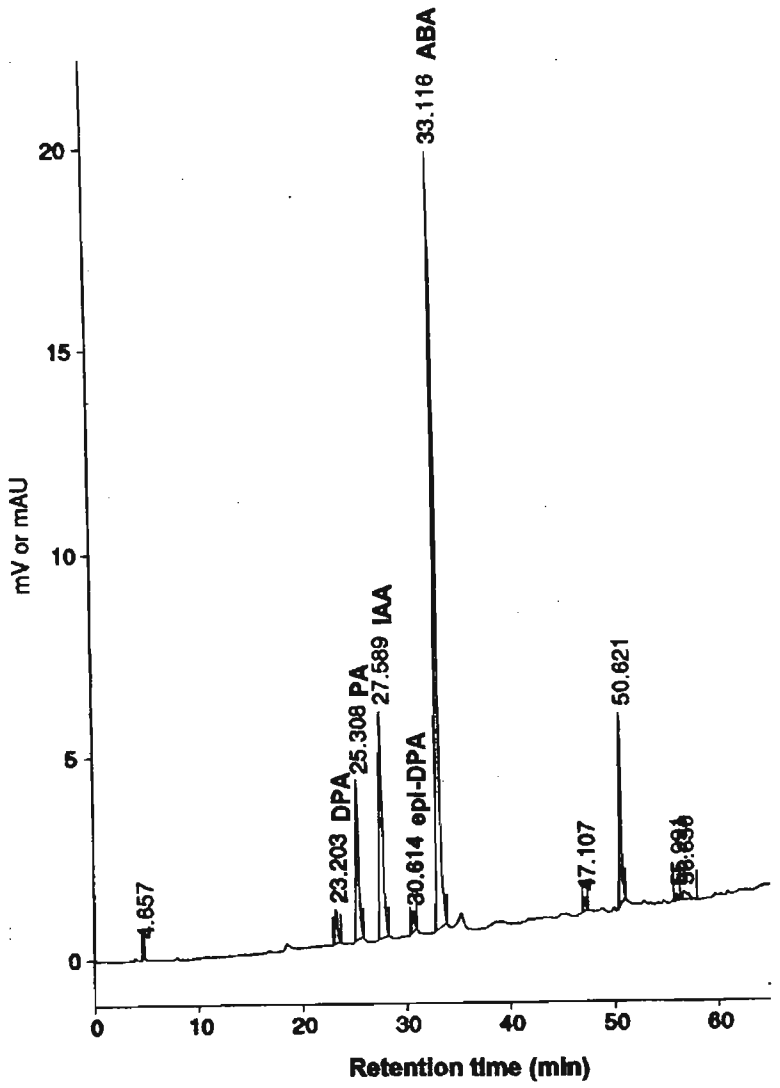

Figure (ii) **Chromatogram of standard solution of DPA, PA, IAA, epi-DPA and ABA. The standards were separated with a linear gradient of 20 % - 100 % methanol, in 0.005 % acetic acid (System A) over 55 minutes at 260 nm on a 5 μm C_{18} column (250 x 10 mm i.d., ODS1) at a flow rate of 2 mL min $^{-1}$.**

4 ABAMe and IAAMe STANDARDS

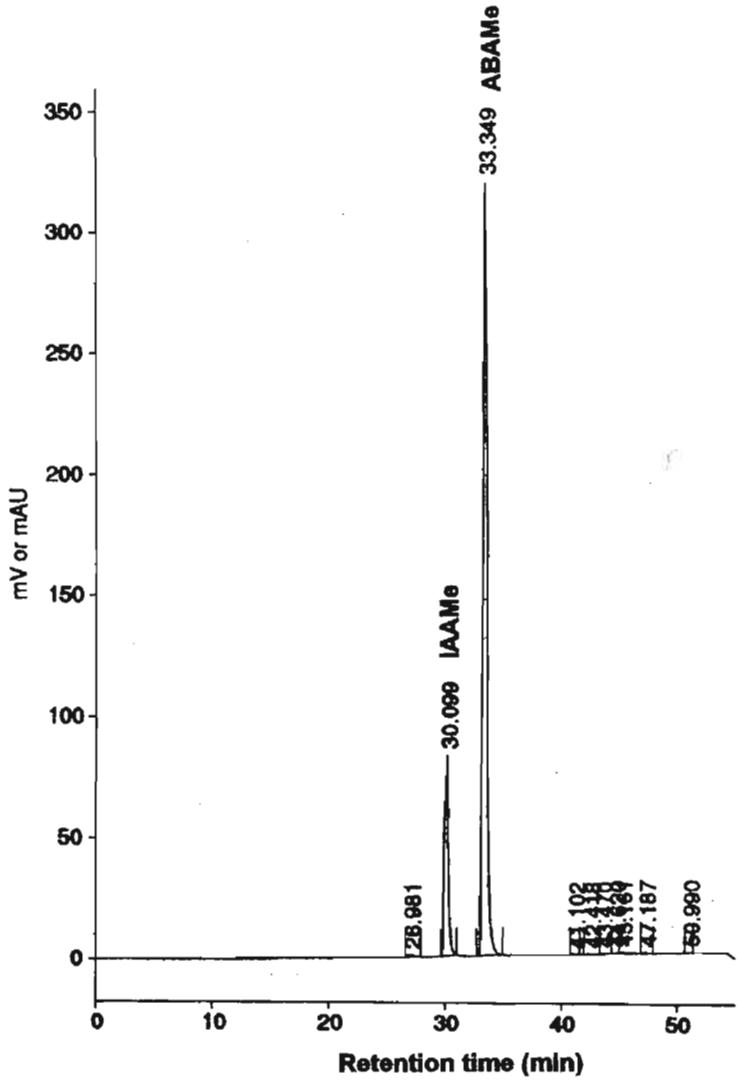

Figure (iii) **Chromatogram of standard solution of IAA-methyl ester (IAAMe) and ABA-methyl ester (ABAMe). The standards were separated with a linear gradient of 20 % - 100 % methanol in water (System B) over 55 minutes at 260 nm on a 5 μm C_{18} column (250 x 10 mm i.d., ODS1) at a flow rate of 2 mL min^{-1}.**

APPENDIX II

CYTOKININ STANDARDS (paper chromatography)

Several CK standards (iP, iPA, Z, ZR and DHZ) were prepared (concentration 0.5 mg L^{-1} or 0.5 µg CK per chromatogram) and loaded onto Whatman® No. 1 chromatography paper. The chromatograms were developed once to a height of 40 cm using descending paper chromatography in the solvent system described in section 2.9.1. The R_f fractions from these chromatograms were used as indicators of the CK standards in the soybean callus bioassay, to facilitate comparisons on the basis of co-chromatography (Figure (iv)).

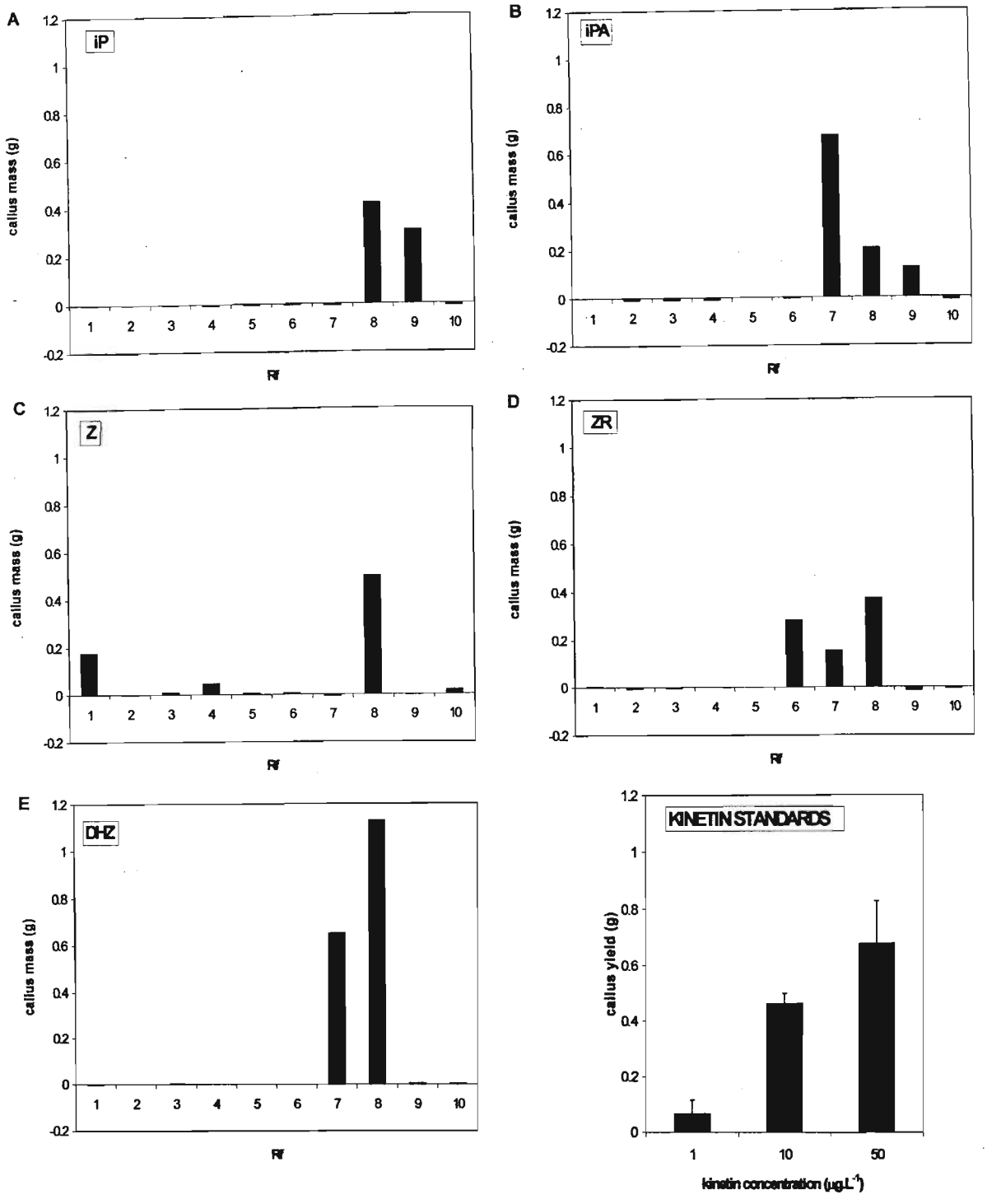

Figure (iv) **Soybean callus bioassays CK standards separated using paper chromatography. (A) isopentenyladenine; (B) isopentenyladenosine; (C) zeatin; (D) zeatin riboside; (E) dihydrozeatin.**

APPENDIX III: PAPERS PUBLISHED AND PRESENTED

PEER REVIEWED JOURNALS

T.B. Campbell; E.W. Richings; R.F. Cripps; N.J. Taylor & A.K. Cowan. 2000. The plant SnRK1 complex: sugar sensing and cell metabolism in avocado fruit growth. S. Afr. J. Bot. 66(2) 104-111.

A.K. Cowan; R.F. Cripps; E.W. Richings & N.J. Taylor. 2001. Fruit size: towards an understanding of the metabolic control of fruit growth using avocado as a model system. Physiol. Plant. 111(2) 127-136.

N.J. Taylor & A.K. Cowan. 2001. Plant hormone homeostasis and the control of avocado fruit size. Plant Growth Regulation 35(3): 247-255.

OTHER POPULAR JOURNALS

R.F. Cripps; E.W. Richings; N.J. Taylor & A.K. Cowan. 1999. The 'Hass' small fruit syndrome: solving a 50 million rand per season problem. South African Avocado Growers' Association Yearbook 1-6.

N.J. Taylor & A.K. Cowan. 2000. The role of cytokinin, auxin and abscisic acid metabolism in the control of 'Hass' avocado fruit size. South African Avocado Growers' Association Yearbook 66-69.

PAPERS PRESENTED AT CONFERENCES

N.J Taylor & A.K. Cowan. Purine metabolism in 'Hass' avocado fruit. South African Society of Horticultural Science Conference. Cape Town, South Africa, January 1999.

N.J Taylor & A.K. Cowan. Purine metabolism in small and normal 'Hass' avocado fruit. South African Avocado Growers' Association Research Symposium. Magoebaskloof, South Africa, February 1999.

N.J. Taylor & A.K. Cowan. The role of cytokinin and abscisic acid metabolism in the control of 'Hass' avocado fruit size. 4th World Avocado Congress. Uruapan, Mexico, October 1999.

T. Dennison; N.J. Taylor; R.F. Cripps; E.W. Richings & A.K. Cowan. Factors affecting fruit growth in 'Hass' avocado. 4th World Avocado Congress. Uruapan, Mexico, October 1999.

N.J. Taylor & A.K. Cowan. The relationship between cytokinin and abscisic acid metabolism in the control of fruit growth. 26th Annual Conference of the South African Association of Botanists. Potchefstroom, South Africa, January 2000.

N.J Taylor & A.K. Cowan. Plant hormone homeostasis and the control of avocado fruit size. 2nd Annual Meeting of the Research Centre for Plant Growth and Development. Pietermaritzburg, South Africa, November 2000.

N.J Taylor; A.K. Cowan and J. van Staden. Plant hormone homeostasis and the control of avocado fruit size. 17th International Conference of Plant Growth Substances. Brno, Czech Republic, July 2001.

N.J Taylor; A.K. Cowan; J. van Staden and A.L.P. Cairns. Avocado fruit size and the role of plant hormone homeostasis. 3rd Annual Meeting of the Research Centre for Plant Growth and Development. Pietermaritzburg, South Africa, November 2001.

The plant SnRK1 complex: sugar sensing and cell metabolism in avocado fruit growth

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The sucrose non-fermenting-1-related protein kinase (SnRK1) complex is considered to play a central role in sugar sensing and signalling in growth and development of plants/plant parts. This paper reviews the SnRK1 complex and its role in the regulation of key metabolic pathways contributing to cell growth and development. Based on results from studies aimed at elucidating the metabolic control of 'Hass' avocado fruit growth, using normal and phenotypically small fruit, a central role for SnRK1 activity in the control of final fruit size is proposed. The model developed in this paper links sugar sensing/signalling with plant hormone signalling pathways and suggests that alterations in these processes lead to changes in isoprenoid metabolism, plant hormone homeostasis and diminished cell division cycle activity resulting in the arrest of fruit growth.

Keywords: avocado, *Persea americana*, plant hormones, sugar sensing, sucrose non-fermenting-1-related protein kinase.

