BIOSYNTHETIC ORIGIN OF ABSCISIC ACID IN RIPENING AVOCADO FRUIT

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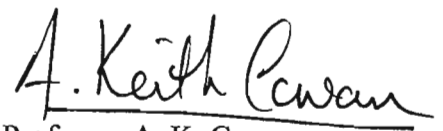
Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Agriculture in Horticultural Science, School of Agricultural Sciences and Agribusiness, University of Natal, Pietermaritzburg, R.S.A.

I hereby certify that this research is the result of my own investigation, except where acknowledged, and that it has not been submitted to any other University for degree purposes.

J. C. Guillaume Maurel

I certify that this statement is correct.



Professor A. K. Cowan

Supervisor

Acknowledgements

I would like to express my sincere thanks to Professor A. Keith Cowan for taking the time to introduce me to the world of plant physiology research. Keith, I am grateful to have had the honour of being part of your research team and to have been able to witness – and share some of - your passion for rigorous scientific research, especially with regards to the unravelling of the plant ABA biosynthetic pathway. Keep the good work going.

My warmest thanks also to my parents, and family in Mauritius and overseas, for their continuous support, affection and hospitality during these years spent in South Africa. You are ever present in my life, regardless of the distances.

My gratitude also goes to the van der Linde and Tweedie families. Your warm hospitality and numerous kindnesses have been very much appreciated. A special thank you to Marine, whose delightful companionship I have had the pleasure to enjoy for many years now.

I would also like to thank the academic staff of the ex-Faculty of Agriculture, Pietermaritzburg, which I met during my years at UNP. Thank you for your valuable discussions and guidance during these 'Varsity' years.

My thanks also go to my friends and colleagues of the 'Physiology team', Bob Kalala, Evelyn Richings, Ryan Cripps and Renate Oberholster. Thanks for all the great times, and all the best for the future.

Finally, thank you to all the other wonderful people that I have had the pleasure to meet and appreciate during my studies. Although your names are not listed here, I would like to thank you my friends. God bless you all.

Learning follows various roads.
We note the start but not the end.
For Time and Fate must rule the course,
While we see not beyond the bend.

The best of knowledge is a dream
The gainer holds steadfast, uncowed
By ridicule, and moves serene,
Despised and lowly in the crowd.

Kahlil Gibran

LIST OF ABBREVIATIONS USED

ABA:	Abscisic acid
AB-ald:	Abscisic aldehyde
ABAMe:	Abscisic acid methyl ester
ACC:	1-amino-cyclopropane-1-carboxylic acid
CH_4:	Methane
CK:	Cytokinin
DPA:	Dihydrophaseic acid
DPAMe:	Dihydrophaseic acid methyl ester
FPP:	Farnesyl pyrophosphate
GC-EI-MS:	Gas chromatography electron impact mass spectrometry
GC-MS:	Gas chromatography mass spectrometry
GC-PICI-MS:	Gas chromatography positive ion chemical ionisation mass spectrometry
GGPP:	Geranyl geranyl pyrophosphate
HCl:	Hydrochloric acid
He:	Helium
HMG-CoA:	3-hydroxy-3-methylglutaryl-coenzyme A
HMGR:	3-hydroxy-3-methylglutaryl reductase
HPLC:	High performance liquid chromatography
IDP:	Isopentenyl diphosphate
MVA:	Mevalonic acid
MVL:	Mevalonolactone
PA:	Phaseic acid
PAMe:	Phaseic acid methyl ester
TLC:	Thin layer chromatography
UV:	Ultraviolet
XAN:	Xanthoxal (xanthoxin)

ABSTRACT

Mesocarp of ripening avocado fruit incorporated label from [2-^{14}C]mevalonolactone, [1-^{14}C]acetic acid, [1-^{14}C]glucose and [1-^{14}C]pyruvate into ABA, although incorporation from mevalonolactone was significantly higher. Inhibition of the mevalonate pathway at the HMGR level using mevastatin reduced incorporation from acetate and MVL, while increasing incorporation from pyruvate and glucose. The carotenoid biosynthesis inhibitors AMO 1618 (inhibitor of lycopene cyclase) and fluridone (inhibitor of phytoene desaturase) both decreased incorporation of MVL into ABA, while the plant growth regulators ancymidol (inhibitor of GA synthesis and cytochrome P450) and jasmonic acid (senescence stimulator reducing the carotenoid content of plants) both increased incorporation of MVL into ABA. Tungstate was found to reduce incorporation from all four substrates into ABA, although more significantly from MVL and acetate. Further investigation revealed that the tungstate-induced decrease in MVL incorporation into ABA occurred concomitantly with increased label incorporation into XAN. Cobalt, an inhibitor of ACC oxidase and therefore of ethylene production, increased incorporation of MVL into ABA. Nickel had a similar effect. Analysis of the methyl ester of ABA extracted from avocado mesocarp supplied with either [1-^{13}C]acetic acid or [1-^{13}C]glucose revealed incorporation of label from acetate consistent with formation of ABA *via* the acetate/mevalonate pathway whereas glucose was incorporated *via* the triose phosphate pathway of isopentenyl pyrophosphate formation. Methane, positive ion-chemical ionisation-mass spectrometry of the *cis,trans* and *all- trans* isomers of ABA indicated more intense labelling of *trans,trans*-ABA, irrespective of substrate used. These results indicate that *trans,trans*- and *cis,trans*-ABA are derived by different pathways and that ABA is formed in avocado by both the mevalonate and non-mevalonate pathways of isopentenyl diphosphate synthesis.

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CHAPTER 1

GENERAL INTRODUCTION

The avocado *(Persea Americana* Mill.) originates from Central America, but the exact area of origin remains unknown due to its long history of cultivation and utilisation (Whiley and Schaffer, 1994). The ability of avocado to adapt to different climatic conditions has led to its classification into three distinguishable races named after their presumed centres of origin viz. Mexican, Guatemalan and West Indian (Storey *et al.*, 1986). Free hybridisation between these races has led to a large number of cultivars adapted to climatic conditions ranging from hot, humid tropical to cool, dry subtropical. While tropical avocado production is mostly concerned with green-skinned West Indian hybrids, subtropical production areas deal with green- or black-skinned Mexican and Guatemalan hybrids. In the past, 'Fuerte' has been the choice cultivar for subtropical avocado production areas due to its ability to withstand wide temperature variation. However, producers have been losing interest in this early maturing cultivar in favour of the late maturing 'Hass' cultivar, which is derived from the Guatemalan (85-90%) and Mexican (10-15%) races (Bergh and Ellstrand, 1986). Due to its mostly Guatemalan origin, 'Hass' is more sensitive to temperature extremes (cold and heat) than 'Fuerte'. However, the A-type flowers of 'Hass' are more temperature-resistant than the B-type flowers of 'Fuerte', and ensure better fertilisation and higher fruit set. As a result, 'Hass' yields are *circa* 25% higher than 'Fuerte' under subtropical conditions (Wolstenholme, personal communication)[1]. In South Africa, 'Fuerte', which accounted for 57% of avocado exports in 1995 was down to 42% in 1997 while the 'Hass' share rose from 27% in 1995 to 43% in 1997 (Korsten, 1997). This trend is expected to continue in the future, following in the wake of other

[1]: *Professor B.N. Wolstenholme, School of Agricultural Science and Agribusiness, University of Natal, Pietermaritzburg, South Africa.*

avocado producing areas like California and Israel, where 'Hass' is by far the preferred cultivar. However, this shift in favour of 'Hass' has slowed due to the high occurrence of small-fruit produced by 'Hass' trees. Because of their size, these fruit are not accepted for the export markets, and therefore represent a major economical concern for growers. The best price for 'Hass' avocados is obtained for count 14-18 (Kremer-Kohne and Kohne, 1995), while count size o 24 is considered not suitable for export (Moore-Gordon, 1997). In any season, 5-20% of the total crop falls into the latter category and these are considered to be small-fruit (Cowan, 1997). In addition, the proportion of small-fruit can be up to 40% of the crop on older, more stressed trees, especially in the warmer, drier areas of cultivation (Hilton-Barber, 1992; Kohne and Schutte, 1991; Kremer-Kohne and Kohne, 1995).

1.1 THE HASS AVOCADO SMALL-FRUIT SYNDROME

The avocado fruit is classified as a berry with a thick pericarp surrounding a single large seed enclosing the embryo (Valmayor, 1967). The pericarp consists of endocarp, mesocarp and exocarp. The endocarp and the mesocarp can be distinguished from each other during the early stages of fruit growth, but merge into one later (the fleshy part of the avocado will therefore be referred to as mesocarp in the text, although technically endocarp is also present). The exocarp is the smooth, or warty, skin of the fruit (Whiley and Schaffer, 1994). It is green in colour from fruit set to ripening, when it gradually turns purple black. The seed and the pericarp are separated by the seed coat, which is linked to the pedicel *via* a network of vessels running through the mesocarp.

The importance of the seed in determining the sink strength of the fruit has been the subject of many studies (Blumenfeld and Gazit, 1970, 1974). Research on tomato fruit has led to the proposal that auxin produced in the seed may be responsible for both drawing nutrients / assimilates / plant growth regulators to the fruit and promoting cell division in the exocarp (Bangerth, 1989). Even though this might also occur in avocado fruit, it appears that in this fruit the seed coat is also a very important structure (Cowan *et al.*, 1997; Cutting, 1993), as clear correlations have been found between seed coat health and fruit size (Moore-Gordon, 1997). Thus, phenotypically

small fruit are characterised by early seed coat senescence / death, while normal fruit retain healthy seed coats for most of the growth period.

Avocado fruit growth is the result of cell division and cell expansion (Schroeder, 1958; Valmayor, 1967; Zilkah and Klein, 1987). Recent results have shown that in avocado it is cell number (not cell size) and thus cell division which determines final fruit size (Cowan *et al.*, 1997). This is in accordance with results obtained from studies on tomato fruit development (Bohner and Bangerth, 1988). Jacobs (1995) has reported on the role of cytokinins (CKs) in promoting cell division cycle activity, thus cell number and final fruit size. Studies pertaining to the CK complex of small- and normal 'Hass' fruit have shown significant quantitative and qualitative differences (Cutting, 1993), suggesting the role of a yet unidentified CK in promoting cell division and thus fruit size in avocado. On the other hand, ABA has been linked to inhibition of cell division (Meyers *et al.*, 1994; Muller *et al.*, 1994; Artlip *et al.*, 1995). Moore Gordon (1997) has further shown that increasing endogenous ABA concentration resulted in decreasing avocado fruit size, while increasing endogenous CK concentration resulted in increasing fruit size. These results seem to indicate that fruit size is regulated by a balance between cell division promoting (CK) and inhibiting (ABA) plant hormones. These two plant hormones arise from the isoprenoid pathway (see Figure 1.1). While the CK pathway originates from IDP, the ABA pathway occurs later, either from FPP (direct pathway) or from GGPP and carotenoid metabolism (indirect pathway). Although many theories have been proposed, exactly how the isoprenoid pathway is regulated is still uncertain. The conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonic acid (MVA) by the enzyme 3-hydroxy-3-methylglutaryl reductase (HMGR) is reportedly the first committed step in isoprenoid biosynthesis (Goldstein and Brown, 1990) and is believed to be a major point in the regulation of the isoprenoid pathway in plants (Bach, 1987; Gray, 1987; Gondet *et al.*, 1992; Moore and Oishi, 1994; Chappell *et al.*, 1995). In tomato, a well studied model system in which cell division is limited to the two weeks following anthesis, HMGR expression and activity is required during early fruit development. *In-vivo* inhibition of HMGR during early fruit development retarded fruit growth, while inhibition during the later cell expansion stage had no significant effect (Narita and Gruissem, 1989). Russell and Davidson (1982) suggested that HMGR activity is regulated *via* hormonal mediation of enzyme activity, which was not allosteric, but *via* some unknown phosphorylation

system. This hypothesis was later supported by Moore and Oishi (1994) working on ABA inhibition of HMGR during maize seed maturation. CKs have been reported to promote sucrose transport and to be involved in nutrient metabolism (Hare and Van Staden, 1997), thus providing the fruit with energy and building blocks necessary for growth. Assuming normal invertase activity (Walker and Ho, 1977), this imported sugar would be expected to increase HMGR activity, and thus fruit size. Cowan *et al.* (1997),using carefully selected chemicals to manipulate the isoprenoid pathway while monitoring endogenous ABA levels, found evidence to support that a relationship exists between accumulation of ABA and inhibition of HMGR. These authors proposed that high levels of ABA decrease the CK:ABA ratio, causing a decline in HMGR activity and thus reducing fruit growth (see Table 1.1). Physiological responses to ABA are determined either by changes in tissue sensitivity to this hormone or changes in ABA concentration. Endogenous ABA concentration, which has been reported to differentiate small- from normal fruit (Moore-Gordon, 1997), is determined by the rate of ABA synthesis and catabolism in the fruit as well as the rate of import into and export from the fruit. When leaves of mesophytic plants are water-stressed, ABA levels have been reported to rise 10- to 50-fold within a few hours due to an increase in biosynthetic rate (Li and Walton, 1987). The high ABA concentration in the mesocarp of the small-fruit may similarly be the result of active *in-situ* ABA biosynthesis. A strong understanding of the ABA biosynthetic pathway in avocado mesocarp is therefore one of the prerequisites to understanding the small-fruit syndrome. Subsequent manipulation of this pathway, and thus ABA levels, might shed some light on the nature of the involvement of ABA in the small-fruit syndrome.