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Introduction

For most plants, sucrose is the major transported sugar linking source organs (e.g. mature leaves) with sink organs (e.g. developing leaves, roots, seeds and fruits) and the path of sucrose movement into terminal sinks is illustrated in Figure 1. This scheme, adapted from reviews on phloem unloading in sink tissues by Ho (1988) and Patrick (1997) proposes that during the early stages of development solute flow is symplastic, and imported sucrose is stored as starch. Towards the conclusion of (fruit) development, transport becomes apoplastic and the switch from symplastic to apoplastic transport is associated with increased extracellular acid invertase (β-D-fructofuranosidase, EC 3.2.1.26) activity which is accompanied by an accumulation of imported carbohydrate as soluble sugars. Symplastic transport occurs via plasmodesmata and is driven by diffusion along gradients of changing osmotic/solute potential. By comparison, apoplastic transport is an energy-dependent process that relies on activation of a plasma membrane-localized hexose H^+ symporter (Lalonde *et al.* 1999). Clearly biochemical and physiological events that occur within the major phloem unloading region of terminal sinks can change carbohydrate status and thereby exert control over seed and fruit growth.

Control of plant growth and development by changes in carbohydrate content and composition is believed to be the result of induction/repression of sugar-sensitive genes (Jang & Sheen 1994, 1997; Jang *et al.* 1997; Sheen 1994; Smeekens 1998; Smeekens & Rook 1997). Thus, it has been suggested that sugar-responsive genes provide a means of adjusting resource allocation in plants/plant parts and may contribute to adaptive changes in form (Koch 1996). In terms of fruit growth, changes in form might include a reduction in size and the appearance of phenotypically (e.g. 'Hass' avocado) or genotypically (e.g. melon) small fruit (Cowan *et al.* 1997; Higashi *et al.* 1999). To affect long-term changes in metabolism, carbon allocation and plant part form, mechanisms must exist to sense and transduce carbohydrate signals to responsive genes. One contemporary hypothesis favours hexokinase (HXK; EC 2.7.1.1) as the primary sugar sensor in plant cells (Jang & Sheen 1994; Smeekens 1998; Dai *et al.* 1999) wherein flux and phosphorylation of hexoses in the cytoplasm is thought to signal carbohydrate status. However, as pointed out by Halford *et al.* (1999) the idea that HXK is involved in sugar sensing in plants remains equivocal. For example, transgenic tobacco expressing a yeast acid invertase in the cytosol was unable to sense the increased hexose content, whereas expression in the apoplast/vacuole resulted in alterations in gene expression (Heineke *et al.* 1994; Herbers *et al.* 1996). Furthermore, antisense repression of HXK in transgenic potato led to over accumulation of starch without significantly changing carbohydrate metabolism (Veramendi *et al.* 1999). Since these authors found no evidence to suggest that HXK is a key regulatory element in sugar sensing they concluded that sucrose level rather than HXK is central to the control of carbohydrate partitioning. In fact, Halford *et al.* (1999) have argued the importance of sucrose and provided convincing evidence that sucrose affects sugar sensing in plants differently from hexose (glucose).

In this paper we focus attention on the SnRK1 complex in plants and briefly review the role of this complex in sugar sensing. We also examine the potential involvement of SnRK1 in

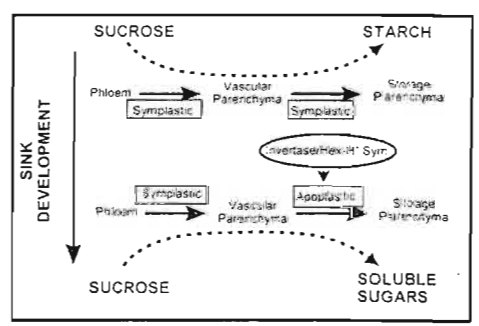

Figure 1 Scheme illustrating the path of sucrose movement into developing fruit and the transition from accumulation of starch to accumulation of soluble sugars.

the regulation of metabolism in cells of developing terminal sinks. In addition, based on recent studies using normal and phenotypically small 'Hass' avocado, a model is developed to describe the potential role of SnRK1 in the metabolic control of fruit growth.

The sucrose non-fermenting 1 (SNF1) family of protein kinases

Protein kinases are believed to play an important role in the regulation of plant development (Thummler *et al.* 1995). Also known as phosphotransferases, these enzymes catalyse the transfer of phosphate groups from one molecule to another and physiologically it is extremely important that they transfer these groups very specifically to the appropriate substrate. Members of the sucrose non-fermenting 1 (SNF1) family form a distinct subgroup of the protein kinase superfamily, based on the sequence of their kinase domains (Halford & Hardie 1998). They appear to have an N-terminal protein kinase domain and a less conserved C-terminal region that may be involved in the regulation of activity or, interaction with other proteins such as the SNF4 protein (Stone & Walker 1995). Additionally, these enzymes form the central components of highly conserved protein kinase cascades that now appear to be present in most, if not all, eukaryotic cells (Hardie *et al.* 1998). SNF1 protein kinases, along with the calmodulin-like domain protein kinases, fall under the group of calcium/calmoduin-independant protein kinases or CaMK group (Stone & Walker 1995). Because the downstream targets of action of these enzymes are many and varied, they have been discovered and rediscovered several times in recent years in different biochemical assays and/or genetic screens. Only when DNA and amino acid sequences became available was it realized that all of the ascribed regulatory functions were carried out by members of the same class of protein kinase. The SNF1 protein kinase family currently comprises SNF1 in the yeast *Saccharomyces cerevisiae*, the AMP-activated protein kinases (AMPK) in mammals and the SnRK1 complex in higher plants. The physiological roles of the SNF1 family are currently better defined in yeast and animals and an enhanced level of understanding of their biochemical function is possible, by a synthesis of these two approaches, in essence pooling knowledge about the animal and yeast systems. The insights obtained also provide guidance in investigating the cellular role of SnRK1 in higher plants, where studies are at a much earlier stage (Hardie *et al.* 1998).

SNF1 protein kinase was originally identified in the yeast *Saccharomyces cerevisiae* as a product of a gene related to the ability of this yeast to ferment sucrose. In yeast, carbon catabolite repression is an important regulatory mechanism that controls the expression of many genes in response to glucose availability (Thevelein 1994). The derepression of glucose-repressible genes requires the function of a complex signal transduction pathway. Genes encoding several members of the pathway have been isolated. One of these genes is SNF1, which encodes a protein serine-threonine kinase and has been shown to be essential for expression of the yeast invertase gene, ~, in response to glucose depletion. Yeast *snf1* mutants are unable to utilize carbon sources (e.g. sucrose, raffinose, galactose, maltose, glycerol and ethanol) that require expression of glucose-repressible genes. Recent molecular and biochemical evidence has shown that SNF1 protein kinase is structurally and functionally related to a mammalian AMP-activated protein kinase (AMPK). AMPK plays a major role in the regulation of lipid metabolism in mammals. When cells are subject to stress ATP levels decline and AMPK is activated, thus inhibiting several biosynthetic pathways in order to maintain adenylate status. AMPK phosphorylates and inactivates acetyl-CoA carboxylase (EC 6.4.1.2), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC 1.1.1.34) and hormone-sensitive lipase (EC 3.1.1.3, 3.1.1.13). The sequence of AMPK shows 46% homology to SNF1 which has also been shown to regulate acetyl-CoA carboxylase *in vivo*. Therefore, in yeast and mammalian cells, SNF1/AMPK seems to regulate fundamental metabolic pathways in response to nutritional and environmental stresses that deplete ATP (Hardie *et al.* 1998).

The plant SnRK1 sub-family

SNF1-related sequences have been isolated from several plant species (for review see, Halford & Hardie 1998). The first plant SNF1-related gene to be characterized was RKIN1, isolated from a rye endosperm cDNA library (Alderson *et al.* 1991). Transformation of an *snf1* mutant strain of yeast with a low copy RKIN1 plasmid restored the ability of this organism to grow on non-fermentable carbon sources, showing that RKIN1 is functionally as well as structurally related to SNF1. SNF1-homologues have subsequently been isolated from *Arabidopsis thaliana* (AKIN 10), barley (BKIN2, BKIN12), sugar beet (SBKIN154), tobacco (NPK5), spinach (HRK) and potato (PKIN1).

In the early 1990s a homologue of AMP-activated protein kinases was isolated from plant tissue extracts and shown to inactivate mammalian HMGR and rat acetyl-CoA carboxylase (MacKintosh *et al.* 1992). However, when this kinase activity was challenged with target proteins purified from plant sources, only HMGR was inactivated, suggesting that the enzyme was related to HMGRkinase (EC 2.7.1.109). Further evidence for the existence of plant HMGRkinase activity, with similar properties to AMP kinase, included the purification and characterization of HMG-CoA reductase kinase-A (HRK-A) from cauliflower inflorescences (Ball *et al.* 1994; Ball *et al.* 1995) and the isolation of a protein kinase from barley endosperm capable of phosphorylating *Arabidopsis* HMGR (Dale *et al.* 1995; Hannappel *et al.* 1995; Barker *et al.* 1996). The success of these endeavours was made possible by the development of the SAMS peptide, a synthetic peptide based on the sequence required for AMP-catalysed phosphorylation of rat acetyl-CoA carboxylase, which is suitable for use in the analysis of kinase activity in plant extracts (Halford & Hardie 1998). Kinase activities that phosphorylate the SAMS peptide have since been detected in extracts of several monocotyledonous and dicotyledonous species. Although HRK-A from cauliflower was not activated by AMP, in many other respects its biochemical properties were very similar to AMPK and SNF1 protein kinase, suggesting HRK-A to be a higher plant homologue of AMPK. Although this plant kinase was not purified to homogeneity, the catalytic subunit was identified, using [^{14}C]FSBA labelling, as a polypeptide of 58 kDa, the predicted mass for higher plant SNF1 homologues that cross-react with this polypeptide. These results suggested that the cauliflower HRK-A was encoded by a homologue of rye RKIN1 and yeast SNF1 (Halford *et al.* 1994; Hardie *et al.* 1998). Recently, Barker *et al.* (1996), partially purified HMGR kinase from barley endosperm and showed that it was recognized by antiserum raised against RKIN1 protein. Thus, there seems little doubt that the protein kinase purified as HMGR kinase corresponds to the SNF1 gene product of plants. This is particularly so given the recent report of the isolation of four Ca^{2+}-independent kinases from spinach leaves (Sugden *et al.* 1999). The major activities (HMGR kinase-A and kinase-C; HRK-A and HRK-C) were extensively purified and shown to be members of the plant SnRK1 family of protein kinases. HMGR is a key enzyme in isoprenoid biosynthesis and the regulation of isoprenoid synthesis is important in the control of plant growth (Dickinson *et al.* 1999) and fruit development (Narita & Gruissem 1989; Gillaspy *et al.* 1993; Cowan *et al.* 1997; Jelesko *et al.* 1999). Even so, it is very

likely that, as in yeast and animal systems, plant SNF1 homologues have other important roles in the regulation of sink cell metabolism. Therefore, it is not surprising that in addition to HMGR kinase activity, plant SnRK1 homologues rapidly phosphorylate and inactivate NADH-dependent nitrate reductase (NR, EC 1.6.6.1) and sucrose phosphate synthase (SPS, EC 2.4.1.14) *in vitro*.

Both NR and SPS are important cytosolic enzymes in plants and catalyse fundamental steps in nitrate assimilation and carbohydrate metabolism respectively. NR is phosphorylated by SnRK1 (Sugden *et al.* 1999) and inactivated following the binding of a 14-3-3 protein to the phosphorylation site (Bachmann *et al.* 1996; Moorhead *et al.* 1996). Another important enzyme involved in primary metabolic processes in plants is sucrose synthase (SSyn, EC 2.4.1.13). SSyn catalyses the reversible conversion of sucrose and UDP to UDP-glucose and fructose, and represents an important sucrose cleavage enzyme in sink tissues. Expression of antisense SnRK1 in potato tubers resulted in decreased SSyn gene expression and loss of sucrose-inducible SSyn transcripts (Purcell *et al.* 1998). This observation was the first demonstration of a role for SnRK1 in the regulation of carbohydrate metabolism in higher plants. Even so, the presence of a kinase that co-purifies with spinach SPS had been reported earlier and identified as the activity responsible for phosphorylation and inactivation of SPS (Toroser & Huber 1997). The major SPS-inactivating kinases in spinach leaves and cauliflower florets were recently shown to be strictly Ca^{2+}-independent (Toroser & Huber 1998; Sugden *et al.* 1999) confirming the earlier classification of this kinase activity as SnRK1 (Douglas *et al.* 1997).

To date, the physiological circumstances under which members of the plant SnRK1 subfamily are regulated and the intracellular signals responsible, remain unknown (Halford & Hardie 1998). Like the yeast system, plant kinases are not allosterically activated by AMP. They are inactivated by protein phosphatases, however, and can be reactivated by mammalian AMPKK and by a putative upstream kinase in plant extracts that can be removed from the downstream protein kinase on further purification. Therefore, plant protein kinases are probably regulated in a manner similar to that of their animal and yeast counterparts.

SnRK1 and the regulation of plant cell metabolism

The expression of a number of genes in plants is repressed by high glucose or sucrose in the cell medium. There is evidence for the regulation of gene expression by carbon metabolites in plants (Halford *et al.* 1994) and the recent and continual isolation of SNF1 homologues, invites the intriguing question: Does a carbon catabolite repression and de-repression system, similar to that of yeast, exist in plants? For example, rye RKIN1 and tobacco NPK5 complement *snf1* mutants which are unable to utilize sucrose and other sugars as a carbon source demonstrating that these plant proteins are functionally similar to SNF1 (Stone & Walker 1995). Also, expression of osk3 cDNA from rice seed in yeast *snf1* mutants restored SNF1 function (Takano *et al.* 1998). Further evidence for the role of SNF1 homologues in plant sugar response comes from an experiment where antisense expression of a putative SNF1 homologue in potato resulted in a loss of the sugar inducible expression of SSyn (Purcell *et al.* 1998). SSyn is involved in the degradation of sucrose, the carbon source supplied to sink tissues such as the potato tuber storage organ. Therefore, SSyn occupies a position in plants that is analogous to the position of the classical glucose-repressed enzyme, invertase, in yeast. The importance of SSyn in carbohydrate metabolism in plants is enough in itself to suggest that SnRK1 in plants could be a significant regulatory complex (Hardie *et al.* 1998). As outlined above, the phosphorylation sites on the plant enzymes, HMGR, SPS and NR, conform to the AMPK/SNF1 recognition motif. HMGR catalyses the committed step in cytosolic isoprenoid biosynthesis leading to the formation of mevalonic acid which, following activation by phosphorylation, is incorporated into terpenyl pyrophosphates and later, sterols. Both mevalonic acid and terpenyl pyrophosphates are required for cell proliferation and growth (Jelesko *et al.* 1999; Yalovsky *et al.* 1999). SPS catalyses net sucrose synthesis in plants and is usually high in source tissues but low in sink organs. Nevertheless, recent information suggests that over-expression of SPS in tomato increases sink strength and fruit number (Nguyen-Quoc *et al.* 1999) illustrating the potential importance of this enzyme in fruit development. NR, which also appears to be subject to SnRK1 regulation, is involved in the initial reduction of nitrate to nitrite in the cytoplasm. Nitrite is then taken up by the chloroplast and assimilated into amino acids and other nitrogen containing compounds. Clearly, these apparently SnRK1-regulated plant enzymes are involved in major biosynthetic processes contributing to plant/plant organ growth and development.

Multiple SPS and NR kinases are present in extracts of spinach leaf and at least two of these kinases appear to be members of the SnRK1 complex (Sugden *et al.* 1999). The fact that SnRK1 is involved in the biosynthesis and metabolism of carbohydrates and nitrogenous compounds underlines the potential role that SnRK1 plays in the control of sink strength, carbon partitioning and the interaction between nitrogen and carbon metabolism. Monger *et al.* (1997), measured SnRK1 activity using the SAMS peptide phosphorylation assay and found that the highest activity occurred in young storage roots of sugar beet. In potato, highest SnRK1 activity is in the stolons and developing tubers. Takano *et al.* (1998) found two forms (group 1, osk1; and, group 2, osk2–5) of SnRK1 in rice seed. Group 1 was expressed uniformly in growing tissues whereas group 2 was strongly expressed in immature seeds. Interestingly, expression of group 2 genes (osk2) was transiently increased during early seed maturation suggesting that SnRK1 plays an important role in endosperm development of rice seeds. Taken together, these observations strongly suggest that the SnRK1 complex plays an important role in regulating metabolism in developing sink organs.

As mentioned above, in yeast and mammalian cells, SNF1/AMPK seems to regulate fundamental metabolic pathways in response to nutritional and environmental stresses. Thus, SnRK1 protein kinases are likely to be involved in the response of plant cells to such stimuli and/or stresses. The concept is emerging that the SnRK1 sub-family of protein kinases protects cells against nutritional and/or environmental stresses, particularly those which compromise cellular energy status, by regulating both metabolism and gene expression (Halford & Hardie 1998). It has been proposed that SnRK1 plays a role in stress adaptation in plants by responding to stress-induced alterations in AMP/ATP ratios. This response involves post-translational control, via phosphorylation, of a number of proteins. The affected proteins include key biosynthetic enzymes, thus allowing cellular metabolism to adapt to the imposed stress. This role of SnRK1 raises the possibility that some SnRK1-mediated responses to sugar may result indirectly from stress signaling as opposed to direct signal-response coupling. For instance, some SnRK1-mediated responses to high sugar concentrations could be the result of osmotic shock-induced stress and/or hormonal imbalance rather than a specific response to sugar (Gibson & Graham 1999). Some plant SnRK1 genes have been shown to be transcriptionally regulated by environmental stimuli, for example, PKABA1 transcript levels increase in response to low levels of abscisic acid (ABA) and water stress in wheat. The mRNA levels of a further wheat SNF1 homologue, wpk4, increase upon exposure to light and cytokinin (CK) as well as nutrient deprivation (Stone & Walker 1995). Thus, there is growing evidence to support crosstalk between environmental cues, plant hormone homeostasis and sugar signals (Mason *et al.* 1992; DeWald *et al.* 1994; Dijkwel *et al.* 1997; Mita *et al.* 1997; Perata *et al.* 1997; Wingler *et al.* 1998; Xu *et al.* 1998; Zhou *et al.* 1998). Furthermore, plant SNF1 kinase (or SnRK1) is apparently regulated by the *pleiotropic regulatory locus 1* (PRL1), an evolutionary conserved α-importin-binding nuclear WD-protein (Salchert *et al.* 1998). These authors state that the *prl1* mutation results in hypersensitivity to glucose and sucrose, it augments the sensitivity of plants to CK, ethylene, indole-3-acetic acid (IAA) and ABA, and derepresses genes that are positively or negatively regulated by glucose, or CK, or both. Interestingly, the *prl1* mutation is exacerbated by *amp1* (CK overproducing), a mutation which regulates CK production and cell division (Deikman 1997).

As the PRL1 protein appears to interact with SnRK1, it has been postulated to function in plant sugar-regulated gene expression by acting as a negative regulator of SNF1 homologues (Gibson & Graham 1999). One such example suggests that PRL1 is a potential subunit *of Arabidopsis* SnRK1 homologues that bind to conserved C-terminal sequences of these proteins (Bhalerao *et al.* 1999). Another example exploited to identify a number of protein factors that interact with yeast SNF1 and used to identify components of the signaling pathway associated with the barley endosperm SnRK1, includes BKIN12. Several proteins which interact with BKIN12 have been identified and two of these encode putative transcription factors, providing evidence of a role for plant SnRK1 in transcriptional regulation (Halford & Hardie 1998).

It is becoming increasingly apparent that protein kinases of the SnRK1 sub-family are ubiquitous and that they have an important role to play in plant metabolism and growth via the regulation of several fundamental metabolic pathways. This makes them extremely interesting to study, however, there is still a great deal of research that needs to be conducted to determine their exact targets and functions in plants. An important gap in our knowledge at present is the identification of the signal which activates SnRK1. However, it can generally be said that at present it appears that the overall function of the SnRK1 cascades in plants is to control metabolism, gene expression and perhaps cell proliferation in response to the varying energy states of the cell and the external signals stresses it receives. It appears that SnRK1 achieves this regulatory role by directly phosphorylating proteins in the target pathways and indirectly by regulating gene expression.

SnRK1 and Avocado fruit growth: An hypothesis

Plant hormone homeostasis and control of fruit size

'Hass' avocado presents an ideal system with which to study the metabolic control of fruit growth and hence elucidate biochemical processes contributing to final fruit size. This cultivar routinely produces both normal and phenotypically small fruit. The latter is characterized by arrested development and early senescence and or death (apoptosis) of the seed coat. We have shown that growth of the 'Hass' small-fruit variant is limited by cell number and that in these fruit, microsomal HMGR activity is reduced whereas ABA content is increased (Cowan *et al.* 1997). Further biochemical characterization of the 'Hass' small-fruit phenotype indicated reduced SSyn activity, increased insoluble acid invertase activity, decreased sucrose content and an increase in glucose as a proportion of the total soluble sugar fraction. Interestingly, ABA, glucose and mevastatin (a competitive inhibitor of HMGR) treatment caused similar biochemical changes, suggesting that sugar and ABA signals act in concert to modulate expression and or activity of HMGR and so affect cell division and final fruit size (Richings *et al.* 2000). Other studies demonstrated that injection of fruit with ABA in the linear phase of growth retarded development and caused the appearance of symptoms typical of the small-fruit variant (Moore-Gordon *et al.* 1998). In this study, the reduction in final fruit size was attributed to ABA-induced inhibition of symplastic solute transport (plasmodesmatal structure/function and cell-to-cell communication) further supporting a relationship between elevated ABA and alterations in carbohydrate metabolism. In all experiments, the deleterious effects of ABA and mevastatin were negated when either compound was co-injected with the CK, isopentenyladenine (iP). Since a previous study had shown CK inhibition of ABA biosynthesis in avocado (Cowan & Railton 1987), a biochemical interaction between ABA and CK in the metabolic control of fruit growth was proposed. Subsequent studies enabled us to describe details of the proposed biochemical basis for CK/ABA antagonism in avocado fruit (Cowan *et al.* 1999). Thus, CK promoted the oxidative catabolism of ABA in a process considered to be associated with CK-induced CK oxidase (CKOX) activity. The resulting rise in levels of adenine decreased activity of the molybdenum-requiring enzyme, xanthine dehydrogenase (XDH; EC 1.2.1.37) thereby increasing the availability of molybdenum (as the molybdenum-cofactor: MoCo) for sulphurylation and activation of the aldehyde oxidase (AO: EC 1.2.3.1) which converts xanthoxal to ABA. Confirmation of this interaction was obtained using tungstate (an inhibitor of AO activity) and allopurinol (an inhibitor of XDH activity) which caused inhibition of xanthoxal oxidation and accumulation of ABA and its acidic catabolites respectively (Cowan *et al.* 1999). The overall scheme is illustrated in Figure 2. This scheme depicts the three major proteins that incorporate molybdenum as the MoCo in relation to CKOX. The scheme proposes that elevated CKOX activity increases the adenine content of tissue leading to inhibition of XDH. As a consequence there is a build up of purines. By feedback, the MoCo then becomes available for incorporation into the AO for ABA (and IAA) biosynthesis. The conversion of indole-3-acetaldehyde to IAA is also dependent on a MoCo-containing AO (Sekimoto *et al.* 1998).

While confirmation of the above scheme is still awaited, what is particularly interesting about the proposal is that NR, which is the first enzyme in the MoCo-apoprotein 'pathway' to incorporate MoCo, is regulated by SnRK1 activity. Furthermore, both AO activity and ABA levels are reduced in plants in which NR

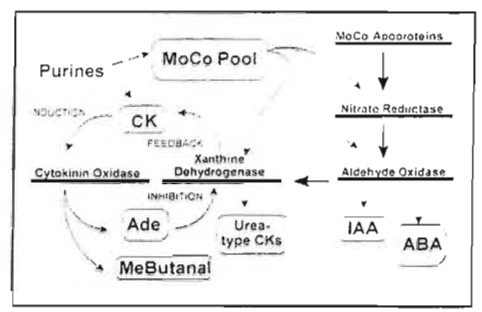

Figure 2 Scheme illustrating the proposed relationship between activation of MoCo-containing enzymes and the biochemical basis for CK antagonism of ABA metabolism in the control of avocado fruit growth. For details, see Cowan *et al.* (1999). Abbreviations: ABA= abscisic acid; Ade = adenine; CK = cytokinin; IAA = indole-3-acetic acid; MoCo = molybdenum-cofactor

has been induced by its substrate (Omarov *et al.* 1998). Implicit in this observation is that control of NR activity by SnRK1 will impact on the regulation of ABA (and IAA) metabolism and hence plant hormone homeostasis, via the differential utilization of MoCo by MoCo-requiring enzymes.

SnRK1 activity, isoprenoid metabolism and fruit growth

In avocado, fruit size is a function of cell number and not cell volume (Cowan *et al.* 1997). Recent observations indicate that a primary, although not exclusive, function of HMGR is to supply mevalonic acid required for cell division and growth (Jelesko *et al.* 1999). Thus, it is not surprising that activity of SnRK1 has also been associated with the cell division cycle (Dickinson *et al.* 1999), particularly as HMGR is an efficient substrate for the SnRK1 sub-family of protein kinases. In view of the results obtained to date from the 'Hass' avocado fruit system, the model illustrated in Figure 3 is used to describe the potential role of SnRK1 activity in the metabolic control of fruit growth. This model is consistent with recent reports that ABA retards cell division cycle activity (Meyers *et al.* 1990; Müller *et al.* 1994) whereas CK promotes this process and does so by regulating the G_2 to M transition, i.e. stimulating tyrosine dephosphorylation and activation of $p34^{cdc2}$-like H1 histone kinase (Zhang *et al.* 1996). Similarly, withdrawal of CK causes cessation of the cell cycle and cells accumulate in M, S and G_1 (Mander & Hanke 1996). An imbalance in the CK:ABA ratio, through reduced CK levels or increased ABA, might, therefore, be expected to impact on avocado fruit cell division cycle activity and sink strength.

Assuming CK is derived *in situ* by isoprenylation of purine, inhibition of isopentenyl diphosphate (IDP) synthesis might limit the amount of dimethylallyl pyrophosphate available for CK biosynthesis. IDP is formed from mevalonic acid, the product of the reaction catalysed by HMGR, and inhibition of HMGR by mevastatin is reversed by both mevalonic acid and CK (Bach 1987; Crowell & Salaz 1992; Cowan *et al.* 1997). HMGR is subject to regulation by phytochrome, reaction end product feedback and post-translational modification (Bach 1987). The latter process is a well documented regulatory system in mammalian cells where enzyme activity is inactivated by a reversible phosphorylation mechanism involving an AMP- or ADP-stimulated kinase (Chappell 1995). As outlined above, there is now substantial biochemical evidence for the existence of plant HMGR kinase activity, with similar properties to AMP kinases. Inhibition of HMGR activity is known to impact on mammalian cell division cycle activity and similar findings have been obtained for higher plants using cultured tobacco and tomato cells (Crowell & Salaz 1992; Jelesko *et al.* 1999). In one instance, inhibition of HMGR activity and cell growth was attributed to reduced CK biosynthesis. Recent information suggests that in addition to CK, pyrophosphorylated intermediates in isoprenoid synthesis are equally important. Thus, isoprenylation of Rab and GTP-binding proteins has been shown (Morehead *et al.* 1995; Biermann *et al.* 1996; Yalovsky *et al.* 1996) and farnesyl protein transferase (FPTase), biochemically characterized in tomato (Schmitt *et al.* 1996) and pea (Qian *et al.* 1996). More importantly, however, inhibition of FPTase by manumycin completely blocked mitosis when added at the S stage but not when added at G_2 (Qian *et al.* 1996). This observation suggests that FPTase is required for cell division cycle activity and that it modulates progression of the cycle through S and in the transition from G_1 to S. A similar role for FPTase in avocado fruit ontogeny seems likely in view of our recent observation that farnesyl diphosphate, co-injected with mevastatin, was able to negate mevastatin-induced retardation of 'Hass' fruit growth (Richings *et al.* 2000). Moreover, this finding supports a relationship between HMGR activity, CK biosynthesis and protein farnesylation in the metabolic control of avocado fruit growth. Furthermore, it has been demonstrated that mutations that confer enhanced response to ABA (*era1* mutants) arise due to perturbed farnesylation of a protein(s) that negatively regulates ABA signaling (Cutler *et al.* 1996). Thus, the appearance of an ABA supersensitive *Arabidopsis* phenotype. Whether a similar perturbation is responsible for the appearance of phenotypically small 'Hass' fruit is currently unknown. Nevertheless, the accumulated information strongly suggests that the aforesaid molecular responses could be manifestations of altered HMGR and SnRK1 activity mediated by carbohydrate and adenylate status.