1.2 ABA BIOSYNTHESIS IN PLANTS

1.2.1 OVERVIEW OF ABA RESEARCH

In the early 1960s, two groups of researchers, one working on leaf abscission (Addicott and colleagues) and the other on bud dormancy (Wareing and associates), each identified a growth inhibiting substance involved in the physiological processes they were studying (Addicott and Lyon, 1969). According to their apparent physiological role, these substances were named 'abscisin' and 'dormin' respectively.

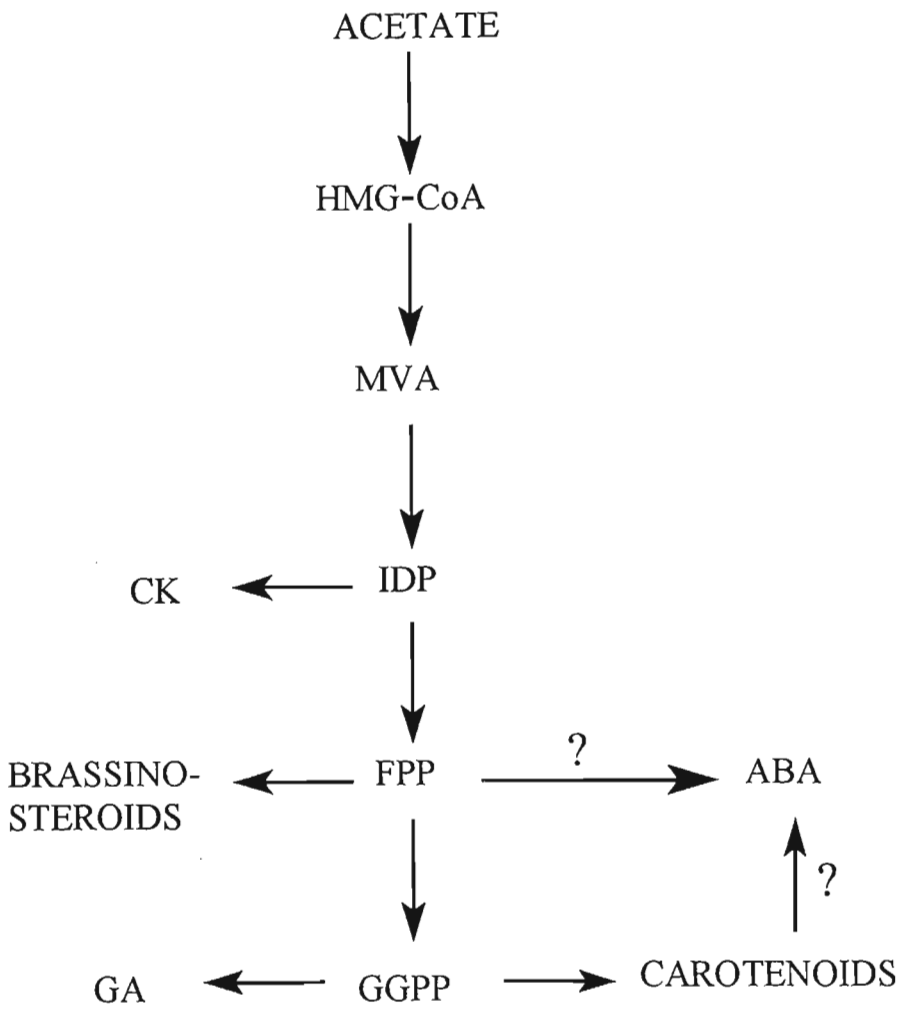

Figure 1.1 - The isoprenoid pathway gives rise to four groups of plant hormones: ABA, CK, GA and brassinosteroids.

The chemical identification of these compounds was not completed until 1965, at which time it was realised that they were the same (Cornforth *et al.*, 1965 a; 1965 b). A molecular structure (see Figure 1.2) was proposed by Okhuma *et al.* (1965) which was later confirmed by synthesis (Cornforth *et al.*, 1965b). By 1966, this growth inhibiting substance had been identified in several plant species, and the need to settle the dormin / abscisin debate became clear. The Sixth International Conference on Plant Growth Substances held in 1967 saw the concerned scientists propose the name 'abscisic acid' and the abbreviation 'ABA' for the newly identified phytohormone.

Table 1.1 - Mesocarp ABA concentration of developing small and normal avocado fruit, and effect of isoprenoid biosynthesis manipulation on ABA concentration (after Cowan et al., 1997).

	Time after full bloom (days)		
	163	216	290
Treatment	Mesocarp ABA concentration ($ng.g^{-1}$ dry weight)		
Control	29.3	12.2	10.9
Small fruit	63.6	81.8	75.5
Mevastatin	66.9	39.0	67.3
Mevastatin + MVL	33.0	16.1	11.1
Mevastatin + iP	14.1	26.4	24.9

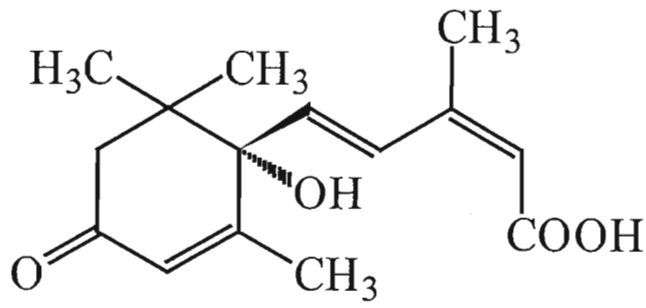

Figure 1.2 - Chemical structure of the biologically active cis-trans-(+)-(S)-abscisic acid.

Although chemical synthesis produces both (R)- [or (-)-] ABA and (S)- [or (+)-] ABA in a 1:1 ratio, only the (S)- [or (+)-] enantiomer occurs naturally (Cutler and Krochko, 1999). This newly discovered plant hormone attracted much attention. By the early 1970s, ABA catabolism had been elucidated (Milborrow, 1968; Milborrow, 1969; Milborrow, 1970; Gross, 1972; Walton and Sondheimer, 1972; Tinelli *et al.*, 1973), and the findings were subsequently confirmed (Loveys and Milborrow, 1984; Murphy, 1984; Cowan and Railton, 1987 a; Zeevaart and Creelman, 1988). In sharp contrast, ABA biosynthesis was still the subject of intense debate. Two observations relating to the structure of the ABA molecule were to lead scientists on different routes. First, the C15 structure of ABA led some scientists to believe that ABA was a sesquiterpenoid derived from MVA by the well known isoprenoid pathway (Popjack and Cornforth, 1966; Goodwin and Mercer, 1983). Other scientists, noting the similarities between ABA and the end group of some carotenoids, postulated that ABA may be an apocarotenoid (Taylor and Smith, 1967; Goodwin, 1971). Further research (Taylor and Burden, 1970b; Firn and Friend, 1972; Burden and Taylor, 1976) supported that ABA may be an apocarotenoid derived from a C40 carotenoid *via* a C15 intermediate characterised as xanthoxin[2]. However, following reports that when [^{14}C]-phytoene (carotenoid precursor) and [^{3}H]-MVA (precursor to all terpenoids) were applied to plant tissues only [^{3}H]-ABA, but both [^{14}C]- and [^{3}H]-carotenoids could be identified (Milborrow, 1970), the interest shifted towards the terpenoid origin of ABA. However, it was later realised that this experiment did not exclude an apocarotenoid origin for ABA, especially considering the fact that the large phytoene molecule would need to get into the chloroplast to be further converted. In the 1980s, improved analytical techniques, the advent of molecular biology, the availability of inhibitors of carotenogenesis and carotenoid mutants led to a resurgence of interest in plant ABA biosynthesis *via* the indirect route.

[2]: *Milborrow et al. (1997a) proposed to rename this compound 'xanthoxal', and their recommended abbreviation 'XAN' will be used in the text to designate this compound.*

1.2.2 CURRENT STATE OF ABA BIOSYNTHESIS UNDERSTANDING

1.2.2.1. THE DIRECT (SESQUITERPENOID) PATHWAY

The biosynthesis of ABA has been extensively studied using classical radiolabelling techniques (Milborrow and Robinson, 1973). Since ABA is structurally a C15 terpenoid compound (or sesquiterpenoid), it was assumed to be derived from MVA *via* the isoprenoid biosynthetic pathway. However, details of the pathway from MVA to ABA and the identity of the endogenous intermediates, particularly those post farnesyl pyrophosphate, have remained elusive. It is generally accepted that formation of MVA occurs *via* the condensation of three acetyl CoA units to HMG-CoA, which is then reduced to MVA by HMGR. Incorporation of label from MVA into ABA has been reported for many plants, plant tissues and plant organelles (Noddle and Robinson, 1969; Milborrow and Noddle, 1970; Milborrow and Robinson, 1973; Milborrow, 1974; Loveys *et al.*, 1975; Milborrow, 1983 a, b ; Cowan and Railton, 1986; Hirai *et al.*, 1986), although always in very low yields. In addition, MVA has been reported to be the precursor of sesquiterpenes in fungi (Arigoni, 1975; Baker *et al.*, 1975; Bradshaw *et al.*, 1978; Cane, 1983; Evans and Hanson, 1975), with FPP as the key intermediate in the biosynthesis of these compounds (Banthorpe and Charlwood, 1980; Loomis and Croteau, 1980). Therefore, when MVA was reported to be a precursor to sesquiterpenoids in higher plants too (Banthorpe *et al.*, 1985; Coolbear and Threlfall, 1985; Croteau *et al.*, 1972; Croteau and Loomis, 1972; Gliezes *et al.*, 1984; Loomis and Croteau, 1980), it was logical to expect that ABA in plants could be derived from MVA *via* FPP and the sesquiterpenoid (C15 terpenoid) pathway. This theory received major support when Robinson and Ryback (1969), feeding [^{3}H]-MVA to avocado mesocarp, found that the pattern of ABA labelling supported the existence of a C15 intermediate in the pathway. However, other scientists working on the indirect (apocarotenoid) pathway also identified a C15 intermediate (XAN), with a structure very similar to carotenoid end-groups, which could be formed by photolytic cleavage of violaxanthin (Taylor and Burden, 1970 a). Further research also demonstrated that XAN could be formed from violaxanthin *via* enzymatic cleavage with lipoxygenase (Firn and Friend, 1972). This was strong evidence against the direct pathway, because the C15 characteristic which gave

strength to the sesquiterpenoid pathway was now in support of an apocarotenoid pathway. In addition, the 'Robinson experiment' mentioned earlier also strongly undermined the possibility of an apocarotenoid origin for ABA, and the interest shifted towards the terpenoid origin of ABA. The ensuing slow progress in identifying pathway intermediates during the last half of the 1970s was mainly due to the very low levels of ABA and ABA-related compounds in plants, which did not enable the labelling and isolation of intermediates without some prior knowledge of these compounds (Neill *et al.*, 1982). Therefore, the discovery of the fungus *Cercospora rosicola*, which produces large amounts of ABA (Assante *et al.*, 1977), was seen as an opportunity for more detailed studies of the ABA biosynthesis pathway. However, unlike the GAs, it was later discovered that the ABA biosynthetic pathway in plants and fungi was very different (Zeevaart and Creelman, 1988). Feeding of sesquiterpenoid-like C15 compounds isolated from fungi, or chemically synthesised, and their resultant conversion into ABA was used to support the sesquiterpenoid pathway (Milborrow and Noddle, 1970; Milborrow and Garmston, 1973; Oritani and Yamashita, 1979; Oritani and Yamashita, 1987), but remained inconclusive overall because the compounds used were not naturally occurring in plants. When carotenogenesis inhibitors and carotenogenic and wilty mutants became available in the 1980s, they provided major support in favour of the indirect pathway, although not allowing for definite dismissal of the sesquiterpenoid route.

1.2.2.2. THE INDIRECT (APOCAROTENOID) PATHWAY

The possibility of ABA arising from carotenoids was first postulated by Taylor and Smith (1967) after they recognised structural similarities between ABA and the end-groups of certain xanthophylls. Further studies showed that photo-oxidation of xanthophylls such as violaxanthin and neoxanthin gave rise to a substance that inhibited cress seed germination. Although this substance was neutral, and thus not ABA, it was postulated that it was closely related to ABA and easily converted to the latter by cress seeds (Taylor and Smith, 1967). Upon further analysis, this substance was identified as a mixture of the *cis*- and *trans*- isomers of XAN (Taylor and Burden, 1970a; 1970b). Numerous bioassays subsequently indicated that the *cis* isomer had biological activity, while *trans*-XAN was biologically inactive (Burden *et al.*, 1971; Taylor and Burden, 1972). To ascertain the possible metabolic relationship between

XAN and ABA, Taylor and Burden (1972, 1973, 1974) applied non-labelled and labelled XAN to various plants. They observed that endogenous ABA concentration was increased in both cases. In addition, in tissues treated with labelled XAN, ABA was also found to be labelled, supporting a precursor-to-product relationship between XAN and ABA. As the probability of XAN being a xanthophyll-derived precursor of ABA increased, more attention was directed to its possible formation from violaxanthin / neoxanthin. In addition to photolytic cleavage of these compounds, Firn and Friend (1972) demonstrated that enzymatic cleavage of these xanthophylls by lipoxygenase also yielded XAN. In the early 1980s, chemical inhibitors of carotenogenesis as well as carotenogenic and ABA mutants had become available. This triggered a resurgence of interest in plant ABA biosynthesis, especially regarding its possible derivation from carotenoids. Both fluridone and norflurazon are inhibitors of carotenogenesis, blocking the dehydration of phytoene to phytofluene (Ridley, 1982). When applied to green plant tissue, these carotenogenesis inhibitors have been reported to only slightly reduce stress-induced ABA accumulation (Gamble and Mullet, 1986; Henson, 1984; Li and Walton, 1987; Quarrie and Lister, 1984). Walton *et al.* (1985) and Gamble and Mullet (1986) postulated that this did not dismiss the apocarotenoid hypothesis, as the pool size of xanthophyll in green tissue is likely to be sufficient to support ABA biosynthesis even if carotenogenesis is inhibited. This was supported by an experiment in which mature green leaves pre-treated with fluridone or water were incubated in a $^{14}CO_2$ environment and then water-stressed (Li and Walton, 1987). Analysis showed a similar dramatic reduction in specific activity in both xanthophylls and ABA in fluridone-treated leaves, although ABA accumulation was not affected. This not only confirmed that the xanthophyll pool is large enough to support ABA biosynthesis even when carotenogenesis is inhibited, but the similarities in specific activity of xanthophylls and ABA further cemented the precursor-product relationship. Further experiments using non-green tissue clearly showed a strong link between carotenogenesis and ABA production. Fluridone-treated, developing wild-type maize seeds produced seedlings that were white and viviparous, illustrating the relationship between lack of pigmentation and reduced ABA (Fong *et al.*, 1983). Young seedlings, poor in xanthophylls, were shown to be impaired in ABA biosynthesis after treatment with fluridone (Moore *et al.*, 1985; Moore and Smith, 1984) or norflurazon (Henson, 1984; Quarrie and Lister, 1984).