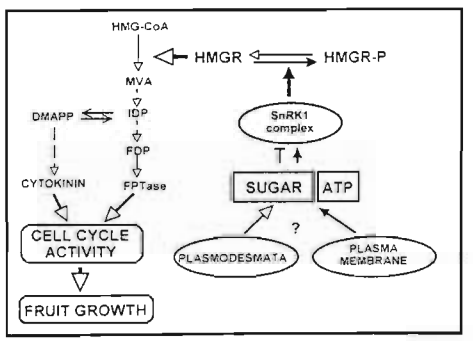

Figure 3 Relationship between sugar sensing and signalling by SnRK1, activity of HMGR and isoprenoid metabolism, and cell division in 'Hass' avocado fruit. Alterations in the supply of sucrose via post-phloem solute transport pathway switching from symplastic to apoplastic, and changes in adenylate status activate the SnRK1 complex. SnRK1 phosphorylates and inactivates HMGR (and SSyn and NR). The resulting depletion of mevalonic acid and isoprenyl pyrophosphates limits substrate for farnesyl protein transferase and cell cycle activity to slow or arrest fruit growth. Abbreviations: DMAPP = dimethylallyl pyrophosphate; FDP = farnesyl diphosphate; FPTase = farnesyl protein transferase; HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A; IDP = isopentenyl diphosphate; MVA = mevalonic acid.

Conclusion

The above review is concerned with the potential regulatory role of SnRK1 in fruit growth and development, with the emphasis on avocado. It is not our intention to dismiss HXK as an additional important regulator in this process. In fact, HXK activities have been detected in extracts of mature avocado mesocarp and the bulk of activity showed a strong preference for glucose as substrate (Copeland & Tanner 1988). SnRK1 activity has also been detected in extracts of avocado mesocarp tissue (MacKintosh *et al.* 1992). Interestingly, *manno*-heptulose, which is a competitive inhibitor of HXK (Pego *et al.* 1999) is present at high concentrations in avocado mesocarp (Ogata *et al.* 1972; Richtmyer 1970; Shaw *et al.* 1980) which might suggest that in this tissue there exists a 'division of labour' between activity of, and sugar signalling by, HXK and SnRK1 during fruit growth. Based on the information presented in this review, it is possible that SnRK1 is sensitive to sucrose availability during the early stages of fruit growth particularly during the phase of cell division and expansion when HMGR and SSyn, which are regulated by SnRK1, are active. During the later stage of this developmental programme, when sucrose import is apoplastic, HXK may (via an active hexose/H^+-symporter) assume the role as the major sugar regulatory element. There is increasing evidence for the existence of multiple sugar-signalling transduction pathways in plants and that these crosstalk with plant hormone signalling pathways (Moore & Sheen 1999). Thus, in association with solute transport pathway switching from symplastic to apoplastic, which has been demonstrated to occur in tomato (Patrick & Offler 1996; Ruan & Patrick 1995), the carbohydrate content and composition in the phloem-unloading region of developing sinks is able to exert control over seed and fruit growth.

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Minireview

Fruit size: Towards an understanding of the metabolic control of fruit growth using avocado as a model system

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Final fruit size is the consequence of complex metabolic events that occur between fruit set and maturation. Disruption of these biochemical and molecular processes at any stage during fruit growth will impact on final fruit size. Because fruit size is a function of cell number rather than cell size, factors affecting cell division cycle activity assume importance. In this paper, we focus attention on the metabolic control of fruit growth using avocado as a model system. Three areas of current interest are highlighted, viz. the contribution by isoprenoid metabolism in the control of cell proliferation, the role played by carbohydrate content and composition in signalling changes in metabolite status and gene expression and maintenance of plant hormone homeostasis. Central to the process of fruit growth and control of final fruit size by cell division is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and activity of the sucrose non-fermenting 1-related protein kinase (SnRK1) complex. It is argued that sugar content and composition of sink cells impact on SnRK1 (and hexokinase) to modulate expression of sugar-metabolizing enzymes, HMGR and molybdenum cofactor (MoCo)-containing enzymes. These changes, in turn, impact on hormone metabolism by affecting allocation of the purine-derived MoCo to aldehyde oxidase and thus the endogenous concentration of indole-3-acetic acid, abscisic acid and cytokinin (CK) to alter plant hormone homeostasis. These aspects are integrated into a model to explain the metabolic control of avocado fruit growth and final fruit size.

Introduction

The intrinsic size of plant organs is determined by internal factors that affect cell cycle activity and cell proliferation. Although the nature of the developmental regulators involved remains obscure, a recent study suggests that regulatory genes like the *Arabidopsis AINTEGUMENATA* gene control cell proliferation and organ growth by maintaining meristematic competence of cells during organogenesis (Mizukami and Fischer 2000). Thus, control of organ size seems to reside either wholly or in part at the level of gene expression, and disparity in size in a given species must consequently be the result of differences in cell number. Control of final fruit size is also dependent on maximization of cell division (Bohner and Bangerth 1988, Higashi et al. 1999).

Developing fruits are terminal sinks and require carbohydrate, other metabolites, mineral nutrients and adequate water to sustain growth. In addition, development of these organs is known to be affected by plant hormones, which act either directly or indirectly to alter gene expression. Appreciation of the pleiotropic effects of plant hormones, however, suggests that no single growth regulator can account for a complex process such as fruit morphogenesis. This is because plant hormones exert multiple control on organ development by alterations in concentration and as a result of changes in sensitivity of the affected tissues (Bradford and Trewavas 1994). Therefore, development must be considered the result of intricate spatial and temporal interactions between the resources required for growth and hormonal mediation through the regulation of gene expression. Even so, the fruit developmental programme remains obscure. As stated by Gillaspy et al. (1993): 'Despite centuries of intensive genetic selection of agriculturally valuable fruit, we still lack most information about how fruits develop, how this development is coordinated with embryonic development and seed formation, and the molecular, cellular, and physiological events that control fruit growth and differentiation.'

In this review, we focus attention on studies initiated to elucidate the metabolic control of fruit growth using avocado as a model system in an effort to explain the appearance of the 'Hass' small-fruit phenotype. Since there is no pattern with respect to the distribution of the 'Hass' small-fruit phenotype on the tree and because growth of this phenotype is limited by cell number and not cell size (Cowan et al. 1997), our studies have concentrated on metabolic events that we believe are closely linked to fruit cell division cycle activity. These are: plant hormone homeostasis; carbohydrate content and composition; and isoprenoid metabolism. Although other factors such as water status and mineral nutrition of the developing fruit are important, a detailed discussion of these aspects is beyond the scope of the present article and the interested reader is referred to Whiley and Schaffer (1994). Based on the results of our studies and the literature, we propose an integrated scheme to explain the metabolic control of avocado fruit growth and suggest that similar biochemical events contribute to the regulation of fruit size in planta.

Avocado fruit development

Fruit growth in most fruits can generally be divided into 3 phases. The first phase (phase I) includes ovary development, fertilization and fruit set; the second (phase II), continued cell division, seed formation and early embryo development; and the third (phase III), cell expansion and embryo maturation (Gillaspy et al. 1993). Avocado fruit development, based on fruit dimensions or mass, follows a single sigmoid curve in which the lag phase persists for approximately 10 weeks after full bloom (Valmayor 1967). The exponential or growth phase lasts for about 30 weeks after full bloom, depending on cultivar and environment, and is followed by a mature phase during which growth slows.

Nothing is known about the molecular signals that control ovary development in avocado. However, light quality and quantity, temperature and plant water and nutrient status seem to be important abiotic factors in determining floral function in avocado (Whiley and Schaffer 1994). Fruit set follows successful completion of pollination and fertilization. Anatomical studies have revealed that within 3 days of pollination, initiation of endosperm formation has occurred, and by 9 days, the endosperm has formed a large cellular body and a pre-embryo of 2–6 cells is evident (Tomer and Gazit 1979). Maintenance of cell division in these structures is required to aid in the establishment of sink strength and for continued growth. Furthermore, Tomer and Gazit (1979) state that formation of the pre-embryo has as one of its functions the supply of chemical triggers for extension expansion growth of the developing fruit. However, the identity of these endogenous chemicals in avocado remains unknown.

In most fruits there exist two general centres of growth, the ovule and pericarp. Growth of the pericarp usually accounts for the early increase in size. Later enlargement is generally associated with seed development. Unlike many fruits in which cell division is confined to the pre-pollination stage (or to a short period following pollination), cell proliferation in avocado continues throughout fruit development, particularly in the mesocarp (Schroeder 1953), albeit at a slower rate as fruits approach maturity. Cell number is a function of the number of mitotic divisions and is thought to be regulated, at least in part, by cytokinin (CK). Mechanisms by which CK promotes cell division are not fully understood, although CK might regulate processes either in G_2 or in the transition from G_2 to mitosis (M) in the cell cycle (Jacobs 1995). In the absence of CK, cells may accumulate in the G_2 to M transition for a period of time and then leave the cell cycle entirely. As pointed out by Ferreira et al. (1994), the G_2 to M transition is crucial for entry into M and if certain conditions are not met differentiation ensues. One of the conditions seems to be the supply of growth-promoting substances.

Avocado seed plays an important role in fruit development (Bower and Cutting 1988). For example, seed-bearing fruits are considerably larger than the seedless fruits ('cukes'), which are frequently found in the cultivars 'Fuerte' and 'Ettinger' (Blumenfeld and Gazit 1974) and which appear to result from early seed degeneration (stenospermocarpy) rather than stimulative or true vegetative parthenocarpy (Tomer et al. 1980). The prominent seed coat in normal fruit is interpreted as a pachychalaza, and its non-development in seedless fruits is suggested to inhibit meristematic activity in the chalazal region and weaken sink strength (Steyn et al. 1993). Thus, a close correlation between seed size and final fruit size is implied. In general, high indole-3-acetic acid (IAA) and gibberellin (GA) levels are associated with active seed growth by cell expansion and fruit growth (Khan 1982). These hormones are at a maximum in mid-embryo growth when CK content is rapidly declining and there is little or no abscisic acid (ABA). Even so, CK is apparently required for most of the fruit developmental programme (Gillaspy et al. 1993), and mitotic activity of meristematic cells requires the presence of both CK and IAA (Ferreira et al. 1994). Whether CK is produced in situ or imported during fruit development is currently unresolved. GA-like activity has been detected in the endosperm and the seed coat of developing fruits (Blumenfeld and Gazit 1972). Since no measurable GA activity was associated with either the mesocarp or embryo, these authors assumed the seed coat to be the site of GA biosynthesis in avocado. Whether GAs are necessary for avocado fruit growth is still unclear. Although GA content does not apparently correlate with total fruit growth in other species, the influence of GAs might be exerted via IAA, particularly as treatment of fruit with GA stimulates endogenous IAA levels and increases fruit length (Goodwin 1978).

The above discussion reflects the rudimentary knowledge of the role of plant hormones in early avocado fruit growth, and in particular, the dearth of information on the metabolic control of avocado fruit growth. While the balance of evidence indicates that CK exerts its effect on fruit set, whereas GAs affect fruit growth and IAA both fruit set and growth, it is still unknown how these plant hormones interact and contribute to the regulation of fruit size.

'Hass' avocado trees produce two distinct populations of fruit, i.e., normal and small fruit (Zilkah and Klein 1987). The small-fruit phenotype has been defined as a physiological phenomenon occurring along the continuum: abortion of embryo → seed coat senescence → cessation of mesocarp cell division → slowing of growth → small fruit, a process that can be initiated at any stage in the 'Hass' fruit development programme, but which typically characterises horticultural maturity (Cowan et al. 1997). In addition, seed coat and mesocarp tissue of the small-fruit phenotype display aberrant plasmodesmatal structure-function and loss of cell-to-cell chemical communication (Moore-Gordon et al. 1998). At the biochemical level, the small-fruit variant has reduced seed 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and sucrose synthase (SuSy) activity, increased seed insoluble acid invertase (AI), decreased seed sucrose content and elevated levels of ABA and its catabolites in seed and mesocarp tissue (Richings et al. 2000). In the absence of evergreen tree-crop mutants with aberrant fruit growth, the 'Hass' small-fruit phenotype provides an ideal system with which to probe more detailed aspects of the control of final avocado fruit size.

Cell division cycle activity is dependent on HMGR and the supply of isoprenoid compounds. In fact, chemical inhibition of HMGR arrests avocado fruit growth (Richings et al. 2000), indicating that formation of mevalonic acid (MVA) and high concentrations of isoprenoid compounds are a requirement for continued cell division. Similar findings have been reported for other species, callus tissue and cell suspension cultures (Crowell and Salaz 1992, Bach 1995, Hemmerlin and Bach 1998). More recently, Jelesko et al. (1999) demonstrated that a primary function of *HMG1* (one of the 4 tomato genes encoding HMGR activity) was to supply the MVA demand associated with cell division and growth.

HMGR catalyzes the irreversible reduction of 3-hydroxy-3-methylglutaryl coenzyme A to MVA and represents the first committed step in cytosolic isoprenoid biosynthesis. Cowan et al. (1997) observed that microsomal HMGR activity in mesocarp of the small 'Hass' phenotype was 70% lower than in normal fruit. Similarly, this variant has low seed HMGR activity (Richings et al. 2000). In situ treatment of normal fruit in the linear phase of growth with a competitive inhibitor of HMGR reduced overall fruit growth and caused the appearance of symptoms usually associated with the small-fruit phenotype. These effects were negated when either MVA lactone, isopentenyl adenine (iP), farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP) were used as co-treatments (Cowan et al. 1997, Richings et al. 2000). These observations suggest that sufficient HMGR activity and isoprenoid metabolism are crucial for cell division and the development of normal-sized 'Hass' avocado fruit.

Several products of the isoprenoid pathway are potentially involved in the control of cell division, fruit growth and fruit size. These include FPP and GGPP (Gillaspy et al. 1993) and end products such as phytosterols (Narita and Gruissem 1989), CK (Zhang et al. 1996) and ABA (Himmelbach et al. 1998).