This relationship between carotenogenesis, xanthophylls and ABA production was consolidated through the use of carotenogenic and ABA mutants. Viviparous (vp) maize mutants, which lack the ability to synthesise carotenoids due to specific defects in their biosynthetic pathway have been identified and shown to have much reduced ABA levels compared to the wild-type (Moore and Smith, 1985; Neill *et al.*, 1986 a). Similarly, further study of the ABA mutant (*aba*) of *Arabidopsis thaliana* revealed that in addition to its low ABA concentration, it also had very low levels of antheraxanthin, violaxanthin and neoxanthin, while zeaxanthin was accumulated, probably due to impaired epoxy-carotenoid metabolism (Duckham *et al.*, 1991; Rock and Zeevaart, 1991).

Furthermore, studies using stable isotopes (^{18}O and ^{2}H) further supported a carotenoid precursor for ABA production. Mass spectrometry analysis showed that the pattern of labelling of xanthophylls was consistent with that of stress induced ABA. Creelman and Zeevaart (1984a) used $^{18}O_2$ to study the possibility that 1' deoxy ABA, a precursor of ABA in fungi, was also an ABA precursor in plants. If this was the case, they would find an ^{18}O atom at the 1' hydroxyl group of the ABA ring. However, the results showed that ^{18}O had been incorporated in the carboxyl group and not at the expected (1') position. Not only did this suggest that 1'-deoxy ABA was not the immediate precursor of ABA in plants, but it also supported the possibility that a xanthophyll was cleaved to produce an ^{18}O-labelled aldehyde which was then converted to ABA. This was in perfect accordance with the xanthophyll-XAN-ABA pathway. The existence of such a pathway was further supported by a similar experiment (Li and Walton, 1987). *Phaseolus* leaves were monitored for incorporation of ^{18}O into the violaxanthin epoxide oxygen. The leaves were then water-stressed, and both violaxanthin and ABA were isolated and analysed. The presence of ^{18}O in the epoxide of XAN and in the ring structure of ABA (probably at the 1'-hydroxyl position) gave additional support to the apocarotenoid origin of ABA. Only 25% of the ABA was found to be labelled with ^{18}O however, making it impossible to unequivocally conclude that all ABA is produced *via* violaxanthin. However, this possibility cannot be dismissed, as it has been suggested that there are two different violaxanthin pools in the chloroplast, only one of which participates in the xanthophyll cycle (Li and Walton, 1987). While these ^{18}O labelling studies were restricted to water-stressed leaves, Zeevaart *et al.* (1989) investigated ABA labelling in water-stressed and turgid leaves, as well as fruit tissue of several plant species.

Their findings were in accordance with the carotenoid origin of ABA. Tissues with low carotenoid content had to synthesise new xanthophyll precursors *de novo*, leading to the incorporation of ^{18}O in the ABA ring as well as in the carboxyl group. In contrast, carotenoid-rich tissue appeared to have a large enough xanthophyll (and precursors) pool to provide for ABA synthesis, the latter compound being labelled only in its carboxyl group, as expected following cleavage of a xanthophyll precursor. Nonhebel and Milborrow (1987), incubated green tomato tissue in 70% ^2H_2O to compare deuterium content of various carotenoids and ABA. Although ca. 20% incorporation (3 or more ^2H) was recorded in ABA, very low incorporation in xanthophylls and XAN led the authors to conclude that neither xanthophylls nor XAN were precursors of ABA. However, the xanthophyll pool in green tissue is a few orders of magnitude larger than that of ABA, making it difficult to link changes in xanthophylls to changes in ABA in these tissues. Parry *et al.* (1990) used etiolated tissue in order to overcome this limitation. Etiolated leaves of *Phaseolus* seedlings grown from seeds which had been imbibed, germinated and grown in the dark in a ^2H_2O-containing environment were excised and either frozen in liquid N or water-stressed and then frozen. Extraction and analysis of carotenoids and ABA showed that the pattern of deuterium incorporation in xanthophylls and ABA was consistent with a C40 pathway. In addition, the extent of labelling of neoxanthin and ABA suggested a precursor-product relationship. Cell-free studies have shown that *trans*-XAN is converted to *trans*-ABA, therefore indicating that isomerisation of the ABA precursors does not occur after cleavage (Sindhu and Walton, 1987). Thus, the xanthophyll precursor must have a 9-*cis* configuration to produce physiologically active *cis*-ABA. Carotenoid studies showed that these compounds exist predominantly in the thermodynamically more stable all-*trans* configuration. For example, more than 99% of the violaxanthin found in plants is in the *trans* configuration (Parry *et al.*, 1990). The exception though is neoxanthin, which occurs mainly as the 9'-*cis* isomer (Khachik *et al.*, 1986; Goodwin and Britton, 1988; Li and Walton, 1989). This led to the postulation that 9'-*cis*-neoxanthin is the direct precursor of XAN and biologically active ABA (Li and Walton, 1990; Parry *et al.*, 1990). Recent studies using a *Citrus* exocarp cell-free system support this hypothesis, showing that neoxanthin is the preferred xanthophyll precursor for XAN and ABA production (Cowan and Richardson, 1997). In addition, in an avocado cell-free system fed [^{14}C] MVL, the addition of naproxen, a lipoxygenase inhibitor, raised [^{14}C] incorporation in 9'-*cis*

neoxanthin by 178%, supporting suggestions that it is neoxanthin that is cleaved to eventually produce ABA.

1.2.2.2.1 XAN AS AN ABA PRECURSOR

The involvement of XAN as an intermediate between xanthophylls and ABA has been widely studied since the 1970s. In addition to its formation following photolytic cleavage of a xanthophyll (Taylor and Burden, 1970; Burden *et al.*, 1971), it was shown that XAN could also arise *via* enzymatic cleavage of the latter compound (Firn and Friend, 1972). Further conversion of XAN to ABA has been reported following application of exogenous XAN to cell-free systems (Sindhu and Walton, 1987; Sindhu and Walton, 1988) or plant tissues (Taylor and Burden, 1974). There are however no reports regarding the possible *de novo* synthesis of XAN from known ABA precursors such as MVA. In addition, there is at least one report that oxidative xanthophyll degradation - whether photolytic or enzymatic - is not a major biosynthetic pathway for XAN formation (Wagner and Elstner, 1989). Therefore, although there is much evidence to support that XAN can be produced from 9' *cis*-xanthophylls in plants, the endogenous conversion of XAN to ABA has still not been established. XAN is reportedly present at very low levels in plants and does not increase significantly in water-stressed plants even when the ABA level rises dramatically (Sindhu and Walton, 1987). It is therefore possible that the pathway producing XAN and the pathway producing ABA are in fact two different pathways.

1.2.2.2.2 THE ABSCISIC ALDEHYDE (AB-ald) CONTROVERSY

In addition to the XAN dilemma, the post-XAN steps toward ABA production are the subject of much debate. Following the Creelman and Zeevaart (1984) experiment mentioned earlier, it became accepted that a xanthophyll was cleaved to form an aldehyde, which was then converted to ABA. Although XAN is an aldehyde, the molecule needs to go through two oxidations and one isomerisation to form ABA (Sindhu and Walton, 1987). It therefore seems likely that more than one enzyme is involved and that there is at least one intermediate between XAN and ABA. The publication of a pathway for the chemical synthesis of ABA revealed that an aldehyde, called AB-ald, was formed as the immediate precursor to ABA (Mayer *et al.*, 1976). As the conversion of AB-ald to ABA involves only one oxygenation step, this aldehyde was postulated to be the immediate precursor to ABA in plants (Rock

and Zeevaart, 1990). This hypothesis was supported by the inability of the ABA-deficient tomato mutants *flacca* and *sitiens* to convert exogenous AB-ald to ABA *in-vivo* (Taylor *et al.*, 1988) or *in-vitro* (Sindhu and Walton, 1988), while the *not* mutant and wild-type tomato showed high conversion rates (Sindhu and Walton, 1988). In addition, Rock and Zeevaart (1990), working with apple fruit cortical tissue treated with $^{18}0_2$, reported more $^{18}0$ incorporation in the *cis* isomer of AB-ald than in the *trans*, which would be in accordance with enzyme specificity toward production of biologically active *cis*-ABA. However, aside from the work of Rock and Zeevaart (1990), AB-ald has not been reported to occur naturally in plants. Furthermore, recent studies have shown that the molybdoenzyme AB-ald oxidase responsible for the conversion of AB-ald to ABA can be inhibited by tungstate, leading to accumulation of XAN (Lee and Milborrow, 1997b). If AB-ald was indeed the substrate for the AB-ald oxidase, inhibition of AB-ald oxidase would be expected to cause the accumulation of AB-ald. However, in the two previously mentioned reports, AB-ald did not accumulate nor was it detected. In addition, tomato leaves supplied with [^{2}H]AB-ald *via* the xylem and stressed in the presence of $^{18}O_2$ produced ABA that was labelled with either ^{18}O or ^2H but not both (Netting and Milborrow, 1994). Considering the efficient incorporation of ^{18}O from $^{18}O_2$ into stress-induced ABA (Creelman and Zeevaart, 1984), the previous authors concluded that AB-ald is unlikely to be the endogenous precursor of stress-induced ABA. The possible involvement of AB-ald in the plant ABA pathway therefore remains to be unequivocally established.

1.2.2.2.3 THE 'ABA ADDUCT' THEORY

Mild base hydrolysis of the conjugated ABA fraction of onion seeds showed that the total amount of ABA so released was much larger than that which could be attributed to the two known conjugates, ABA glucose ester and ABA glucoside (Netting and Milborrow, 1988, Vaughan and Milborrow, 1984). The unknown source (compound or group of compounds) of hydrolysable conjugates was given the vague name 'ABA adduct', so as not to imply any structural characteristics. Further investigation showed that the adduct also occurs naturally in peas and barley, and can yield ABA and methyl abscisate (MeABA) at extremes of pH. The adduct was not significantly labelled following feeding of [2-^{14}C] ABA or Me[2-^{14}C]ABA, suggesting that the adduct is not a catabolite of these compounds. Attempts to label the adduct by feeding

labelled AB-ald were similarly unsuccessful. In addition, feeding of AB-ald caused a 20-25 fold drop in tissue adduct concentration, implying some kind of feedback inhibition from AB-ald and/or the ABA produced from the latter, therefore suggesting that adduct components could comprise the precursor pool (Netting *et al.*, 1992). However, recent results from the same authors do not support this possibility. Based on the calculations by Nonhebel and Milborrow (1986), ABA is reportedly produced from a precursor pool that is 35 times the size of the ABA pool. The adduct is not present in sufficiently large amounts for it to be this precursor, and therefore has subsequently been proposed to be an intermediate between a still unknown precursor pool and ABA (Netting *et al.*, 1997).

1.2.2.2.4 MOLECULAR BIOLOGY AND GENETIC ENGINEERING

The most recent development in ABA biosynthesis research is linked to the identification of DNA lesions in ABA mutants of various plant species, and the subsequent isolation of the recombinant DNA from the wild type. Marin *et al.* (1996), working with the *Nicotiana* ABA-deficient mutant *aba2* which shows very low levels of epoxy xanthophylls, established *via* recombinant DNA that the mutant was impaired in zeaxanthin epoxidase, therefore being unable to synthesise epoxy xanthophylls from zeaxanthin. These findings are in agreement with the widely accepted role of violaxanthin / neoxanthin as ABA precursors. Similar studies by Schwartz *et al.* (1997a) and Burbidge *et al.* (1997) on the *vp14* mutant of *Zea mays* and the ZE mutant of *Lycopersicon* respectively, reported the presence of a similar lesion in these two ABA-deficient mutants. They both showed no impairment in carotenoid synthesis or in the conversion of XAN to ABA. Recombinant DNA studies showed that they were unable to synthesise the VP14 enzyme responsible for the specific oxidation of 9-*cis* xanthophylls. The cloned VP14 protein was subsequently shown to cleave 9'-*cis* violaxanthin and 9'-*cis* neoxanthin to *cis*-XAN (Cutler and Krochko, 1999). Similar studies further led to the characterisation of the *Arabidopsis* ABA-deficient mutants *aba2* and *aba3* (Schwartz *et al*, 1997b). The former was found to be unable to convert XAN to AB-ald, while the latter could not convert AB-ald to ABA. Therefore, ABA-deficient mutants and advances in plant genetics are opening the door to a new concept in ABA biosynthesis research. Until now, this type of research provided support in favour of the indirect (carotenoid) pathway for ABA synthesis.