Narita and Gruissem (1989) were the first to argue the importance of HMGR activity and expression in the early development of tomato fruit and suggested this was due to a requirement for phytosterol synthesis. Similarly, Rodríguez-Concepión and Gruissem (1999) demonstrated a close correlation between *HMG1* expression and tomato fruit growth, suggesting that a major function of *HMG1* is to ensure adequate phytosterol production for cell division and expansion. However, addition of stigmasterol to avocado fruit during phase I reduced growth and accelerated abscission (Cowan et al. 1997). In phases II and III, however, stigmasterol was observed to negate the growth-retarding affect of HMGR inhibition. Stigmasterol supports plant cell division (Haughan et al. 1987), but inhibits cytosolic HMGR activity (Russell and Davidson 1982). Thus, it is plausible that the excess stigmasterol during the early stage of avocado fruit growth may, via feedback, have inhibited HMGR activity. This assumes that maximal HMGR activity coincides with maximum sterol synthesis during early avocado fruit growth. In support of this assumption, addition of a constitutively expressing hamster *HMGR* gene to tobacco resulted in increased total HMGR activity and increased sterol production (Chappell et al. 1995). Conversely, inhibition of HMGR reduces endogenous sterol content (Bach 1995). Thus, both HMGR activity and phytosterol synthesis appear to be vital for normal fruit growth.

In addition to sterols, pyrophosphorylated intermediates in isoprenoid synthesis are also required for cell division and plant organ growth. For example, heterotrimeric G-proteins and members of the RAS superfamily of proteins need to be farnesylated to function correctly, and inhibition of protein farnesylation prevents the correct localization of RAS proteins to the plasma membrane. The modification of regulatory proteins by the addition of isoprenoids (farnesol and geranylgeraniol) is catalyzed by prenyl:protein transferases. In plants, isoprenylation of Rab and GTP-binding proteins has been demonstrated and farnesyl protein transferase (FTase), biochemically characterised in tomato, pea and *Arabidopsis* (Nambara and McCourt 1999). Plant FTase uses MVA-derived FPP as a substrate, and inhibition of either HMGR or prenyl transferase activity retards cell growth (Morehead et al. 1995). Activity of FTase is highest in early tomato fruit growth and declines as maturity is approached (Schmitt et al. 1996). Furthermore, the presence of both geranylgeranyl protein transferase (GGTase) and FTase in extracts of tomato tissue led to the suggestion that these enzymes are integrated with sterol biosynthesis in the control of plant cell and organ growth (Schmitt et al. 1996). The ability of FPP and GGPP to override inhibition of avocado fruit growth would seem to support, at least in part, a role for FPTase and GGTase activity in plant organ growth (Richings et al. 2000). Moreover, these observations confirm a relationship between HMGR activity and a requirement for isoprenyl pyrophosphates in the metabolic control of avocado fruit growth.

Given the complexity of the process of plant organ development, it is perhaps not surprising that FTase activity seems inextricably linked to plant hormone action. Expression of the pea β-subunit gene of FTase appears to be positively regulated by IAA and negatively influenced by sugar and light (Zhou et al. 1997). The statement by Nambara and McCourt (1999) that this indicates that auxin action requires farnesylation is intriguing, as auxin plays a role in plant cell division and inhibition of FTase inhibits cell cycling. Similarly, there is evidence to suggest that ABA action involves farnesylation of a signalling protein. Selection for an inability to germinate in low concentrations of ABA led to the isolation of enhanced response to ABA (*era1*) mutants of *Arabidopsis*, which are defective in the β-subunit of FTase (Cutler et al. 1996). This mutation displays an exaggerated response to ABA, and the *era1* phenotype indicates that a negative regulator of ABA signalling must be farnesylated to function. Thus, a decrease in FTase activity increases or amplifies the ABA signal leading to ABA super-sensitivity. Whether a similar perturbation is responsible for arresting growth of phenotypically small 'Hass' avocado fruit is currently unknown.

Studies on the effect of fruit size on ABA and CK content revealed that mesocarp ABA level was negatively correlated with avocado fruit size, whilst the iP ABA ratio was positive (Moore-Gordon et al. 1998). Furthermore, the characteristic features of the small-fruit variant are phenocopied in fruit treated with ABA, and these effects are negated in the presence of equimolar exogenous iP. This observation is consistent with reports that ABA retards cell division cycle activity (Mambelli and Setter 1998), whereas CK promotes this process (Zhang et al. 1996). Similarly, withdrawal of CK causes cessation of the cell cycle (Mander and Hanke 1996). An imbalance in the CK ABA ratio, through reduced CK synthesis or increased ABA, might, therefore, be expected to impact on cell division activity and sink strength of developing 'Hass' avocado fruit.

As noted above, inhibition of HMGR activity is known to impact on cell division cycle activity in higher plants. This effect has also been attributed to reduced CK biosynthesis (Crowell and Salaz 1992). Assuming CK is derived in situ by isoprenylation of adenosine monophosphate (AMP), inhibition of isopentenyl diphosphate (IDP) synthesis will reduce CK biosynthesis. IDP is formed from MVA, the product of the reaction catalyzed by HMGR. Although HMGR is subject to regulation by reaction end product feedback, post-translational modification has garnered much recent attention. Post-translational modification is a well-documented regulatory system in mammalian cells where enzyme activity is inactivated by a reversible phosphorylation mechanism involving an AMP- or adenosine diphosphate-stimulated kinase (Chappell 1995). Biochemical evidence for the existence of plant HMGR kinase activity, with similar properties to AMP kinases, includes the purification and characterization of HMGR kinases from cauliflower and avocado (MacKintosh et al. 1992). Analysis of the partially purified protein kinase revealed in vitro phosphorylation of HMGR, confirming the activity to be HMGR kinase (Barker et al. 1996). The authors also presented convincing evidence to support the hypothesis that HMGR kinase is a member of the sucrose non-fermenting 1-related protein kinase (SnRK-1) family of protein kinases in plants. These potentially regulate several major biosynthetic pathways, including isoprenoid synthesis, sucrose synthesis and nitrogen metabolism (Sugden et al. 1999) and have been implicated in the control of plant cell cycling (Dickinson et al. 1999). SnRK1 appears to regulate plant cell metabolism in accordance with availability of sucrose coupled with adenylate status, indicating that HMGR is subject to control by sink carbohydrate content and composition. Consequently, a central role for SnRK1 in the metabolic control of avocado fruit growth has been proposed (Campbell et al. 2000)

It is generally accepted that sucrose is the major carbohydrate used in assimilate partitioning and that phloem unloading in terminal sinks, during the early stage of development, is symplastic (Patrick 1997). Towards the end of fruit growth, transport becomes apoplastic and is associated with increased extracellular AI activity. Symplastic solute flow occurs through plasmodesmata along gradients of changing osmotic potential and may be modulated by plant hormones (Morris 1996). By comparison, apoplastic transport requires activation of plasma membrane-localised sugar transporter proteins (Lalonde et al. 1999). Studies on solute transport in 'Hass' avocado and its small-fruit variant indicate that sugar transport pathway switching may be exacerbated by an altered CK ABA ratio and that elevated ABA or reduced CK arrests both symplastic and apoplastic transport (Moore-Gordon et al. 1998). Sugar transport pathway switching has been observed during development of tomato fruit (Patrick 1997). Since the 'Hass' small-fruit variant is essentially a horticulturally mature product, it seems likely that the transition from symplastic to apoplastic transport is also part of the normal course of avocado fruit development.

The avocado seed coat is believed to function as the major conduit of photoassimilate supply to the fruit. In addition to sucrose, glucose and fructose, avocado also translocates and accumulates substantial quantities of the C7 sugar alcohol perseitol and its reduced form D-mannoheptulose (Liu et al. 1999). Although the significance of the C7 sugars in avocado fruit growth remains to be determined, premature seed coat senescence, which characterises the small-fruit phenotype, and mevastatin- and ABA-treated fruit might be expected to induce a state of sugar starvation, which triggers arrest of cell growth, a decline in respiration rate and reduced glycolytic enzyme activity (Yu 1999). These events are typical of mature pre-climacteric fruit, and it is assumed that they occur after expansion and maturation of the cells produced during the preceding period of meristematic growth.

Soluble sugar composition and availability strongly affect cell cycle activity and cell differentiaton. For example, carbohydrate supply is critical for kernel set in maize (Zinselmeier et al. 1995), fruit development in *Trillium* (Lapointe 1998) and fruit size in tomato (Klann et al. 1996). The utilization of sugars during organ development is a function

of sugar-metabolizing enzymes that are encoded by sugar-responsive genes. Current information suggests that regulation of sugar-responsive gene expression involves either a hexose sensor or SnRK1, or both. The hexose sensor (hexokinase, HXK), although ill-defined, seems to comprise phosphorylated fructose and glucose and a putative plasma membrane (plasmodesmatal?) signal and is dependent on both hexose phosphorylation and metabolism (Koch 1996). In contrast, the SnRK1 complex comprises products of several genes (*SNF1*, *SNF4* and either *SIP1*, *SIP2* or *GAL38*) and catalyzes the phosphorylation and inactivation of regulatory enzymes such as HMGR (Halford et al. 1999). It is believed that an increase in hexose content causes repression of sugar-regulated genes. Support for this argument has relied upon photosynthetic gene expression as the markers for glucose repression. However, this hypothesis has recently been challenged. Halford et al. (1999) state that alterations in hexose metabolism impact on adenosine triphosphate (ATP) production and as such could influence the metabolism of many compounds. Furthermore, since sucrose is the principal transport form of sugar in most plants, these authors argue that sucrose acts as the effector, which generates a signal (via SnRK1) that is independent from that induced by glucose. Thus, an active SnRK1 complex is required for sucrose-induced expression of the gene encoding SuSy, which catalyzes the interconversion of sucrose and uridine diphosphate (UDP)-glucose and fructose and plays a role in determining the rate of carbohydrate flow into the fruit and starch and dry matter accumulation.

Although it is difficult to rationalise the relative contribution of HXK and SnRK1 to organ growth and sink strength, it is possible that there exists a 'division of labour' between activity of and sugar signalling by these two components. For example, SnRK1 might assume significance during the early stage of fruit growth (which involves maximum cell proliferation) when HMGR and SuSy are active. During the later stages of fruit growth when sucrose transport is apoplastic, HXK could (via a hexose H -symporter) be the major sugar regulatory element. This idea has been mooted to account for the role of HXK and SnRK1 during avocado fruit growth (Campbell et al. 2000). Both activities have been found in avocado tissue (Copeland and Tanner 1988, MacKintosh et al. 1992). During the course of avocado fruit growth manno-heptulose (a specific inhibitor of HXK) declines (Liu et al. 1999), indicating possible derepression of HXK during the course of fruit growth, which correlates with the proposed temporal switch from symplastic to apoplastic solute transport.

There exist at least 4 possible routes for sieve-element unloading and post-phloem sugar transport in terminal sinks (Herbers and Sonnewald 1998). First, sucrose unloading may be exclusively symplastic (i.e., via plasmodesmata). Elegant studies by Ehlers and Kollmann (1996) indicate that continuous plasmodesmata originate from the fusion of half-plasmodesmata, which persist in the outer walls adjacent to the division wall in cytokinetically active cells. Although specific cells are symplastically isolated following cell division (e.g., during embryogenesis plasmodesmata in the walls separating the developing embryo from the embryo sac cell disappear after first division of the zygote, Schulz and Jensen 1968), a contiguous symplast in the cell division stage of organ development seems essential in the control of organ size to maintain stem cells in an undifferentiated state. Support for this view comes from the maize *KNOTTED1* gene, which encodes a protein (KN1) that alters differentiation within adjacent mesophyll and epidermal cells (Lucas et al. 1995). *KNOTTED1* mRNA is expressed only within the inner cell layer of the meristem (L2), whereas the protein can be detected in all cells (L1 and L2). Microinjection of KN1 revealed that this protein was not only trafficked from cell to cell symplastically, but that it increased the plasmodesmatal size exclusion limit. Since hydrolysis of symplastically imported sucrose by soluble invertase and SuSy generates substrate for metabolism and growth, expression of a *KNOTTED1* homologue in early fruit growth may contribute to sink strength, via maintenance of cells in an undifferentiated state and through increased supply of sugar. Intriguingly, in seed coat and mesocarp tissue of the 'Hass' avocado small-fruit variant and in ABA-treated fruit, the plasmodesmata are occluded by electron-dense material indicative of plasmodesmatal gating, loss of symplastic continuity and a reduction in sink demand (Moore-Gordon et al. 1998).

The remaining 3 pathways of post-phloem transport contain an apoplastic step, which might involve endocytosis-mediated uptake of sucrose (Herbers and Sonnewald 1998), but which is more frequently associated with a cell wall-localised AI. This enzyme hydrolyzes sucrose to its corresponding hexose sugars, which are taken up by an active hexose H -symporter. Interestingly, plasma membrane-localised transporter proteins seem to be sterol-modulated and ABA-sensitive.

Depending on the pathway of sucrose unloading, several enzymatic routes exist for the breakdown of imported sugar. These include invertase (cytosolic and vacuolar) and SuSy and both contribute to sink strength. For example, expression of a constitutive antisense AI gene in tomato fruit resulted in an increased sucrose hexose ratio and a 30% reduction in fruit size (Klann et al. 1996). By comparison, over-expression of AI is associated with morphological changes such as stunted growth (Dickinson et al. 1991) and arrested development of secondary plasmodesmata (Ding et al. 1993). Activity of SuSy has also been correlated with final fruit size and antisense inhibition of tomato fruit SuSy decreases fruit set, sucrose unloading and early fruit development (D'Aoust et al. 1999). Sucrose phosphate synthase (SPS), which is generally more active in source tissue, is also correlated with increased fruit set. SPS catalyzes the synthesis of sucrose from UDP-glucose and fructose-6-phosphate and is responsible for maintenance of the sugar gradient from outside to the inside of sink cells, compartmentalization and sugar accumulation. Over-expression of SPS in transformed tomato plants enhanced activity of SuSy, increased sucrose unloading and stimulated sucrose turnover in the fruit (Nguyen-Quoc et al. 1999). Thus, sucrose-induced sucrose metabolism is clearly linked to increased sink strength.

In avocado fruit, activity of insoluble AI, SPS and SuSy is greater in mesocarp than in seed tissue (Richings et al. 2000). Expression of the small-fruit phenotype, however,

occurs coincident with a dramatic increase in seed insoluble AI and a reduction in activity of SuSy in the mesocarp. These changes are mirrored in normal fruit treated either with an inhibitor of HMGR activity or ABA. The consequence is sucrose depletion and an increase in glucose as a proportion of total soluble sugar. Whether these changes are the result (or cause) of differences in the content and composition of available sugar is currently unknown. Nevertheless, when the endogenous content and composition of soluble sugars is altered, by the pulsed application of either hexose or sucrose, a rapid decline in avocado HMGR activity and altered hormone metabolism are routinely observed (Richings et al. 2000). Since the products of sucrose metabolism, like plant hormones, have an ability to affect gene expression to alter sink metabolism, the sucrose hexose ratio may constitute a homeostat in which the relative amount of each component serves to modulate SnRK1 HXK activity on the one hand and plant hormone metabolism on the other.