In conclusion, two major routes have been proposed for ABA synthesis in plants: the direct sesquiterpenoid and indirect apocarotenoid pathways. However, more than 30 years after the discovery of ABA, even though a vast amount of work has been dedicated to the subject, no unequivocal biosynthetic pathway has yet been established in plants for this phytohormone. It is however 'accepted' that ABA is an apocarotenoid derived from an epoxy-xanthophyll, probably 9'-*cis* neoxanthin, which is cleaved to form a C15 aldehyde compound, most probably XAN. XAN is then converted to ABA *via* AB-ald. It must be kept in mind that although there is evidence to support the existence of the above pathway, the possibility of ABA arising *via* a yet unknown pathway cannot be ruled out.

1.2.2.3 THE ABA PATHWAY IN AVOCADO FRUIT

As can be expected, the volume of work dedicated to the ABA pathway in avocado fruits is but a fraction of the published research on ABA biosynthesis in higher plants in general. In the early years of ABA biosynthesis research, avocado fruits were shown to have a high endogenous ABA content, and to be able to synthesise labelled ABA from applied [^{14}C]- or [^{3}H]-MVA (Noddle and Robinson, 1969; Robinson and Ryback, 1969; Milborrow, 1970). These results supported the sesquiterpenoid origin of ABA, and were in line with the contemporary results obtained with other plant species / tissues. In contrast, a few years later, at a time when mainstream research was accumulating evidence supporting an apocarotenoid origin for ABA, the 'Robinson experiment' mentioned earlier shifted the general direction of ABA research towards the sesquiterpenoid pathway. Consequently, progress in unravelling the pathway was considerably slow, and reached a virtual halt by the end of the 1970s. Most of the work done on avocado during this period was related to feeding labelled compounds to the system and looking for incorporation of label in ABA. However, these compounds did not naturally occur in plants, making them unlikely candidates for the ABA biosynthetic pathway in these organisms.

Mainstream research had come to support a xanthophyll - XAN - AB-ald - ABA biosynthetic route. In contrast, although the work in avocado indicates a possible apocarotenoid origin for ABA, this aspect has never been confirmed. Furthermore, work on avocado has led to the postulation of different intermediates between XAN and ABA. What now appears to have been the most relevant avocado work during the

early 1970s relates to a report of the possible involvement of xanthoxic acid[3] and 1',4'-*trans*-ABA diol in ABA biosynthesis (Milborrow, 1972; Milborrow and Robinson, 1973; Milborrow and Garmston, 1973) . Although these experiments were inconclusive as to the role of the two compounds in ABA biosynthesis, it opened a new research avenue which, surprisingly, was not to be further examined until the late 1990s. XAN-acid, as well as a XAN-acid precursor chemically synthesised from violaxanthin, have been reported to be rapidly converted to ABA in avocado mesocarp (Milborrow and Garmston, 1973; Milborrow *et al.*, 1997b), suggesting that XAN-acid may be a precursor of ABA in plants. In addition, recent studies have shown that the molybdoenzyme AB-ald oxidase, which is believed to convert AB-ald to ABA, is inhibited by tungstate, and that such inhibition leads to accumulation of XAN (Cowan *et al.* 1999, Milborrow and Lee, 1997). If AB-ald was indeed a precursor of ABA in plants, inhibition of AB-ald oxidase would be expected to cause the accumulation of AB-ald. However, in the two previously mentioned reports, AB-ald did not accumulate nor was it detected.

This tungstate-induced accumulation of XAN (Cowan and Richardson, 1997) suggests that XAN, and not AB-ald, is the substrate of the so-called AB-ald oxidase. Theoretical biochemistry suggests that oxidation of XAN would produce XAN-acid, which could be converted to ABA *via* 1',4'-*trans*-ABA diol. However, similar to the AB-ald issue, there is no report of naturally occurring XAN-acid in plants, leaving doubts as to its possible involvement in the ABA pathway. In contrast, the 1',4'-*trans*-ABA diol has been reported to occur naturally not only in avocado, but in all higher plants species and tissues studied (Okamoto *et al.*, 1987; Vaughan and Milborrow, 1987). Studies using avocado show incorporation of ^{2}H from ABA diol into ABA and PA, but incorporation of ^{2}H from ABA into PA only, the ABA diol remaining unlabeled (Okamoto *et al.*, 1987), suggesting that ABA diol is a precursor of ABA. Further studies involving the metabolism of this compound in other plant systems however disagree with the role of the ABA diol as a precursor in the ABA pathway.

[3]: *Milborrow et al. (1997a) proposed to abbreviate this compound as 'XAN-acid', and this short version will be used to designate this compound in the text.*

A more recent report using a *Citrus* exocarp cell-free system, shows strong evidence supporting that 1',4'-*trans*-ABA diol is not a precursor but a catabolite of ABA (Cowan and Richardson, 1993). Other authors have suggested that the 1',4'-*trans*-ABA diol is not a natural metabolite in higher plant ABA biosynthesis (Rock and Zeevaart, 1990; Vaughan and Milborrow, 1988). Therefore, the proposed role of XAN-acid and ABA diol as intermediates between xanthophylls and ABA in avocado-based research is as contentious an issue as the AB-ald postulation in mainstream research. Even though the pathway in avocado has been extensively studied using labelling techniques, leading to the postulation of a chemically sound pathway, the latter has not been unequivocally shown to exist.

1.3 OBJECTIVES

Recent studies have shown that mature, phenotypically small 'Hass' fruit contain substantially more ABA than the 'normal' fruit. The question therefore arises: Are small fruit a consequence of increased ABA content or do levels of ABA rise as a result of 'expression' of the small fruit phenotype? Indications are that, whatever the reason, increased fruit ABA does not occur due to tree stress and subsequent import of ABA synthesised elsewhere in the plant, but is the result of localised ABA metabolism. To investigate *de novo* synthesis of ABA in 'Hass' avocado fruit and to elucidate the underlying mechanism(s) responsible for ABA accumulation in small fruit, this research programme has as its objectives:

(1) the identification of metabolites potentially involved in ABA metabolism in avocado mesocarp;
(2) the manipulation of the ABA biosynthesis pathway using selected chemicals, in an attempt to assess aspects of the accepted pathway, and;
(3) the investigation of the carbon source for ABA biosynthesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

2.1.1 [^{14}C]- and [^{13}C]-LABELLED CHEMICALS

Acetic-[1-^{13}C]acid, sodium salt (99% atom ^{13}C) was purchased from Sigma Chemical Co., USA. [1-^{14}C]acetic acid, sodium salt (2.07 GBq/mmol), D-[1-^{14}C]glucose (2.04 GBq/mmol), R-[2-^{14}C]mevalonic acid lactone (2.07 GBq/mmol) and [1-^{14}C]pyruvic acid, sodium salt (1.04 GBq/mmol) were obtained from Amersham International, England. D-[1-^{13}C]glucose (99% atom ^{13}C), and DL-[2-^{13}C]mevalonolactone (99% atom ^{13}C) were obtained from Euriso-top, France.

2.1.2 FINE CHEMICALS AND PLANT GROWTH REGULATORS

Authentic abscisic acid (ABA) and ABA methyl ester (ABAMe) standards, allopurinol, ancymidol, fluridone, jasmonic acid and mevastatin were purchased from Sigma Chemical Co., USA. Fluridone was kindly supplied by Professor Andy Cairns, Department of Agronomy, University of Natal, Pietermaritzburg, R.S.A. AMO 1618 was obtained from Pierce Chemical Co. California, USA.

2.1.3 TRANSITION METALS

Cobaltous sulphate, sodium molybdate and sodium tungstate were obtained from Associated Chemical Enterprises, R.S.A. Nickel sulphate was purchased from uniLAB, SAARCHEM, R.S.A. Potassium tungstate was obtained from Aldrich Chemical Co., USA.

2.1.4.SOLVENTS AND GENERAL LABORATORY SUPPLIES

Acetone and polyoxyethylene sorbitan monolaurate (Tween 20) used in the formulation of substrate and treatments were obtained from Associated Chemical Enterprises, R.S.A and uniLAB, SAARCHEM, R.S.A respectively.

Tissue extraction was carried out using insoluble polyvinylpyrrolidone (PVP), Polyclar SB 100, obtained from BDH Laboratory Supplies, England, to reduce lipophilic impurities. Butylated hydroxytoluene (BHT) and diethyldithiocarbamic acid (DDC) sodium salt, used as antioxidants, were obtained from Sigma Chemical Co., USA.

For thin layer chromatography, diethyl ether, *n*-hexane and concentrated hydrochloric acid (HCl) 32.0% were obtained from NT Laboratory Supplies (Pty) Ltd., R.S.A.

Ethyl acetate, methanol and toluene were purchased from Associated Chemical Enterprises, R.S.A. Glacial acetic acid was purchased from Merck, Darmstadt, Germany.

For high performance liquid chromatography (HPLC), acetonitrile (UV cut-off 189 nm), ethyl acetate (UV cut-off 253 nm) and methanol (UV cut-off 193 nm) were obtained from Burdick & Jackson, USA.

Liquid scintillation spectrophotometry was performed using Insta-Fluor scintillation liquid purchased from Packard Instrument Co., USA.

2.1.5 PREPARATION OF STANDARDS

Authentic standards of phaseic acid (PA), dihydrophaseic acid (DPA) and epi-DPA were kindly supplied by Professor A.K. Cowan, Department of Horticultural Science, University of Natal, Pietermaritzburg, R.S.A. Methyl esters of authentic PA and DPA, used as chromatographic standards and for GC-MS analysis, were prepared by addition of ethereal diazomethane to methanolic solutions of each standard compound.

2.1.6 PREPARATION OF ETHEREAL DIAZOMETHANE

Before gas chromatography – mass spectrometry (GC-MS) analysis, the samples containing ABA and related acids were derivatised using ethereal diazomethane. The

latter was generated by hydrolysis of N-nitroso-N-methyl urea (purchased from Sigma Chemical Co., USA.) with NaOH (5N), as described by Fales *et al.*(1973). N-nitroso-N-methyl urea (133 mg) and distilled water (0.5 ml) were placed in the inner tube of a Wheaton Diazomethane generator (obtained from Pierce Chemical Co., Rockford, Ill., USA.) while diethyl ether (3.0 ml) was placed in the outer tube. The Generator was clamped close and placed in an ice bath. After a few minutes, 5N NaOH (0.6 ml) was injected through the Teflon septum into the inner tube. The reaction was allowed to proceed for around 45 min, by what time the ether had developed a deep yellow colour.

2.2 PLANT MATERIAL

Mature avocado (*Persea americana* Miller cv Hass) fruit were obtained from Everdon Estate in the KwaZulu-Natal Midlands, Republic of South Africa. The orchard was cultivated on a well-drained, medium to heavy textured (clay content 35-55%) soil of the Hutton form. Water supply during the dry winter months, characteristic of the prevailing cool mesic 'mist-belt' climate, was achieved through a micro-jet irrigation system (two micro-jets per tree) scheduled according to tensiometer readings. The fruit were picked from eight year old trees grafted on clonal Duke 7 rootstocks, and allowed to ripen in darkness at 25°C for 8-10 days.

2.3 APPLICATION OF CHEMICALS AND INCUBATION PROCEDURE

Soft, ripe fruits were cut in half and de-stoned. Excised blocks of mesocarp were supplied various chemicals, in the presence of specific substrates, at the concentrations specified in the Results. For each experimental treatment, the mesocarp blocks (ca. 20g fresh weight) used were from the same fruit. Chemicals were formulated in Tween20:acetone:water (1:1:8, v/v) and infiltrated *via* a series of cuts in the surface of the tissue. A total volume of 300 µl of solution was applied this way. For each treatment, one block of mesocarp was supplied with 300 µl of Tween20:acetone:water (1:1:8, v/v) only, and served as a control. The mesocarp blocks were subsequently incubated for 24 h, unless otherwise stated, in a water saturated environment at 25°C. Cellular activity was stopped at the end of the

incubation time by immersing the mesocarp blocks into liquid N. The mesocarp was then freeze-dried prior to extraction.

2.4 EXTRACTION AND PURIFICATION OF ABA AND RELATED COMPOUNDS

Freeze-dried tissue was homogenised in darkness for 3 min using an Ultra-Turrax top-drive tissue disperser (model T 25, Janke & Kunkel, GmbH & Co, IKA Labortechnik, Staufen, Germany) in 150 ml of ice-cold methanol:ethyl acetate (1:1, v/v) solution containing the antioxidants BHT (100 mg/l) and DDC (200 mg/l), as well as insoluble PVP (10%, w/w). The homogenate was filtered under vacuum using a Buchner funnel lined with Whatman filter paper No. 1, the filter-cake washed with an additional 100 ml of ice-cold methanol:ethyl acetate (1:1, v/v), and the filtrate was reduced to dryness *in vacuo* at 35°C using a rotary evaporator (Bibby RE 100 B, Bibby Sterilin Ltd, Stone Staffordshire, England). The residue was resuspended in 20 ml of 0.5 M potassium phosphate buffer (pH 8.5) and partitioned six times against equal volumes of *n*-hexane to remove pigments and other lipophilic impurities. To extract XAN, the pH of the aqueous phase was adjusted to 7.0 using conc. HCl, and neutral compounds partitioned twice into equal volumes of ethyl acetate. To extract ABA and related acids, the pH of the aqueous phase was then adjusted to 2.5 using conc. HCl, and the acidic compounds were partitioned four times into equal volumes of ethyl acetate. Water was removed from the XAN- and ABA-containing ethyl acetate fractions by freezing to -20°C followed by filtration under vacuum using a Buchner funnel. The organic phase was then reduced to dryness *in vacuo* at 35°C using a rotary evaporator.

2.5 THIN LAYER CHROMATOGRAPHY (TLC)

XAN and the acidic compounds ABA, PA, DPA and epi-DPA were purified by TLC using silica gel GF_{254} plates (20 cm x 20 cm, 0.20 or 0.25 mm thick) obtained from Macherey- Nagel, Germany. The plates were developed to 15 cm, once in *n*-hexane:ethyl acetate (3:2, v/v; System 1) or three times in toluene:ethyl acetate:acetic acid (25:25:2, v/v; System 2) respectively. The UV-absorbing zones corresponding to XAN, ABA, PA, DPA and epi-DPA were recovered into water-saturated ethyl

acetate, and the samples were reduced to dryness *in vacuo* at 35°C. The Rf values for the above-mentioned compounds are given in Table 2.1.

2.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Reverse-phase HPLC analysis was performed using either a 5 μm C_{18} column (250 x 4.6 mm i.d. ODS 2, Spherisorb, Phase Separation Inc., Clwyd, UK) eluted isocratically over 60 min with methanol:water:acetic acid (40:60:0.5, v/v/v) at a flow rate of 1.5 $ml.min^{-1}$ (System A) or, a 5 μm C_{18} column (250 x 10 mm i.d. ODS 1, Sphereclone, Phenomenex, Torrance, CA) eluted over 45 min using a linear gradient of 20-100% methanol in either 0.5% aqueous acetic acid (System B) or water (System C) at a flow rate of 2 $ml.min^{-1}$. Retention times for ABA, PA, DPA, epi-DPA, ABAMe and XAN were as shown in Table 2.2. The acidic compounds were quantified at 260 nm by peak integration following calibration with authentic standards using a Spectra System UV3000 rapid scanning detector or a Spectra System UV/VIS 1000 detector (Thermo Separation Products, Freemont, CA, USA). XAN was quantified at 260 nm, using the same detectors, by peak integration following calibration with ABAMe standard.

2.7 LIQUID SCINTILLATION COUNTING

Where measurement of ^{14}C radioactivity level in compounds of interest was needed, the HPLC eluate was fractionated into 0.4 ml aliquots in scintillation counter tubes. 'Insta-Fluor' liquid scintillation cocktail (2 ml) was added to each sample. The tubes were vortexed to obtain good mixing of sample and scintillation liquid, and run through a Packard Tri-Carb 1500 liquid scintillation analyser (Packard Instrument Company, USA), set for automatic quench correction and a count time of 2 minutes per sample.

Table 2.1 - Rf values for XAN, ABAMe, PAMe, DPAMe ABA, PA, DPA and epi-DPA separated by TLC as described in Materials and Methods.

Metabolite	Rf value		
	System 1	System 2	System 3
XAN	0.20		
ABA		0.76	
PA		0.50	
epi-DPA		0.45	
DPA		0.40	
ABAMe			0.80
PAMe			0.56
DPAMe			0.47

Table 2.2 - Retention times for XAN, ABAMe, DPA, PA, epi-DPA and ABA separated by HPLC as described in Materials and Methods.

Metabolite	Retention time (min)		
	System A	System B	System C
XAN	16.7		37.6
ABAMe			33.3
DPA	4.1	23.3	
PA	5.2	26.4	
epi-DPA	6.2	29.6	
ABA	12.2	37.8	

2.8 GAS CHROMATOGRAPHY ELECTRON IMPACT MASS SPECTROMETRY (GC-EI-MS)

HPLC-purified acidic metabolites co-eluting with authentic standards were collected, derivatised at room temperature with ice-cold ethereal diazomethane and repurified on silica gel GF_{254} TLC plates run in hexane:ethyl acetate (1:1, v/v; System 3). Rf values for ABAMe, PAMe and DPAMe are given in Table 2.1. HPLC eluate corresponding to XAN was similarly collected. Dr Birgit Bartels-Rahm, Department of Chemistry, University of Natal, Pietermaritzburg, R.S.A., kindly derivatised the XAN fraction in acetic anhydride / pyridine. Unequivocal identification of the methyl ester and ortho-acetyl ester derivatives was achieved by GC-EI-MS using a Hewlett-Packard 5890 gas chromatogram 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, CA, USA) programmed from 120 °C at 5 °C.min^{-1} with He as carrier gas (1.5-2.0 ml.min^{-1}) and EI spectra recorded at 70 eV. The total ion chromatogram and electron impact mass spectrum of standard ABAMe are presented in Figures 2.1 A and B.

2.9 GAS CHROMATOGRAPHY POSITIVE ION CHEMICAL IONISATION MASS SPECTROMETRY (GC-PICI-MS)

GC-PICI-MS was carried out essentially as described by Netting *et al.* (1988). Following derivatisation of ABA, PA and DPA using ethereal diazomethane and repurification by TLC (see section 2.8), the methyl esters of the above compounds were analysed using a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard quadrupole MS system. Analysis was carried out by injecting samples onto a fused-silica capillary column (15m × 0.25 mm i.d., film thickness 3 Φm) of OV-1 (Supelco Inc., Bellefonte, USA). Initial oven temperature was 40 °C for 1 min increasing to 300 °C at 20 °C min^{-1} with He as carrier (head pressure of 30 kPa) and CH_4 as ionising gas (source pressure of 1 torr). The ion source was 220 °C and spectra were recorded at 70 eV. The total ion chromatogram and positive ion (CH_4)

chemical ionisation mass spectra of standard *cis*- and *trans*-ABAMe are presented in Figure 2.2 A, B and C.

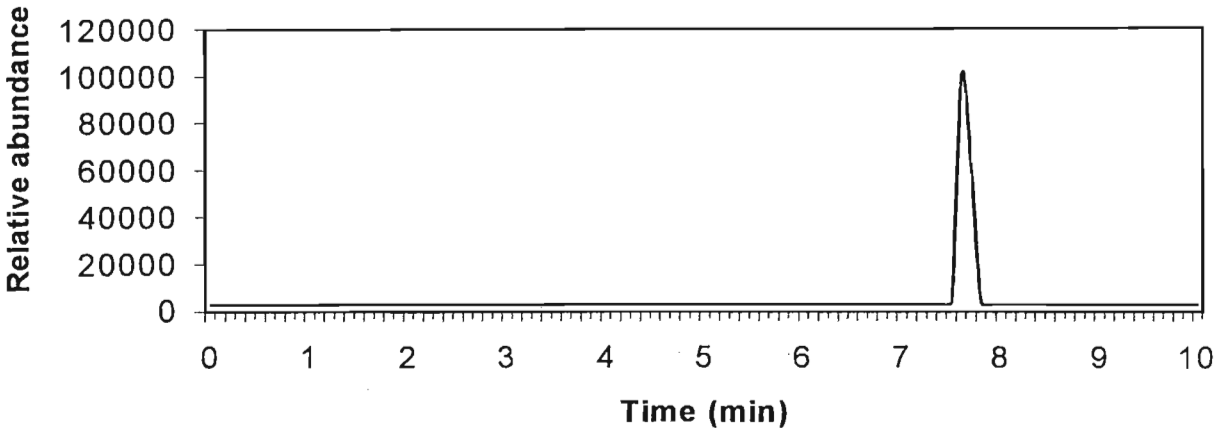

Figure 2.1 A - Total ion chromatogram of standard ABAMe analysed by gas chromatography as described in Materials and Methods.

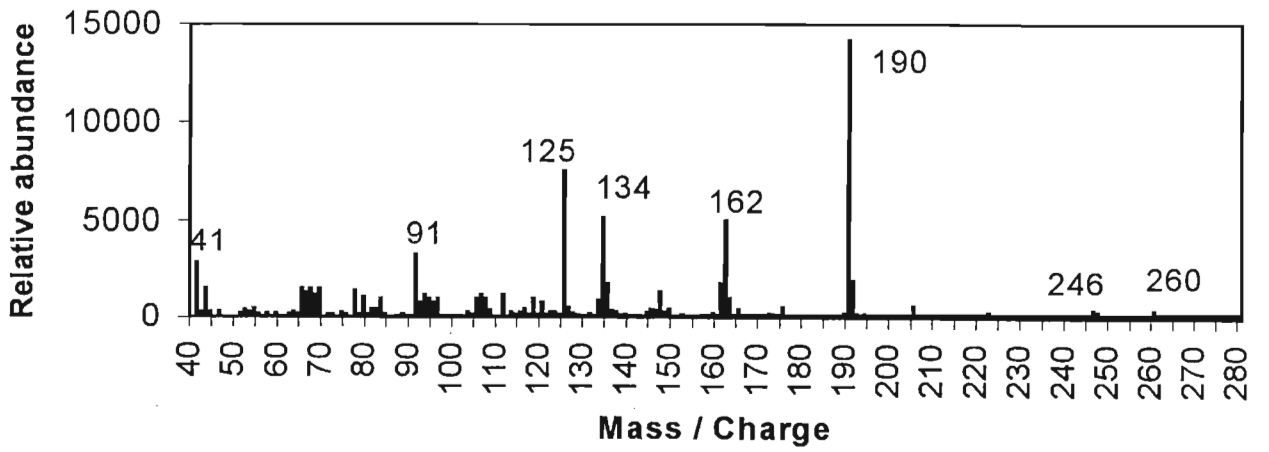

Figure 2.1 B - Electron impact mass spectrum of standard ABAMe analysed by EI-MS as described in Materials and Methods. Both cis- and trans-ABAMe (not present in Figure 2.1 A) give identical mass spectra when analysed by EI-MS.

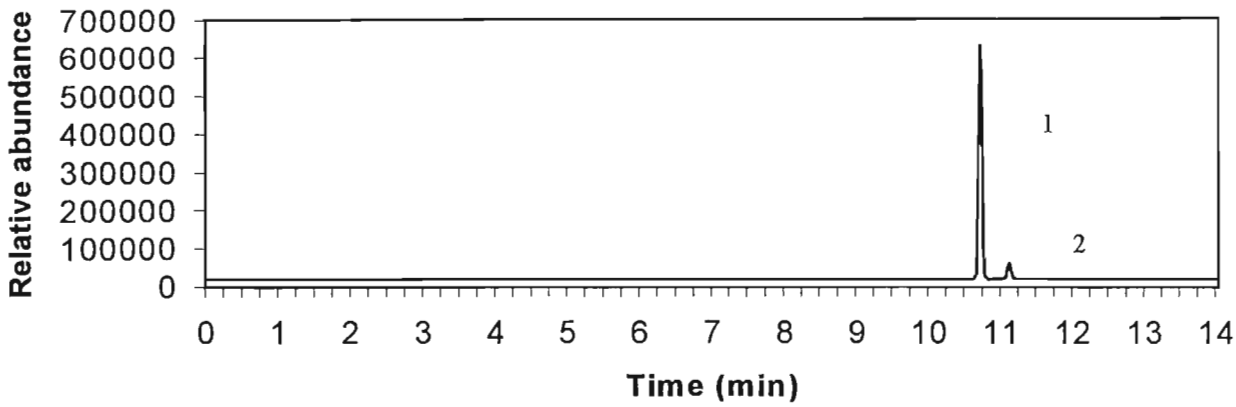

Figure 2.2 A - Total ion chromatogram of standard cis,trans-ABAMe (1) and its all-trans-isomer (2) analysed by gas chromatography as described in Materials and Methods.

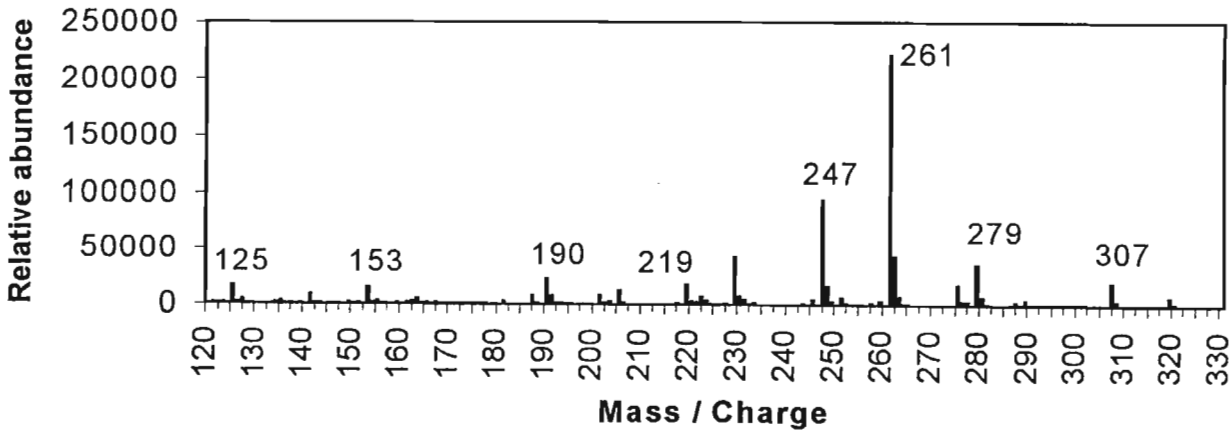

Figure 2.2 B - Positive ion (CH_4) chemical ionisation mass spectrum of standard cis-trans-ABAMe analysed as described in Materials and Methods.