The plant hormone concentration of tissues is a balance between synthesis, catabolism, import and export. Phytohormone action, however, is restricted to competent tissues in which hormones function in signalling pathways that involve perception and transduction of external stimuli. In terms of fruit growth, one potential role of plant hormone signalling could be to detect changes in sugar content and composition, and as a consequence, co-ordinate or redirect development. The corollary is that carbohydrate status of the growing organ impacts hormone metabolism to alter flux through hormone metabolic pathways and signal changes in development. Implicit in this argument is cross-talk between sugar and hormone signalling pathways, which is supported by recent observations that sugars and hormones interact in the control of plant growth. For example, sucrose regulates tuber formation in potato by influencing GA levels (Xu et al. 1998) and overrides auxin-induced *vsp* gene expression in soybean (De Wald et al. 1994), and sucrose negatively regulates the signalling pathway in which transcriptional activation of wheat *WPK4* (which encodes a protein kinase capable of phosphorylation of HMGR in vitro) gene is mediated by CK (Ikeda et al. 1999). Hormones may also interact with each other. Such interaction may reside either at the level of hormone ratio, through changes in the effective concentration or tissue sensitivity of one hormone by another, and by the sequential action of different hormones. In avocado mesocarp tissue, the CK ABA ratio seems critical with respect to post-phloem solute transport, growth rate and final fruit size (Cowan et al. 1997, Moore-Gordon et al. 1998). Moreover, ABA metabolism is greater in tissues of the small-fruit phenotype, while ABA-induced symptoms, which are typical of the small-fruit phenotype, are negated when ABA is applied in the presence of equimolar iP. These findings support the contention that hormone balance contributes to control of plant organ growth.

Antagonistic effects between CK and ABA are well established for processes such a leaf senescence, stomatal closure, leaf and fruit abscission and CK-induced release from seed dormancy. A detailed biochemical study of the metabolic interaction between CK and ABA in mesocarp of ripening avocado fruit revealed that CK stimulated the oxidative catabolism of ABA (Cowan et al. 1999). Chemical dissection of the response of the ABA metabolic pathway to CK, allopurinol (an inhibitor of xanthine dehydrogenase, XDH) and tungstate (an inhibitor of aldehyde oxidase, AO) indicated the involvement of a molybdenum cofactor (MoCo)-containing AO. Plants contain at least 3 MoCo-requiring enzymes. These are nitrate reductase (NR), XDH and AO, and activation of the apoproteins is dependent on MoCo biosynthesis. Thus, NR utilises the dioxo form of MoCo, whereas XDH AO requires the sulphurylated (or desulpho) form. Under conditions where nitrate assimilation is reduced and or XDH inhibited, more MoCo might be expected to be available for the AO required for ABA biosynthesis. In support of this supposition, both AO and ABA levels are reduced in plants in which NR has been induced by its substrate (Omarov et al. 1999). Our studies using avocado mesocarp tissue showed that allopurinol and adenine, a product of CK oxidase (CKOX) activity and inhibitor of XDH activity, promoted ABA catabolism. Since CKOX is a substrate-inducible enzyme, we proposed that CK-induced CKOX activity contributes to the regulation of endogenous ABA during plant and organ growth (Cowan et al. 1999).

Plant tissues exhibit high but transient levels of CK during specific periods of development, and the level of CK is believed to be largely under the control CKOX (Jones and Schreider 1997). CKOX is the only enzyme known to catalyze the irreversible breakdown of CK. Thus, while CK promotes cell division cycle activity, it also appears to stimulate its own degradation and, indirectly, the oxidative catabolism of ABA.

Interestingly, the conversion of indole-3-acetaldehyde in IAA biosynthesis also requires a MoCo-AO (Koshiba et al. 1996). Once produced, IAA diffuses into surrounding tissue and eventually away from the developing organ via the pedicel. It is the transport of IAA out of the fruit that correlates with a rapid increase in organ size. Thus, low extractable auxin coincides with rapid growth rate of avocado fruit (Gazit and Blumenfeld 1972). Coupled with CK- and ABA-stimulated photoassimilate unloading in sink tissue (Brenner and Cheikh 1995), the above strongly suggests that phytohormones play a concerted (interactive) role in mediating fruit growth.

ABA levels are high in developing fruits of avocado (Cowan et al. 1997) and decline over the course of fruit growth, presumably due to oxidative catabolism. Similarly, CK-like activity seems to decline during avocado growth (Blumenfeld and Gazit 1970), implying maintenance of the CK ABA ratio throughout fruit growth and development. Auxin has been shown to stimulate the oxidative breakdown of CK by activating CKOX (Palni et al. 1988). Thus, IAA may also mediate the oxidative catabolism of ABA, albeit indirectly, to maintain plant hormone homeostasis during organ growth. However, there is no evidence in the litera-

ture to indicate auxin-mediated ABA metabolism during fruit growth. Likewise, there is no information on the GA content and composition of developing avocado fruit, although indications are that the endosperm is the site of both GA and IAA biosynthesis. Even so, an increase in GA content precedes the rise in auxin content prior to or at fruit set in many species (Goodwin 1978). Thus, it is likely that endosperm-derived GA impacts on IAA metabolism to facilitate fruit set and enhance early fruit growth.

In tomato, early seed growth correlates with an increase in bioactive GAs, but these seem to be necessary for only a short period after fertilization. GA levels are again detectable in immature fruits of 2–3 cm in diameter (Koornneef et al. 1994). This later increase in GA content might suggest that GA accumulation serves to de-repress a signalling pathway that otherwise represses growth. Thus, GA is potentially an inhibitor of inhibitors. Recent implications from work on the *Arabidopsis* GA-insensitive (*gai*) mutant suggest that the *GAI* gene product (GAI) is a nucleus-localised transcription factor that represses elongation growth (Harberd et al. 1998). This protein also shows homology with a protein that regulates the pattern of cell division in *Arabidopsis* roots. In response to accumulated GA and or activation of the GA signalling pathway, activity of GAI is inhibited and growth restored. Thus, the GA response of developing fruits may not be direct, but rather initiated as a result of the opposing action of a GAI-like protein.

Elaboration of the shoot apical meristem (SAM) into a reproductive structure seems to depend on the timing and import of carbohydrates and plant hormones and the expression of homeobox genes (Bernier et al. 1993). In particular, CK and GA have been implicated in the control of floral evocation and morphogenesis, and at least one consequence of elevated CK content of the SAM is a change in gene expression. For example, the *knotted1* (*kn1*) homeobox family of genes is expressed exclusively in the SAM and is involved in its development and maintenance. Transgenic plants over-expressing the bacterial isopentenyl transferase (*ipt*) gene show phenotypes similar to *kn1*, indicative of CK-induced *kn1* expression. Likewise, expression of the *kn1* homologues (*KNAT1* and *STM*) of *Arabidopsis* correlates with CK content implying that CK affects expression of these homeobox genes (D'Agostino and Kieber 1999). Furthermore, CK treatment or *ipt* gene expression causes affected plants to increase the production of active IAA apparently as a result of CK inhibition of IAA conjugation (Coenen and Lomax 1997). As previously pointed out, polar auxin transport is largely responsible for conveyance of this hormone from its site of synthesis in apical tissue to basal target tissue. This movement of IAA may serve to enhance the sensitivity of tissues to other hormones (e.g. CK and GA) to facilitate growth. Such synergism between hormones is well documented, but may depend on a reduction in the endogenous concentration of an inhibitor (e.g., ABA) so that growth rate of the organ is determined and maintained by plant hormone homeostasis. As outlined above, this might be a function of CK (and IAA), which appears to stimulate the oxidative catabolism of ABA.

Development of the embryo is coordinated not only with that of the endosperm, but with the fruit itself, which is formed by the ovary and sometimes other associated tissues. The signals that initiate fruit growth seem to do so by impacting on phytohormone concentration and would appear to emanate from the developing embryo and endosperm. Thus, it is the developing seed that regulates division and expansion of fruit cells, and it is cell number that determines final fruit size. For example, hexose was suggested to promote cell division in *Vicia faba* seeds, whereas sucrose caused a switch to a non-proliferating storage stage (Weber et al. 1996). Thus, a seed coat associated invertase was correlated with embryo cell number, seed storage capacity and seed size. In contrast, the maize *miniature1* mutant is deficient in the extracellular invertase gene (Cheng et al. 1996). In addition, sugar content and composition coupled with isoprenoid biosynthesis seem inextricably linked to the fruit developmental programme. The results of our studies using avocado and those of others using tomato have enabled us to propose an integrated scheme in an attempt to explain the metabolic control of fruit growth (Fig. 1). This scheme illustrates the major physiological

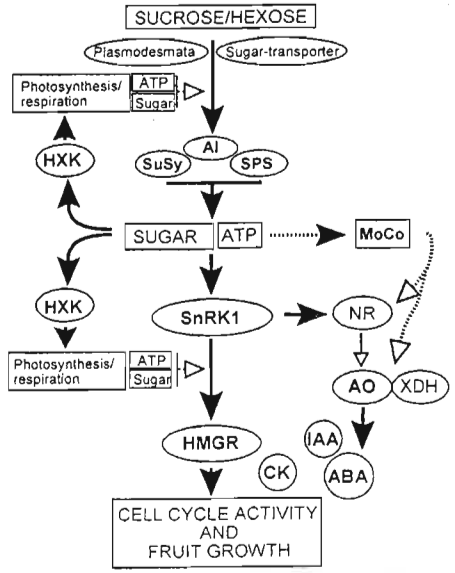

Fig. 1. Hypothetical scheme illustrating the temporal relationship between sugar sensing and signalling by SnRK1 HXK, activity of HMGR and plant hormones in sink cells of developing avocado fruit. Alterations in sugar content and composition coupled with changes in adenylate status impact on activity of HXK, SnRK1 and MoCo biosynthesis and allocation to maintain the supply isoprenoid compounds and optimise the sucrose hexose ratio and plant hormone homeostasis for active cell proliferation, sustained sink strength and fruit growth.

processes in relation to potential regulatory molecules considered to be important in the control of fruit growth and final fruit size. Thus, the sucrose hexose ratio (and AMP ATP ratio) and phytohormone homeostasis are assigned major signalling roles in this developmental programme and are thought to be linked via the activity of sugar-metabolizing enzymes, HMGR and AO.

Based on recent observations, HMGR is clearly central in the metabolic control of fruit growth. Activity of HMGR determines availability of regulatory isoprenoid products (e.g., MVA, prenyl pyrophosphates such as FPP and GGPP, phytosterols and phytohormones) required for cell division cycle activity, sink strength and fruit growth. HMGR is apparently modulated by SnRK1 protein kinase in concert with changes in sugar content and composition. A decrease in the sucrose hexose ratio impacts on activity of either SnRK1 or HXK (possibly via alterations in the AMP ATP ratio) to redirect the sugar-induced signalling cascade. In avocado, exogenous sucrose and glucose effect changes in HMGR activity (Richings et al. 2000). Two other SnRK1 or sugar-regulated enzymes are SPS and NR. This is perhaps not surprising given that carbon and nitrogen metabolism are tightly linked in terms of the requirement for carbon skeletons and competition for energy derived from either photosynthesis or respiration. Both SPS and NR are substrates for kinase activities. Addition of phosphate to SPS reduces enzyme activity, whereas phosphorylation of NR facilitates binding of an inhibitor protein. NR is a MoCo-containing enzyme and the endogenous MoCo pool varies in response to nutritional status. The scheme illustrated in Fig. 1 proposes that, as a result of inactive NR, available MoCo is allocated preferentially to the remaining MoCo-requiring enzymes, XDH and AO. As a consequence, IAA and ABA biosynthesis are stimulated. The increase in IAA in situ contributes to activation of CKOX, and together with elevated ABA and reduced endogenous CK content, these processes combine to reduce cell division cycle activity. The effect is exacerbated by the product of CKOX activity, adenine, which inhibits XDH further increasing allocation of MoCo to the AO for ABA (and IAA) biosynthesis. Sustained sucrose starvation triggers the arrest of cell growth and the onset of characteristics typical of mature (pre-climacteric) fruit. Interestingly, elevated AO activity is expected to increase the production of reactive oxygen species, and oxidative damage is typically evident in seed coats of horticulturally mature avocado fruit, irrespective of final fruit size.

Knowledge of the metabolic control of fruit growth has been enhanced by detailed biochemical analyses of the process and the use of molecular biology. The outcome has clearly been a greater understanding of the complex network of events associated with this important developmental programme. Armed with this information, it is now possible to evaluate factors involved in the regulation of final fruit size. Thus, it has emerged that isoprenoid and carbohydrate metabolism are critical components. The former supplies products that regulate, co-ordinate and direct cell division and development, whereas the latter supplies substrate for metabolism and affects the expression of sugar-regulated genes. Of these, the sugar-metabolizing enzymes AI, SuSy and SPS are clearly important, and activity is largely responsible for determining sink strength and final fruit size. Likewise, control of nitrogen metabolism via phosphorylation and inactivation of NR is integral and apparently linked to phytohormone homeostasis via allocation of the purine-derived MoCo. Activity of carbon- and nitrogen-metabolizing enzymes is linked to either HXK or SnRK1 or both. These putative sugar sensors signal changes in carbohydrate status to redirect metabolism and growth in accordance with prevailing nutritional conditions. Surprisingly, there is very little information on the biochemical and molecular role of plant hormones in the control of fruit growth and final fruit size. Nevertheless, it is assumed that they play an integral part, presumably by altering gene expression, but also through direct hormone-hormone interaction and the maintenance of hormone homeostasis. Cross-talk between sugar and phytohormone signalling is evident, indicating that these two apparently parallel signal transduction pathways interact at the level of gene expression. The cross-talk hypothesis is supported by the identification of sugar-insensitive mutants that display aberrant hormonal responses. However, indications are that hormones predominate over sugars to initiate gene expression. Thus, the idea of a two-component signalling system involving CK and sugars in the control of cell proliferation and plant organ size seems attractive (D'Agostino and Kieber 1999). In such a system, CK might be expected to induce gene expression and activate sugar metabolism, while changes in sugar content and composition modulate CK signalling and endogenous IAA and ABA.