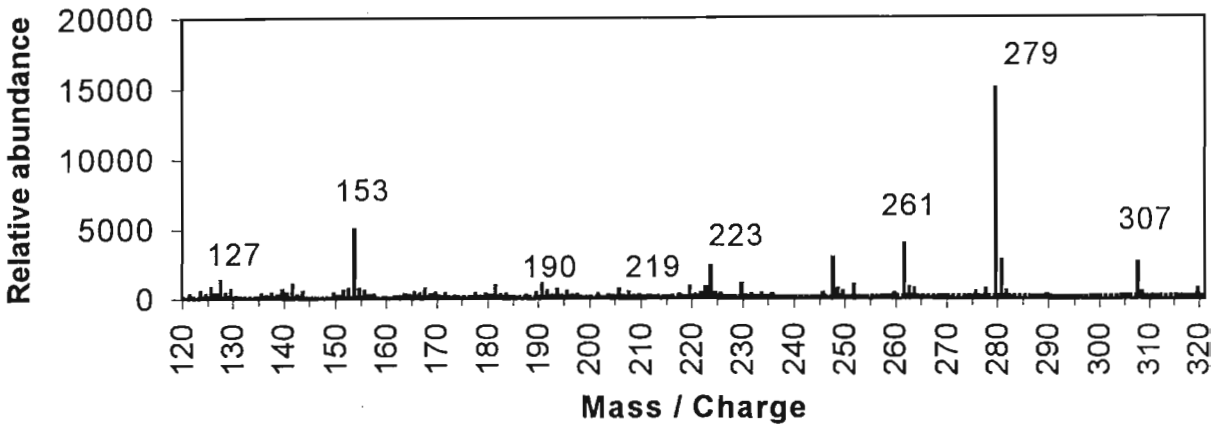

Figure 2.2 C - Positive ion (CH_4) chemical ionisation mass spectrum of standard all-trans-ABAMe analysed as described in Materials and Methods.

CHAPTER 3

CHEMICAL DISSECTION OF ABA BIOSYNTHESIS IN MESOCARP OF RIPENING AVOCADO FRUIT

3.1 INTRODUCTION

Work done in the late 1960s and early 1970s (Noddle and Robinson, 1969; Robinson and Ryback, 1969; Milborrow, 1970) indicated that avocado mesocarp converts supplied MVA into ABA. Similar feeding studies published a few years later (Milborrow, 1972; Milborrow and Robinson, 1973; Milborrow and Garmston, 1973) also showed that the system converted applied xanthophylls derivatives into ABA. Thus, as in other higher plant systems, most of the investigations using avocado support a carotenoid origin for ABA, but the last biosynthetic steps are still being debated. While the commonly accepted, but not unequivocally established, last steps in ABA biosynthesis are 9'-*cis*-neoxanthin → XAN → AB-ald → ABA in higher plant tissues (Li and Walton, 1990; Parry *et al.*, 1990), in avocado mesocarp XAN-acid and 1',4'-*trans*-ABA diol have been proposed as intermediates between xanthophylls and ABA. Following on their work dating nearly 30 years (Milborrow and Garmston, 1973), Milborrow *et al.* (1997b) again showed that XAN-acid is rapidly converted to ABA. According to theoretical biochemistry principles, these researchers elaborated that the conversion of XAN-acid to ABA would take place *via* 1',4'-*trans*-ABA diol. Further studies (Milborrow and Lee, 1998b) however led to a rebuttal of this theory, the 1',4'-*trans*-ABA diol being identified as a metabolite of ABA rather than as a precursor. According to these authors, theoretical biochemistry dictates that conversion of XAN-acid to ABA involves (1) oxidation of the C-4'-hydroxyl group to give 4'-keto-XAN-acid, and (2) isomerisation of the 1',2'-epoxide group to yield ABA. If (2) happened before (1), 1',4'-*trans*-ABA diol would be the immediate precursor to ABA. However, while naturally occurring 1',4'-*trans*-ABA

diol has been identified in avocado mesocarp (Okamoto *et al.*, 1987), to date there has been no report of naturally-occurring XAN-acid or 4'-keto-XAN-acid in this system. The post-XAN ABA biosynthetic steps in avocado are therefore still very unclear. Furthermore, ^{14}C-labelled MVL fed to ripening avocado mesocarp reportedly gives very low incorporation into ABA (Milborrow, 1976), while there is no published study showing incorporation of label from MVL into XAN in this system. In fact, there is no report of the unequivocal identification of XAN in avocado mesocarp. Moreover, an unequivocal link between MVL, XAN and ABA biosynthesis has yet to be established.

It is surprising that after 30 years of research, the ABA biosynthetic pathway in avocado mesocarp is still mostly unknown, the whole issue resting on a number of 'accepted assumptions' which have never been unequivocally demonstrated. The publication by Rohmer *et al.* (1993) of evidence supporting the biosynthesis of IDP *via* a non-mevalonate pathway in eubacteria has highlighted the long-disregarded possible existence of such a pathway in plants.

In this light, a series of experiments aimed at investigating the incorporation of a range of possible precursors into ABA was designed. Furthermore, endogenous *de novo* MVA biosynthesis was manipulated using mevastatin, a known HMGR inhibitor which blocks the conversion of HMGCoA to MVA (Cowan *et al.*, 1997). This inhibition has a two-fold effect: (1) it reduces *de novo* MVA production, therefore theoretically enabling better incorporation (because of reduced dilution) of supplied labelled MVA, and (2) when other possible precursors are used, it indicates whether these precursors are metabolised to ABA *via* the mevalonate pathway or not.

Other chemical modifiers were also used to obtain information on specific parts of the pathway. The carotenoid biosynthesis inhibitors AMO 1618 and fluridone respectively inhibit lycopene cyclase and phytoene desaturase (Sandmann *et al*, 1985; Gamble and Mullet, 1986). Therefore, if the system is supplied labelled MVA in the presence of either of these inhibitors, and the pathway proceeds *via* carotenoids, these inhibitors should reduce incorporation of label into ABA. The plant growth regulators ancymidol, a CK analogue which inhibits GA synthesis (Grossmann, 1990) and cytochrome P450 (Davis and Curry, 1991), and jasmonic acid, which has been reported to stimulate senescence with the effect that it enhances carbon flux through carotenoids (Sanioewski and Czapski, 1983; Perez *et al.*, 1993), were similarly used to dissect the pathway. While, in theory, ancymidol would be expected to favour

incorporation of substrate into ABA by (1) diverting more MVA to its biosynthetic pathway and (2) inhibiting ABA catabolism, it has been reported to have the reverse effect (Cowan and Railton, 1987 b; Norman *et al.*, 1983 a, b). Jasmonic acid, by stimulating carbon flux through the carotenoid pathway, should yield higher label incorporation into ABA if the pathway involves carotenoids.

Finally, as enzymes reportedly involved in ABA biosynthesis require transition metal ions as cofactors, these metal ions can be used to obtain information on the activity of such enzymes in the pathway. As mentioned above, the biosynthetic step between XAN and ABA is still a matter of intense debate. It is however reported that an aldehyde oxidase enzyme requiring a molybdenum cofactor is involved (Cowan *et al.*, 1999; Lee and Milborrow, 1997; Milborrow *et al.*, 1997b). This enzyme has been found to be inactivated by tungstate (Rajogopalan and Johnson, 1992; Omarov *et al.*, 1999). Tungstate can thus be used to investigate this area of the pathway in avocado fruit mesocarp. Another heavy metal ion, cobalt, has been reported to inhibit ethylene production by inactivating ACC oxidase, the enzyme responsible for the conversion of ACC to ethylene (Yu and Yang, 1979). Ethylene production is widely accepted as preceding or coinciding with ABA biosynthesis during the climacteric (Lee and Milborrow, 1997b). It would therefore be interesting to know whether ABA biosynthesis would be affected by a cobalt-impaired production of ethylene. The effect of nickel - a transition metal ion with very similar physical and chemical characteristics as cobalt in nature - on ABA biosynthesis was also investigated.

This chapter is therefore aimed at investigating the effect of various chemicals on the ABA biosynthetic pathway in ripening avocado mesocarp.

3.2 RESULTS

3.2.1 IDENTIFICATION OF ABA AND RELATED METABOLITES IN RIPENING AVOCADO

In order to unequivocally establish the identity of the compounds XAN, ABA, PA and DPA in this study, and to establish their natural occurrence in ripening avocado mesocarp, untreated tissue was extracted and purified by partitioning, TLC and HPLC as described in sections 2.4, 2.5 and 2.6. The HPLC eluate corresponding to XAN was

derivatised in acetic anhydride / pyridine, while the ABA, PA and DPA eluates were derivatised using ethereal diazomethane, and the methyl ester derivatives purified by TLC. The esters were subsequently analysed by GC-EI-MS as described in section 2.8. The electron impact mass spectra of the ester derivatives of naturally occurring XAN, ABA, PA and DPA in ripening avocado mesocarp are presented in Figures 3.1 A, B, C and D.

Figure 3.1 A shows a weak molecular ion peak at *m/z* 292, which is consistent with the compound being O-acetyl XAN. The major ion peaks at *m/z* 232, 176, 149 (base peak), 133, 121, 105, 95 and 43 further support the identity of the compound (Neill and Horgan, 1987). Figure 3.1 B shows a weak molecular ion peak at 278, which is consistent with the compound being ABAMe. This is further supported by the fragmentation pattern, with major ions at *m/z* 260, 246, 190 (base peak), 162, 134, 125 and 91. The weak *m/z* 260 ion corresponds to the loss of water from the molecular ion. The main fragmentation pathway is initiated by cleavage of the 2',3'-bond with elimination of isobutylene, producing the *m/z* 190 ion. Subsequent eliminations and cyclisations give rise to the other ions. The molecular ion of ABA also fragments *via* an independent pathway involving cleavage of the 1',5'-bond, giving rise to the 125 ion. Figure 3.1 C shows a molecular ion at 294, and major ions at *m/z* 244, 217, 167, 139, 125 (base peak), 122, 121, 94 and 43, fragmentation pattern consistent with the compound being PAMe. Losses of H_2O, CH_3O and CH_3OH give rise to ions at *m/z* 276, 263, 262 and in combination *m/z* 244. The *m/z* 263 ion looses the bridging CH_2O group to give the *m/z* 139 ion. Loss of the side chain with retention of the tertiary oxygen gives rise to the *m/z* 167 ion. Elimination of CO from the 4'-keto group yields the *m/z* 139 ion. The base peak ion (*m/z* 125) derives from the side chain. Figure 3.1 D shows a molecular ion at *m/z* 296, and major ions at *m/z* 278, 246, 220, 188, 154, 122 (base peak), 121, 94 and 43. This fragmentation pattern is consistent with the compound being PAMe. The fragmentation pathway of DPAMe is very similar to that of PAMe. The characteristic ion cluster at 121, 122, 125 has the same origin as that in the PAMe spectrum, except that the intensity is shifted in favour of the 121, 122 pair (Neill and Horgan, 1987).

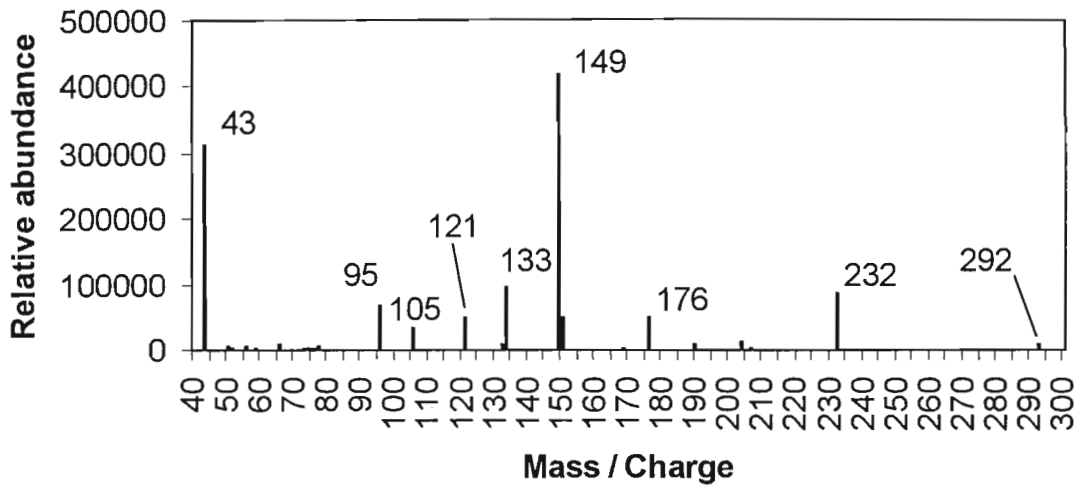

Figure 3.1 A - Electron impact mass spectrum of the O-acetyl ester derivative of XAN. XAN was extracted from ripening avocado mesocarp, purified, derivatised and analysed as described in Materials and Methods.

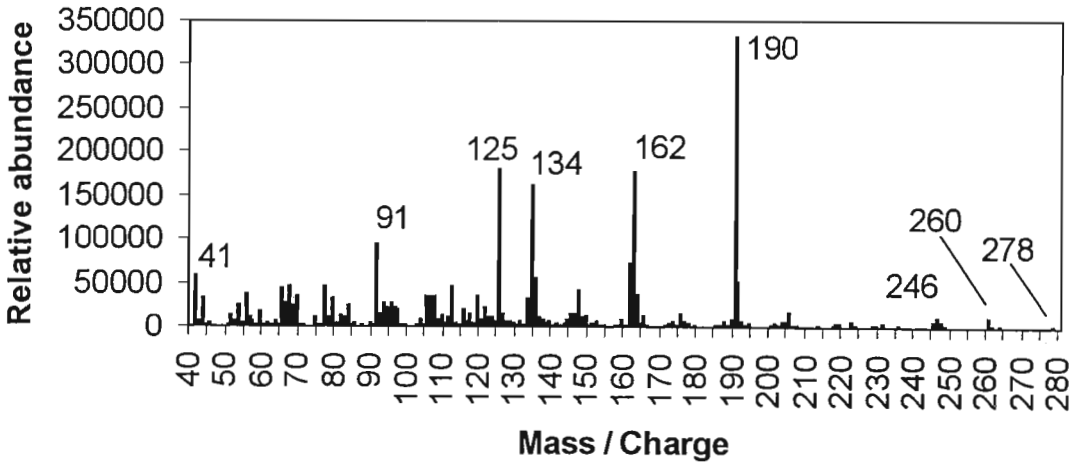

Figure 3.1 B - Electron impact mass spectrum of cis, trans-ABAMe. ABA was extracted from ripening avocado mesocarp, purified, derivatised and analysed as described in Materials and Methods.

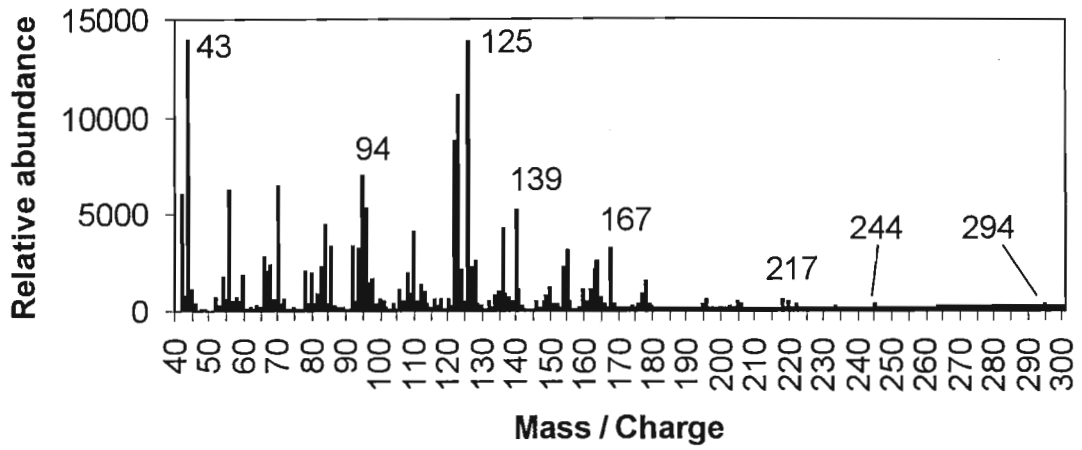

Figure 3.1 C - Electron impact mass spectrum of PAMe. PA was extracted from ripening avocado mesocarp, purified, derivatised and analysed as described in Materials and Methods.

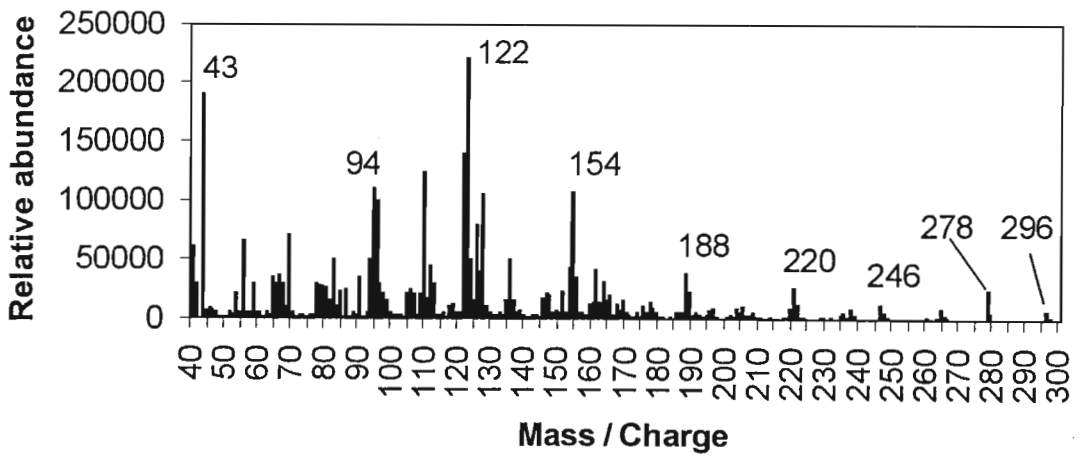

Figure 3.1 D - Electron impact mass spectrum of DPAMe. DPA was extracted from ripening avocado mesocarp, purified, derivatised and analysed as described in Materials and Methods.

3.2.2 SUBSTRATE SPECIFICITY FOR ABA PRODUCTION IN RIPENING AVOCADO

Studies on both the direct or the indirect ABA biosynthetic pathways in higher plants and avocado mesocarp support the earliest precursor of ABA as being MVA. Labelling studies using either [^{14}C]- or [^{3}H]-labelled MVL however show very low label incorporation into ABA by avocado mesocarp, seldom exceeding 0,1% (Milborrow, 1976). This might either be attributed to the large spectrum of isoprenoids produced by avocado fruit from MVA, or to the large endogenous pool of an intermediate along the pathway (e.g. carotenoids) which dilutes the labelled substrate, or to the existence of another source of carbon for ABA biosynthesis. Rohmer *et al.* (1993) reported the existence of a pathway of IDP formation in bacteria that did not involve the mevalonate pathway. This pathway reportedly uses pyruvate and glyceraldehyde-3-phosphate to form IDP. This pathway was subsequently reported in the unicellular alga *Scenedesmus*, where it was found to produce carotenoids (Schwender *et al.*, 1996). This pathway is also reportedly used for carotenoid and other isoprenoid lipids synthesis in higher plants chloroplasts (Lichtenthaler *et al.*, 1997). Milborrow and Lee (1998a), using a cell-free system from avocado fruit, reported that more pyruvate than MVL was incorporated into ABA, suggesting that most of the ABA in avocado is formed *via* the triose-pyruvate pathway, and not *via* the accepted mevalonate route. There is however, no report of the situation in intact avocado fruit. In order to establish the relative contribution of the two pathways of IDP formation in ABA biosynthesis in intact avocado fruit, [^{14}C]-labelled acetate, glucose, MVL and pyruvate were fed to slices of ripening avocado mesocarp, and incorporation into ABA measured after a 24 h incubation. Results shown in Table 3.1 clearly illustrate that of the precursors tested, pyruvate is the one yielding the lowest incorporation into ABA. This result must however be taken with care. The pyruvate was labelled at position 1, and this carbon would have been lost if pyruvate was converted to ABA *via* the triose / pyruvate pathway. The interesting aspect of the pyruvate result though is the remarkably higher incorporation into XAN. Although this experiment was not repeated, the result seems to indicate that XAN and ABA might not be formed by the same pathway.

Table 3.1 - Incorporation of radioactivity from [^{14}C]-labelled acetate, glucose, MVL and pyruvate into XAN and ABA in ripening avocado mesocarp. In each experiment substrates (5.5 MBq) were applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) via the cut surface and incubated at 25^{o}C for 24 h in a water-saturated environment. ABA and XAN were extracted, purified and analysed as described in Materials and Methods.

	Substrate	Radioactivity in ABA (Bq)	% incorporation	Radioactivity in XAN (Bq)	% incorporation
Experiment 1					
	[1-^{14}C]Glucose	3.22	5.85E-05	3.50	6.36E-05
	[2-^{14}C]MVL	18.43	3.35E-04	4.27	7.76E-05
Experiment 2					
	[1-^{14}C]Acetate	3.63	6.60E-05	2.12	3.85E-05
	[1-^{14}C]Pyruvate	1.58	2.87E-05	9.56	1.74E-04
Experiment 3					
	[1-^{14}C]Acetate	16.11	2.93E-04		
	[1-^{14}C]Glucose	21.16	3.85E-04	N/D*	N/D*
	[2-^{14}C]MVL	411.42	7.48E-03		
Experiment 4					
	[1-^{14}C]Acetate	11.35	2.06E-04	5.09	9.25E-05
	[1-^{14}C]Glucose	10.25	1.86E-04	5.18	9.41E-05
	[2-^{14}C]MVL	42.20	7.67E-04	5.34	9.70E-05

*: N/D = not determined

Glucose and acetate were better incorporated into ABA, although significantly less so than MVL. It therefore appears that MVL is the preferred precursor. Thus MVL was used in the subsequent experiments aimed at dissecting the ABA biosynthetic pathway, except where the effect of mevalonate pathway inhibition was investigated,

using acetate as substrate. It is also worth noting that although incorporation into ABA was much higher with MVL, incorporation into XAN was similar for acetate, glucose and MVL.

3.2.3 EFFECT OF INHIBITION OF THE MEVALONATE PATHWAY ON ABA BIOSYNTHESIS

If the mevalonate pathway is the main supplier of precursor (MVA) for ABA biosynthesis in avocado, its inhibition should have a marked effect on *de novo* ABA production. Acetate is converted *via* acetyl CoA to HMGCoA, which is converted to MVA by the enzyme HMGCoA reductase (HMGR). Mevastatin is a known inhibitor of HMGR, and may therefore be used to block the production of endogenous MVA. If the tissue is fed with [^{14}C]-labelled acetate and mevastatin simultaneously, and assuming that acetate is incorporated into ABA *via* the mevalonate pathway and not *via* the triose phosphate pathway, one would expect the labelling of ABA to be much lower than when the tissue is fed [^{14}C]-labelled acetate in the absence of mevastatin. If this were not the case, acetate would then appear to be converted to ABA *via* a non-mevalonate pathway. Table 3.2 shows the effect of mevastatin on incorporation of label from acetate into ABA. These results indicate that incorporation of acetate into ABA is slightly decreased following HMGR inhibition. The fact that incorporation into ABA is not severely reduced by mevastatin might indicate that only very little of the acetate is converted to ABA *via* the mevalonate pathway, while the bulk of it becomes incorporated into ABA *via* a different route.

Mevastatin was also used to investigate ABA biosynthesis from [^{14}C]-labelled acetate, glucose, MVL and pyruvate. A decrease in label incorporation from acetate, glucose or pyruvate into ABA due to the presence of the HMGR inhibitor might suggest that the precursor is converted to ABA *via* the mevalonate pathway. With regards to MVL, HMGR inhibition would be expected to increase label incorporation into ABA, the reduction in *de novo* MVA biosynthesis reducing the dilution of labelled precursor. Results are shown in Table 3.3. Mevastatin was found to slightly increase incorporation from glucose and pyruvate, while slightly reducing incorporation from acetate and MVL. This could indicate that acetate and glucose are metabolised to ABA *via* a non-mevalonate pathway.

Table 3.2 - Effect of mevastatin on ABA biosynthesis from ^{14}C-labelled acetate in ripening avocado mesocarp. In each experiment the substrate was applied (5.5 MBq) to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) with or without mevastatin via the cut surface and incubated at 25°C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

	Mevastatin (μg)	Radioactivity in ABA (Bq)	% incorporation
Experiment 1			
	0	2.11	3.83E-05
	200	1.83	3.32E-05
Experiment 2			
	0	1.92	3.49E-05
	200	1.44	2.61E-05

Regarding acetate, these results support the hypothesis proposed in the previous paragraph. In the case of MVL, the result is unexpected: not only did the incorporation into ABA not rise, but it decreased slightly. Either this result is erroneous, or it might indicate that the endogenous MVA pool is rather small, and therefore there is no label dilution problem at this level.

3.2.4 EFFECT OF CHEMICAL MODIFIERS ON ABA BIOSYNTHESIS IN RIPENING AVOCADO

3.2.4.1 EFFECT OF CAROTENOID BIOSYNTHESIS INHIBITORS

Table 3.3 - Effect of mevastatin on ABA biosynthesis from ^{14}C-labelled acetate, glucose, MVL and pyruvate in ripening avocado mesocarp. In each experiment substrates (5.5 MBq) were applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) with or without mevastatin via the cut surface and incubated at 25°C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

	Substrate	Mevastatin (μg)	Radioactivity in ABA (Bq)	% incorporation
Experiment 1	Glucose	0	3.22	5.85E-05
	Glucose	200	3.24	5.89E-05
	MVL	0	18.43	3.35E-04
	MVL	200	17.30	3.14E-04
Experiment 2	Acetate	0	3.63	6.60E-05
	Acetate	200	3.06	5.56E-05
	Pyruvate	0	1.58	5.74E-05
	Pyruvate	200	2.05	7.44E-05

Following the first reports presenting ABA as a sesquiterpenoid (Noddle and Robinson, 1969; Robinson and Ryback, 1969; Milborrow, 1970), the structural similarities between ABA and the end parts of carotenoid molecules led to the proposal of a carotenoid origin for ABA (Milborrow, 1972; Milborrow and Robinson, 1973; Milborrow and Garmston, 1973). Nowadays, it is widely accepted that ABA is derived from carotenoids in higher plant tissues (Parry, 1993; Cowan and Richardson, 1997; Schwartz *et al.*, 1997a). ABA is also accepted to follow this route in avocado mesocarp, but to date there has been no unequivocal demonstration that ABA is in fact derived from supplied precursors *via* the carotenoid pathway. Feeding of labelled MVA to avocado mesocarp yields low label incorporation into ABA. If the biosynthetic pathway involves carotenoids, it is expected that these compounds would

also show signs of labelling. However, due to the large size of the carotenoid pool in avocado mesocarp compared to the amount of radioactivity supplied, significant label incorporation into carotenoids has not been reported. There are however some chemicals known to affect carotenoid metabolism that may enable more insight into this matter. AMO 1618 and fluridone respectively inhibit lycopene cyclase and phytoene desaturase. Therefore, if *de novo* ABA biosynthesis proceeds from MVA to ABA *via* carotenoids, incorporation of label into ABA is expected to be significantly reduced by both inhibitors. Results are shown in Figures 3.1 and 3.2.