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Plant hormone homeostasis and the control of avocado fruit size

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Abstract

Control of plant hormone homeostasis is crucial for normal organ development in plants. To elucidate the contribution of plant hormone homeostasis to fruit growth, tissue distribution and activity of xanthine dehydrogenase (XDH), abscisic aldehyde (AB-ald)- and indole acetaldehyde (IA-ald) oxidase, and cytokinin oxidase (CKOX) were determined in seed, seed coat and mesocarp of normal 'Hass' avocado and its small-fruit phenotype during the linear phase of growth. Activity of these enzymes was related to the tissue content of indole-3-acetic acid (IAA) and abscisic acid (ABA). IA-ald oxidase was present only in seed tissue whereas AB-ald oxidase and XDH activity was found in seed and mesocarp tissue. Seed of the small 'Hass' fruit had increased XDH and AB-ald oxidase activity and high endogenous ABA, but reduced IA-ald oxidase activity and adenine. There was no difference in seed, seed coat and mesocarp CKOX activity between normal and small fruit. Inhibition of XDH activity in whole fruit by treatment with allopurinol decreased IAA and increased ABA of seed tissue. In mesocarp of ripening fruit allopurinol increased ABA and IAA but had no effect on levels of iP. Results indicate that activity of IA-ald and AB-ald oxidases in avocado fruit contribute to maintenance of the IAA/ABA ratio in seed and mesocarp tissue and that increased AB-ald oxidase, or reduced IA-ald oxidase, may be part of the syndrome associated with the appearance of a small-fruit phenotype.

Abbreviations: ABA – abscisic acid, AB-ald – abscisic aldehyde, AO – aldehyde oxidase, CK – cytokinin, CKOX – cytokinin oxidase, IAA – indole-3-acetic acid, IA-ald – indole acetaldehyde, iP – isopentenyl adenine, MoCo – molybdenum cofactor, XDH – xanthine dehydrogenase

Introduction

Phytohormones are involved in most stages of fruit growth, directing development from fertilization to senescence (Gillaspy et al. 1993; Goodwin 1978). Auxin (IAA), gibberellin (GA), cytokinin (CK), abscisic acid (ABA) and ethylene are produced by fruits in large amounts, and often in a sequence typical for a species. Sometimes they act synergistically in promoting growth, sometimes they substitute for one another and sometimes they act antagonistically. Evidence that plant hormones are involved in the control of fruit growth is demonstrated by the fact that these compounds enhance cell division and promote cell enlargement during different stages of development (Bohner and Bangerth 1988; Mapelli et al. 1978; Mapelli 1981). Furthermore, cell division appears to depend on the maintenance of hormone homeostasis (Barlow 1976). Since final fruit size of avocados is due more to cell division than cell enlargement (Schroeder 1953), any factor that impacts on cell division will affect fruit growth and fruit size. For example, both ABA and water stress are known to retard cell division cycle activity (Artlip et al. 1995; Meyers et al. 1990) whereas CK promotes this event (Jacobs 1995). An imbalance in the CK/ABA ratio,

through reduced CK synthesis or accumulation of ABA, might therefore be expected to impact on cell division cycle activity and final fruit size, e.g. the 'Hass' avocado small-fruit phenotype (Moore-Gordon et al. 1998). Together with CK and ABA, IAA is also known to play a role in cell division (Jacobs 1995) and the highest level of auxin is found in regions of active cell division (Schneider and Wightman 1978). Since IAA is thought to act synergistically with CK in the control of cell division, the decision by a cell to divide might be mitigated by the digital output of analogous phytohormone signals that stimulate and inhibit cell cycle activity (Grill and Himmelbach 1998).

Developing seeds appear to produce the hormones necessary for early fruit growth (Goodwin 1978). This is emphasised by the observation that the developing embryo and seed control the rate of cell division in the surrounding tissue (Gillaspy et al. 1993). Since endogenous hormone concentration of tissues is a balance between synthesis, degradation, transport, and conjugation, determination of plant hormone levels alone is often of limited value. Such data reveals little about the site of hormone metabolism within the tissue under investigation, and very little can be inferred about the contribution of hormone metabolism to changes in net hormone levels. One way to minimize this limitation is to measure the activity of key enzymes involved in phytohormone metabolism, in addition to quantifying endogenous hormone levels. Information about the hormone content and composition of the major tissues of avocado fruit is however rudimentary and most available data is based on bioassay. For example, GA-like activity was observed in extracts of endosperm and seed coat tissue but not in the mesocarp or embryo (Blumenfeld and Gazit 1972). CK activity was high in the embryo, endosperm and seed coat and levels declined over the course of fruit growth (Blumenfeld and Gazit 1970). No CK activity was detected in mesocarp tissue (Gazit and Blumenfeld 1970). Auxin-like activity was higher in seed tissue than in the mesocarp (Gazit and Blumenfeld 1972) while ABA, which declines with fruit growth (Cowan et al. 1997), occurs in similar amounts in seed and mesocarp tissue (Richings et al. 2000). Together, these data suggest that the seed is the primary source of hormones required for avocado fruit growth and development.

Final fruit size of avocado is dependent on sustained cell division in the mesocarp (Cowan et al. 1997) and is negatively affected by increased ABA or reduced CK (Moore-Gordon et al. 1998). Furthermore, since low extractable levels of auxin correlate with rapid growth of the mesocarp (Gazit and Blumenfeld 1972), it is assumed that basipetal movement of auxin is associated with continued fruit growth. In fact, when the growth rate is low, auxin levels are high. Implicit in this assumption is movement of hormones from the site of synthesis (seed) into the surrounding tissues. We therefore hypothesized that activity of key enzymes in hormone metabolism would reflect hormone distribution and content of tissues in developing avocado fruit. This was based on results from a detailed study of the metabolic interaction between CK and ABA in avocado fruit that revealed that CK stimulated the oxidative catabolism of ABA (Cowan et al. 1999). Chemical dissection of the response of ABA metabolism to CK, allopurinol (an inhibitor of xanthine dehydrogenase, XDH; EC 1.1.1.204, formerly EC 1.2.1.37) and tungstate (an inhibitor of aldehyde oxidase, AO; EC 1.2.3.1) indicated the involvement of a molybdenum cofactor (MoCo)-containing AO. These studies also showed that allopurinol and adenine, a product of CK oxidase (CKOX) activity and inhibitor of XDH activity, promoted ABA catabolism. Since CKOX is a substrate-inducible enzyme we suggested that CK-induced CKOX activity contributed to the regulation of endogenous ABA during plant organ growth (Cowan et al. 1999). The requirement for a MoCo-AO in the final step of IAA and ABA biosynthesis (Koshiba et al. 1996; Schwartz et al. 1997; Tsurusaki et al. 1997), coupled with CK-ABA antagonism, suggested that plant hormones play an interactive role in mediating avocado fruit growth. In this paper we extend the argument by reporting on the tissue distribution of XDH, AO and CKOX in relation to hormone levels in normal and phenotypically small 'Hass' avocado fruits.

Materials and methods

Plant material and application of chemicals

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. Fruit was harvested in the early morning and transported to the laboratory where the seed and mesocarp tissue was dissected into liquid nitrogen and freeze-dried immediately. For studies on the

effect of allopurinol and molybdate on ABA and IAA metabolism, pedicels were re-cut under water and fruit supplied, via the transpiration stream, with solutions of allopurinol and molybdate dissolved in 2% DMSO (v/v) and formulated in water. A total volume of 0.5 ml was pulsed into the fruit via the pedicel and the fruit incubated for 24 h at room temperature. Where specified, mature fruit was allowed to ripen in darkness at 25 °C for 8–10 days and excised mesocarp tissue supplied with allopurinol and/or potassium molybdate prepared in 2% DMSO (v/v) and formulated to a final volume of 0.5 ml in Tween 20:acetone:water (1:1:8, v/v/v), which was infiltrated via a series of cuts in the surface of the tissue. A total volume of 0.5 ml was administered in this way and the tissue was incubated for 48 h in a water-saturated environment at 25 °C prior to extraction and analysis.

Enzyme assays

AO and XDH activity were assayed *in vitro* according to the procedure described by Triplett et al. (1982). Freeze-dried tissue was milled to a fine powder and 0.5 g aliquots, together with insoluble polyvinylpolypyrrolidone (Polyclar, 10% w/w), homogenised on ice in 50 m*M* potassium phosphate buffer (pH 7.8) containing 1 m*M* dithiothreitol (DTT) using an Ultra-Turrax top-drive tissue disperser. Extracts were allowed to stand on ice for 20 min prior to centrifugation at 30,000 g for 15 min at 2 °C. The resulting supernatant was brought to 60% saturation with solid ammonium sulfate. After stirring for 30 min the mixture was re-centrifuged at 40,000 g for 20 min at 2 °C. The pellet was resuspended in 2 ml of 50 m*M* potassium phosphate buffer (pH 7.8) and desalted on a 1 × 3 cm Sephadex G-25 (Sigma Chemical Co., St Loius, MO, USA) column equilibrated with 50 m*M* Tris-HCl buffer (pH 7.8). AO activity was determined spectrophotometrically by monitoring the decrease in absorbance of 2,6-dichloroindophenol (DCIP) at 600 nm (Courtright 1967). The reaction mixture consisted of 0.2 ml enzyme extract and 1.8 ml phenazine methosulfate (0.1 m*M*) and either 2 m*M* indole-3-aldehyde (for abscisic aldehyde; AB-ald oxidase activity) or indole-3-acetaldehyde (for IA-ald oxidase activity) in 50 m*M* potassium phosphate buffer (pH 7.4) in a total volume of 2 ml. The reaction was initiated by the addition of 0.002% (w/v) DCIP in 50 m*M* potassium phosphate buffer (pH 7.4) and allowed to proceed for a maximum of 20 min. Specific activity of either AB-ald or IA-ald oxidase is expressed as pmol DCIP reduced mg^{-1} protein min^{-1} (Sagi et al. 1998). For XDH, the reaction mixture included 1 m*M* hypoxanthine and 1 m*M* DTT in 50 m*M* Tris-HCl buffer (pH 6.5). The reaction was initiated by the addition of either 0.002% (w/v) DCIP or 2.5 m*M* NAD^+ in 50 m*M* Tris-HCl buffer (pH 6.5) followed by incubation at 30 °C (Sagi et al. 1998). XDH activity was monitored spectrophotometrically at 340 nm and is expressed as pmol DCIP (NADH) mg^{-1} protein min^{-1}. Where specified, and to determine the effect of allopurinol and adenine on XDH activity *in vitro*, these purines were formulated in 2% DMSO (v/v) and 50 m*M* Tris-HCl (pH 7.8), and added directly to crude enzyme preparations before reaction initiation.

Extracts for measurement of CKOX activity were prepared essentially as described above. Tissue was homogenised on ice in 50 m*M* potassium phosphate buffer (pH 7.4), containing 2 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 0.5 m*M* dithiothreitol, Complete® protease inhibitor tablets (1 tablet/50 mL, Roche Diagnostics GmbH, Mannheim, Germany) and 1% (v/v) polyethylenimine (Polymin P, 50% aqueous solution) and allowed to stand for 20 min on ice. The suspension was centrifuged at 20,000 g for 20 min at 2 °C. CKOX activity was assayed in the supernatant using the method of Liberos-Minotta and Tipton (1995). The reaction mixture in 0.2 *M* imidazole-HCl buffer (pH 7.5), contained 80 μM iP and 1 m*M* $CuCl_2$, and was initiated by the addition of enzyme and incubated at 37 °C for 30 min. The reaction was terminated using 40% (w/v) trichloroacetic acid. Following the addition of *p*-aminophenol reagent (3% (w/v) *p*-aminophenol in 6% (w/v) trichloroacetic acid) and development of colour for 10 min at room temperature, the absorbance at 352 nm was determined. CKOX activity is expressed as μmol 3-methyl-2-butenal produced mg^{-1} protein, interpolated from a standard curve prepared by reacting 3-methyl-2-butenal with *p*-aminophenol reagent. Results were confirmed by monitoring the conversion of [2-^{3}H]iP to [^{3}H]adenine according to the method of Motyka and Kamínek (1994). The assay mixture (50 μl final volume) contained 50 m*M* Tris-HCl buffer (pH 8), 10 μM substrate ([2-^{3}H]iP, 1.25 TBq $mmol^{-1}$ purchased from Academy of Sciences of the Czech Republic Isotope Laboratory IEB, Czech Republic) and 25 μl of enzyme preparation. After incubating the reaction mixture for 4 h at 37 °C, the reaction was terminated by the addition of 10 μl Na_4EDTA (200 m*M*) and 120 μl of cold 95% (v/v) ethanol containing unlabelled iP and adenine (0.75 m*M* each). Residual substrate and

labelled product were subsequently separated on thin layers of silica gel GF_{254} (Merck, Darmstadt Germany), developed once to 10 cm in chloroform/methanol/ammonium hydroxide (25%, w/v) (9:2:0.1, v/v/v) (Redig et al. 1997). Radioactivity in the zones corresponding to authentic iP (R_f 0.7–0.8) and Ade (R_f 0.3–0.4) zones was determined by liquid scintillation spectrometry using a Packard Tri-Carb® 1500 Scintillation Counter.

For all assays, protein concentration of the enzyme preparations was determined using Bradford's dye-binding assay (Bradford 1976).

Determination of ABA, IAA, iP and adenine

Tissue together with Polyclar (10%, w/w) was homogenised in ice-cold methanol:ethyl acetate (50:50, v/v) containing butylated hydroxytoluene and diethyldithio-carbamate (both 100 mgl^{-1}) as antioxidants and [G-^{3}H]ABA and 3-[5(n)-^{3}H]IAA (both 10 000 dpm/sample, obtained from Amersham International, UK), added to correct for losses, and extracted overnight in darkness at 2 °C. Homogenates were filtered under vacuum and the filtrate reduced to dryness *in vacuo* at 35 °C. The residue was resuspended in water (adjusted to pH 8 with 0.05 *N* NaOH) and loaded onto a Sephadex A-25 (Pharmacia) anion exchange column (5 ml bed volume) pre-equilibrated with water (pH 8). The column was washed with 10 ml aliquots of water (pH 8) and the acids eluted onto a pre-wetted C_{18} solid phase extraction (SPE) column (Isolute, International Sorbent Technology Ltd, Glamorgan, UK) with four 5 ml aliquots of 0.2 *M* formic acid. The C_{18} column was washed with 10 ml 80% methanol and the eluate reduced to dryness. Samples were resuspended in methanol and methylated by the addition of excess ethereal diazomethane. After removal of the ether phase under a stream of nitrogen, methylated samples were dried, partitioned three times into ethyl acetate and passed through a nitrile (SPE) cartridge column (Isolute, International Sorbent Technology Ltd, Glamorgan, UK). The ethyl acetate was removed under nitrogen and the sample resuspended, for analysis by reversed phase HPLC in 20% methanol. Chromatography was carried out using a 5 μm C_{18} column (250 × 10 mm i.d., ODS1, Spherecione, Phenomenex, Torrance, CA, USA) eluted with a linear gradient of 20–100% methanol over 55 min at a flow rate of 2 ml min^{-1}. Compounds of interest were detected at 254 nm using a Spectra System UV/VIS 1000 detector (Thermo Separations Products, Freemont, CA, USA) and quantified after calibration with authentic standards of ABA- and IAA-methyl ester. Radioactivity remaining in the HPLC-purified ABA- and IAA-methyl ester fractions was determined by liquid scintillation spectrometry. Confirmation of the identity of ABA- and IAA-methyl esters was established by combined gas chromatography-mass spectrometry using the procedure described by Cowan et al. (1999).

The extraction and quantification of iP by radioimmunoassay was as described by Moore-Gordon et al. (1998). Adenine was extracted and quantified by reversed-phase HPLC (Gilmore and Björkman 1994).

Results and discussion

An earlier study indicated a possible link between ABA metabolism and purine and/or CK-induced suppression of the MoCo-containing enzyme, XDH, in avocado (Cowan et al. 1999). The purine adenine, a product of CKOX activity, is both a precursor and analog to hypoxanthine and an inhibitor of XDH in ureide metabolism (Nguyen 1986). Adenine also stimulates ABA production in plant tissues and in ABA-producing fungi (Cowan et al. 1999). XDH and AB-ald oxidase require a sulfurylated MoCo for catalytic activity. Thus, small changes in the adenine pool for ureide formation might be expected to inhibit XDH resulting in more efficient utilization of the sulfurylated MoCo by the AO in ABA biosynthesis. Since adenine does not affect AO, the relative activity of this enzyme might be enhanced leading to increased ABA and presumably IAA production. The data in Figure 1A and B confirm that XDH activity is present in seed and mesocarp tissue of avocado fruit and, as expected, that *in vitro* activity could be reduced by the addition of either allopurinol, a potent inhibitor of XDH, or adenine (Nguyen 1986). Activity of XDH was ten-fold greater in seed tissue and allopurinol- and adenine-induced inhibition of XDH was more pronounced in these extracts (Figure 1A) than in extracts of mesocarp tissue (Figure 1B). While this observation may indicate differential expression and/or activity of XDH in avocado fruit tissues, the results nevertheless lend support to the hypothesis that adenine-induced alterations in XDH activity might indeed impact on plant hormone homeostasis and final fruit size by influencing activity of other MoCo-requiring enzymes particularly those involved in IAA and ABA biosynthesis.

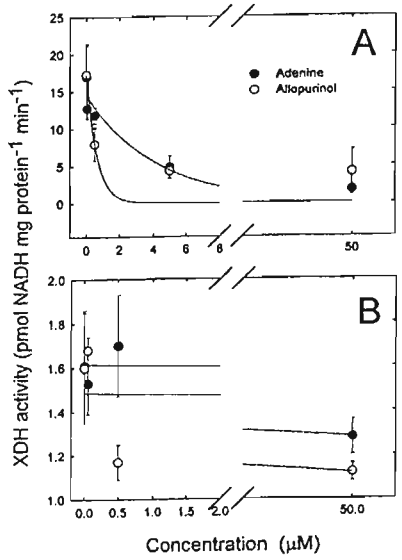

Figure 1. Effect of allopurinol and adenine on *in vitro* activity of XDH in extracts prepared from seed (A) and mesocarp (B) tissue of normal 'Hass' avocado fruit harvested in the linear phase of growth, 256 days after full bloom.

The activity of XDH, IA-ald and AB-ald oxidase in seed, seed coat and mesocarp of normal and phenotypically small 'Hass' fruit is shown in Table 1. No IA-ald oxidase activity was detected in either mesocarp or seed coat extracts. In contrast, extracts of seed tissue contained substantial IA-ald oxidase activity that was ten-fold greater in normal fruit. AB-ald oxidase activity was present in all tissues of avocado fruit. High activity in seed and seed coat tissue of the small-fruit phenotype was not unexpected as appearance of this phenotype occurs coincident with embryo abortion and seed coat senescence, and elevated ABA (Moore-Gordon et al. 1998; Richings et al. 2000). XDH activity was present only in seed and mesocarp tissue and was greater in these tissues from the small-fruit phenotype. The results in Table 1 therefore confirm that a reduction in avocado fruit size is related to activity of MoCo-requiring enzymes insofar as high seed IA-ald oxidase corresponds to low AB-ald oxidase and XDH activity in tissues of normal sized fruit. Furthermore, the model developed by Cowan et al. (1999) proposed that low XDH activity, typical of that observed in seed of normal fruit, would be associated with either increased CKOX activity or increased adenine, or both, with CKOX being the key enzyme for cytokinin degradation in plants. The data in Table 2 show that there was no significant difference between small and normal fruit in terms of CKOX activity. However, the level of adenine was substantially greater in seed and mesocarp of normal 'Hass' avocado fruit, and this would seem to confirm the postulated relationship between adenine content of fruit tissues and activity of XDH alluded to above.

In view of these findings it was envisaged that the IAA content of seed of normal 'Hass' fruit would be greater than that of the small-fruit phenotype. Likewise, ABA content of seed of the small fruit variant would be expected to be greater than that of seed of the normal fruit. The latter was confirmed following analysis of ABA in seed and mesocarp of small and normal fruit (Table 3). Surprisingly, the IAA content of seed of the small-fruit phenotype was two-fold that of the seed of normal fruit. One possible explanation is that early senescence of the seed coat in small fruit (Moore-Gordon et al. 1998) prevents basipetal movement of seed-derived IAA resulting in apparent accumulation, assuming that IAA is derived from IA-ald in avocado tissue. To overcome the confounding influence of seed coat senescence, and to determine whether alterations in XDH activity affect the tissue distribution of IAA and ABA, normal fruit was supplied solutions of allopurinol and allopurinol plus molybdate via the transpiration stream. Following incubation, the seed and mesocarp were dissected and extracted and analysed for ABA and IAA and the results are shown in Table 4. Both molybdate and allopurinol increased the ABA content of the seed but had little or no effect on ABA levels in mesocarp tissue. When applied together, molybdate plus allopurinol had no apparent effect on seed ABA content but increased mesocarp content by 58%. Levels of IAA were reduced in seed tissue by treatment of fruit with allopurinol, but increased by molybdate plus allopurinol. By comparison, IAA in mesocarp tissue increased significantly in fruit treated either with allopurinol or molybdate plus allopurinol. These results suggest that inhibition of XDH increases ABA biosynthesis in affected tissues confirming an earlier report (Cowan et al. 1999). Furthermore, since no IA-ald oxidase activity was detected in the mesocarp (Table 1) an increase in IAA content of mesocarp implies transport

Table 1. Tissue distribution and activity of IA-ald and AB-ald oxidase, and XDH in normal and phenotypically small 'Hass' avocado fruit, harvested 240 days after full bloom.

Tissue	Phenotype	IA-ald oxidase (pmol DCIP mg^{-1} protein min^{-1})	AB-ald oxidase	XDH
Seed	normal	29.78 ± 14.04	6.64 ± 0.30	9.93 ± 5.81
	small	2.51 ± 1.74	19.58 ± 4.44	16.19 ± 1.73
Seed coat	normal	ND[a]	16.76 ± 1.90	ND
	small	ND	43.96 ± 9.22	ND
Mesocarp	normal	ND	7.48 ± 2.84	3.26 ± 1.45
	small	ND	8.76 ± 2.90	23.44 ± 6.80

[a]Not detected.

Table 2. CKOX activity and adenine content of the major tissues of developing normal and phenotypically small 'Hass' avocado fruit, harvested 240 days after full bloom

Tissue	Phenotype	CKOX (μmol 3-methyl-2-butenal mg^{-1} protein)	Adenine (μmol g^{-1} DW)
Seed	normal	250.86 ± 52.86	241.2
	small	256.77 ± 57.29	24.59
Seed coat	normal	32.01 ± 10.79	nd[a]
	small	47.45 ± 18.40	nd
Mesocarp	normal	7.31 ± 3.16	320.7
	small	9.26 ± 3.81	19.66

[a]Not detected.

Table 3. ABA and IAA content of seed and mesocarp tissue from normal and small 'Hass' avocado fruit harvested in the linear phase of growth, 256 days after full bloom

Tissue	Phenotype	ABA	IAA
		(nmol g^{-1} FW)	
Seed	normal	0.85a[a]	7.41a
	small	14.29b	21.56b
Mesocarp	normal	0.67a	0.95a
	small	1.11a	1.68a

[a]Values followed by different letters are significantly different (for ABA $LSD_{(0.05)}$ = 6.57 and for IAA $LSD_{(0.05)}$ = 9.36)

of seed-derived IAA into this tissue which explains the increased levels of IAA detected in seed of the small-fruit phenotype in which seed coat senescence is a typical feature (Table 3). However, whether IAA is derived from IA-ald in ripening avocado remains to be determined.

To evaluate the effect of inhibition of XDH on levels of ABA, IAA and iP we chose to use ripening avocado mesocarp because of its high metabolic activity with respect to ABA synthesis. The results in Table 5 show that allopurinol increased the ABA and IAA content of ripening mesocarp but had little or no effect on levels of iP. Presumably, an increase in IAA reflects inhibition of IAA turnover in this tissue. Interestingly, molybdate caused a decrease in ABA, IAA and iP indicating that overall metabolism of these hormones was stimulated in the presence of exogenous molybdate.

Results from this study indicate that the 'Hass' small-fruit variant possesses elevated AO activity capable of utilising indole-3-aldehyde as substrate, but reduced AO activity when indole-3-acetaldehyde was used as substrate (Table 1). Although AO has low substrate specificity, the observation that two different substrates are utilised by AO to a differing degree in normal and small fruit, coupled with the finding of an altered ABA/IAA ratio in response to allopurinol and molybdate treatment, suggests that different isoforms of the AO enzyme exist in avocado fruit, that these have different affinity for molybdenum, catalyse AO-mediated reactions in ABA and IAA biosynthesis respectively, and that activity is development- and/or tissue-dependent. Similar findings have been reported for maize and *Arabidopsis thaliana* (Koshiba and Matsuyama 1993; Koshiba et al. 1996; Sekimoto et al. 1998). For example, three organ- and substrate-specific AO activity bands were detected in *Arabidopsis* seedlings, namely AOα, AOβ and AOγ (Seo et al. 1998). AOα was abundant in roots, whilst AOγ was most abundant in the cotyledons and leaves. In terms

Table 4. Effect of allopurinol (100 μM), molybdate (100 μM) and allopurinol (50 μM) plus molybdate (50 μM) on ABA and IAA levels in seed and mesocarp of normal 'Hass' avocado fruit harvested in the linear phase of growth, 269 days after full bloom. Solutions of allopurinol and/or molybdate were supplied to intact fruit via the pedicel (in the transpiration stream) and the fruit incubated for 24 h prior to extraction of ABA and IAA

Treatment	ABA		IAA	
	(nmol g^{-1} FW) (% of control)			
	Seed	Mesocarp	Seed	Mesocarp
Control	5.32 (100)	5.32 (100)	6.45 (100)	5.86 (100)
Molybdate	7.65^{*a} (144)	3.84 (72)	5.32 (82)	2.59* (44)
Allopurinol	9.02^{a} (170)	5.13 (96)	3.42* (53)	27.11* (463)
Molybdate + Allopurinol	3.71 (70)	8.42* (158)	8.37* (130)	28.02^{a} (478)

[a]Values followed by * are significantly ($P \leq 0.05$) different from the control.

Table 5. ABA, IAA and iP levels in mesocarp tissue of ripening 'Hass' avocado fruit treated with either molybdate, allopurinol, or molybdate plus allopurinol. Solutions of allopurinol and/or molybdate, at the concentrations specified in Table 4, were supplied to the cut surface of mesocarp tissue which was incubated for 48 h prior to extraction of ABA, IAA and iP.

Treatment	ABA	IAA	iP
	(nmol g^{-1} FW) (% of control)		(pmol g^{-1} DW) (% of control)
Control	4.00 (100)	2.03 (100)	59.8 (100)
Molybdate	1.46^{*a} (36)	1.46 (72)	42.9^{a} (72)
Allopurinol	8.89^{*a} (222)	3.93* (193)	56.4 (94)
Molybdate + Allopurinol	6.09^{a} (152)	3.37* (166)	59.3 (99)

[a]Values followed by * are significantly ($P \leq 0.05$) different from the control.

of substrate specificity, AOα showed a strong preference for indole-3-aldehyde, while AOγ efficiently oxidised 1-naphthaldehyde. AOβ exhibited properties intermediate between AOα and AOγ, in terms of its mobility in native PAGE and substrate preference. Thus, of the three isoforms, AOα seems most likely to be involved in IAA biosynthesis, due to its high affinity for IA-ald and its high expression in the IAA overproducing *sur1* mutant compared to the wild type (Seo et al. 1998). Preliminary studies with *Arabidopsis* revealed that two AO genes, atAO-3 and atAO-4, were rapidly induced after desiccation, suggesting that these genes encoded an AB-ald oxidase (Seo et al. 1999). High AO-type activity has also been detected in tomato fruit, and its expression seems to be related to the biosynthetic capacity required for typical plant metabolic "sink" tissues. Alternatively, it may however catalyse the final step in IAA and ABA biosynthesis (Ori et al. 1997). In the latter case, the tissue specific expression of the *TAO1* (tomato aldehyde oxidase 1) gene, detected by the TAO1 antibody in fruit, may reflect the role ABA plays in seed maturation and dormancy, whilst its expression in apical meristems could reflect the role this tissue plays in auxin biosynthesis (Ori et al. 1997).

Increased activity of the isoform that uses indole-3-aldehyde as the preferred substrate seems to correlate with increased ABA and small sized avocado fruit (i.e. reduced cell division cycle activity), whereas activity of the isoform utilising indole-3-acetaldehyde seems to correlate with sustained cell division cycle activity and therefore normal sized fruit. Thus, AO activity and its substrate specificity seem to play a central role in controlling plant hormone homeostasis, cell division and avocado fruit growth, possibly through the modulation of the IAA/ABA ratio. ABA levels decline over the course of avocado fruit growth (Cowan et al. 1997), presumably due to oxidative catabolism. Similarly, CK-like activity seems to decline during avocado growth (Blumenfeld and Gazit 1970) implying maintenance of the CK/ABA ratio throughout fruit growth and development. We previously demonstrated CK modulation of ABA metabolism in avocado (Cowan et al. 1999). Since auxin has been shown to stimulate the oxidative breakdown of CK by activating CKOX (Palni et al. 1988), it is distinctly possible that IAA also mediates the metabolism of ABA.

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