AMO 1618 and fluridone were found to reduce MVL incorporation into ABA, more so at higher concentrations. The fact that there was still fairly good incorporation into ABA even in the presence of AMO 1618 or fluridone indicates that MVL might be converted to ABA *via* a pathway not involving carotenoids. The reduction in incorporation also seems to indicate that at least some of the MVL is being converted to ABA *via* the carotenoid pathway. There might thus be more than one biosynthetic pathway that can produce ABA from MVL.

3.2.4.2 EFFECT OF PLANT GROWTH REGULATORS

The plant growth regulators ancymidol and jasmonic acid were used to investigate their effect on ABA biosynthesis. The mevalonate pathway provides precursors for not only ABA but also for CK, GA and sterol biosynthesis. Ancymidol is a known inhibitor of GA synthesis, and is therefore expected to reduce the use of MVA for non-ABA production purposes. In theory, this should make more MVA available for ABA biosynthesis, resulting in higher label incorporation into ABA from fed [^{14}C]MVL. Furthermore, the ancymidol-induced inhibition of cytochrome P450 is expected to result in the accumulation of label in ABA.

Jasmonic acid is known to stimulate the synthesis of B-carotene, a precursor to ABA if ABA biosynthesis follows the carotenoid route. The feeding of jasmonic acid to avocado mesocarp is thus expected to increase incorporation of label into the carotenoid pool, and subsequently to yield an increase in label incorporation in ABA if the latter compound is derived from carotenoids. Results presented in Figure 3.3 show that ancymidol increased the incorporation of MVL into ABA. This could be explained by the inhibition of cytochrome P450, which leads to the accumulation of ABA as the catabolic pathway is blocked.

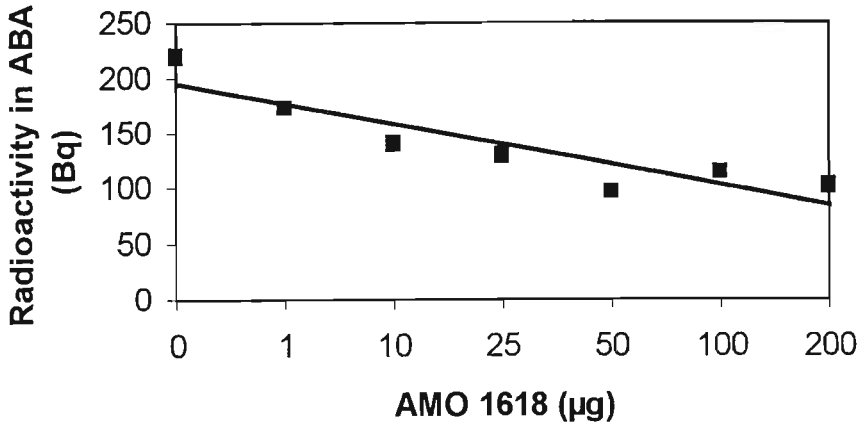

Figure 3.1 - Effect of AMO 1618 on [^{14}C]MVL incorporation into ABA in ripening avocado mesocarp. Substrate (5.5 MBq) was applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) with or without AMO 1618 (0, 1, 10, 25, 50, 100 or 200 µg) via the cut surface and incubated at 25^{o}C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

This increase may also be due to the fact that the part of the MVA pool which is usually used for GA biosynthesis is available for ABA biosynthesis as the GA pathway is also inhibited by ancymidol. As illustrated in Figure 3.4, jasmonic acid causes a marked increase in MVL incorporation into ABA. This is in accordance with MVA proceeding *via* carotenoids *en route* to ABA, and jasmonic acid increasing carotenoid synthesis from the supplied labelled substrate. Such an increase in *de novo* carotenoid biosynthesis from labelled MVL would be expected to result in a carotenoid pool more strongly labelled than normal. If derived from carotenoids, ABA would also be expected to be more strongly labelled.

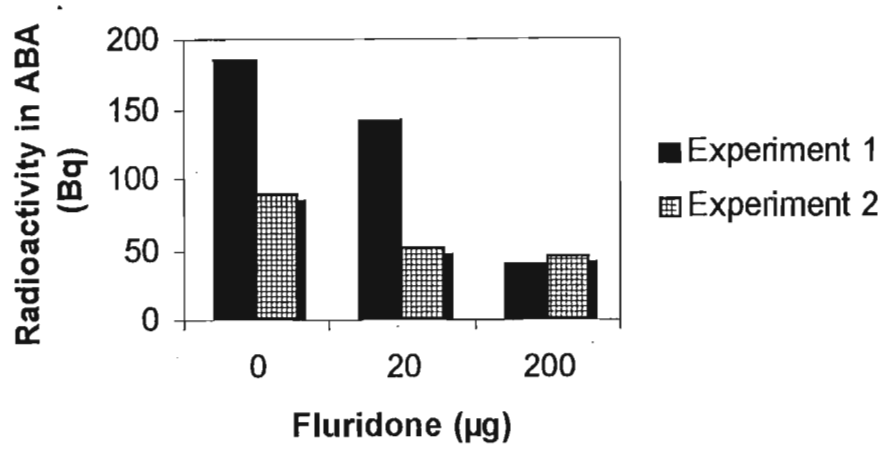

Figure 3.2 - Effect of fluridone on [^{14}C]MVL incorporation into ABA in ripening avocado mesocarp. In each experiment, substrate (5.5 MBq) was applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) with or without fluridone (0, 20 or 200 µg) via the cut surface and incubated at 25°C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

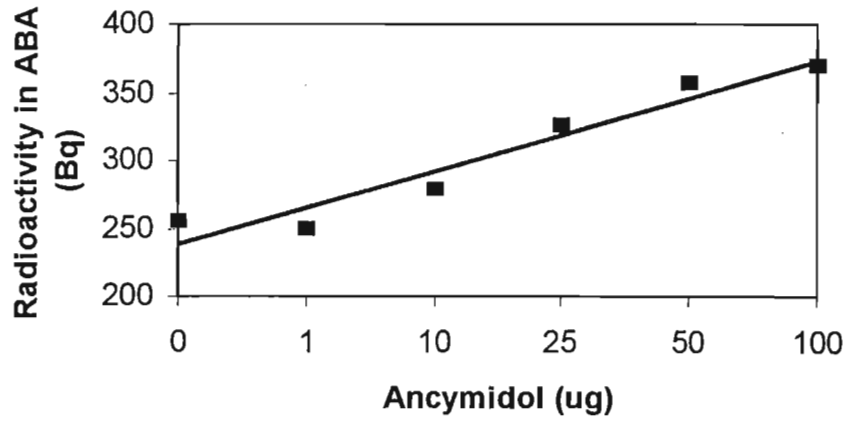

Figure 3.3 - Effect of ancymidol on [^{14}C]MVL incorporation into ABA in ripening avocado mesocarp. Substrate (5.5 MBq) was applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) with or without ancymidol (0, 1, 10, 25, 50, 100 or 200 µg) via the cut surface and incubated at 25°C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

3.2.4.3 EFFECT OF TRANSITION METALS

The last biosynthetic steps between XAN and ABA in avocado mesocarp are still rather unclear. Milborrow *et al.* (1997b) suggested the involvement of a molybdenum-cofactor aldehyde oxidase enzyme at this level. As per its name, this enzyme is activated by molybdenum, but has also been found to be inactivated by tungstate (Rajogopalan and Johnson, 1992). Incubation of mesocarp tissue with labelled MVA together with tungstate would therefore be expected to result in reduced label incorporation in ABA, if such a Mo-Co enzyme is involved in de novo ABA biosynthesis. Furthermore, tungstate is expected to cause the accumulation of an aldehyde that would be a later precursor to ABA. The identification of this compound would enable the localisation of the site of action of this enzyme in the pathway. The effect of tungstate on ABA biosynthesis from various [^{14}C]-labelled precursors was investigated, as this might provide interesting information about the biosynthetic pathway followed from each precursor. The results presented in Table 3.4 show that tungstate only slightly decreased glucose and pyruvate incorporation into ABA.

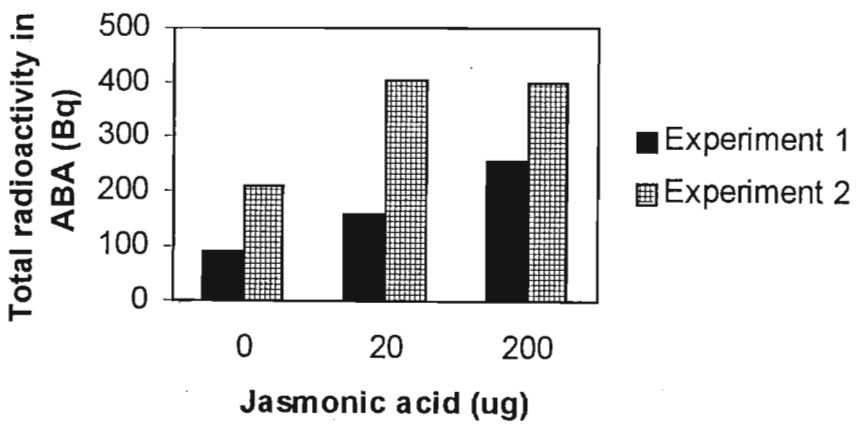

Figure 3.4 Effect of jasmonic acid on [^{14}C]MVL incorporation into ABA in ripening avocado mesocarp. In each experiment, substrate (5.5 MBq) was applied to mesocarp of the same fruit in solutions of Tween20: acetone:water (1:1:8, v/v) with or without jasmonic acid (0, 20 or 200 μg) via the cut surface and incubated at 25^{o}C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

However, acetate incorporation is reduced by about 26%, while incorporation from MVL is reduced by over 55%. These result could indicate that most of the glucose, pyruvate and acetate is converted to ABA *via* a pathway that does not require the use of a MoCo-requiring aldehyde oxidase. In the case of MVL, this substrate seems to be mainly converted to ABA *via* a MoCo-requiring step.

Table 3.4 - Effect of NaW on [^{14}C]acetate, -glucose, -MVL and –pyruvate incorporation into ABA in ripening avocado mesocarp. Substrate (5.5 MBq) was applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) with or without NaW (0 or 200 μg) via the cut surface and incubated at 25^{o}C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

Precursor	NaW (μg)	Radioactivity in ABA (Bq)	% incorporation
[1-^{14}C]Acetate	0	27	4.91E-04
	200	20	3.64E-04
[1-^{14}C]Glucose	0	24	4.36E-04
	200	22	4.00E-04
[2-^{14}C]MVL	0	445	8.09E-03
	200	198	3.60E-03
[1-^{14}C]Pyruvate	0	20	3.64E-04
	200	18	3.27E-04

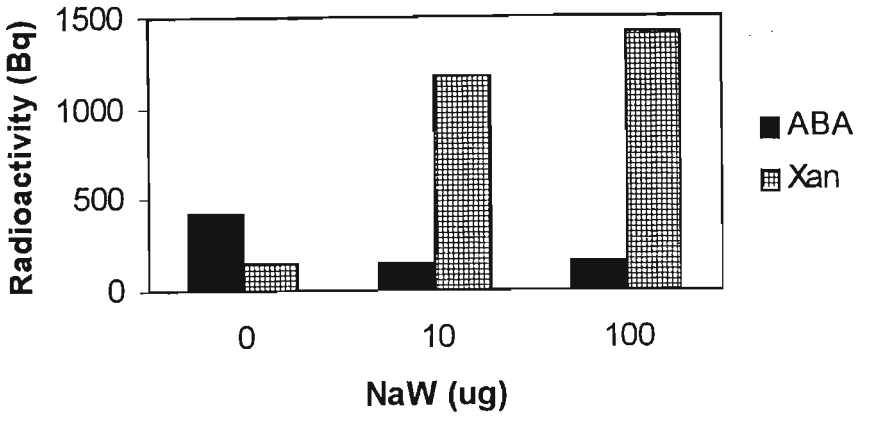

Figure 3.5 - Effect of NaW on [^{14}C]MVL incorporation into XAN and ABA in ripening avocado mesocarp. Substrate (5.5 MBq) was applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) with or without NaW (0, 10 or 100 μg) via the cut surface and incubated at 25^{o}C for 24 h in a water-saturated environment. ABA and XAN were extracted, purified and analysed as described in Materials and Methods.

However, as there is still a large proportion of labelled ABA biosynthesised from [^{14}C]MVL in the presence of tungstate, this might imply that some MVL could be converted to ABA *via* a route not involving a MoCo-requiring enzyme. The accepted biosynthetic pathway according to which MVA is converted to carotenoids, then to XAN and finally to ABA by a MoCo requiring enzyme was then investigated in some detail. [^{14}C]MVL was fed to mesocarp tissue in the presence of various concentrations of tungstate, and ABA and XAN analysed for radioactivity. Data in Figure 3.5 show that tungstate not only reduces the incorporation of MVL into ABA, but also causes a concomitant increase in incorporation in XAN. This result can be interpreted in two ways. First it might be taken as strongly supporting the role of XAN as the last intermediate between MVL and ABA, as the MoCo enzyme is reportedly the last in the pathway, and its inhibition causes label to accumulate in XAN, which is then probably the substrate for this enzyme. Another interpretation is that there is a MoCo enzyme along the ABA biosynthesis pathway, and therefore tungstate blocks label incorporation into ABA. This being said, it is still possible that the supplied labelled

MVL is metabolised into XAN following a pathway not involving a MoCo enzyme. As the ABA pathway is blocked by tungstate, more of the supplied substrate is made available for XAN biosynthesis, resulting in stronger labelling of XAN.

The use of transition metal ions can also shed some light on the widely accepted theory according to which the respiratory climacteric is a prerequisite for ethylene and ABA biosynthesis (Lee and Milborrow, 1997b). ACC is converted to ethylene by the enzyme ACC oxidase, which is inhibited by is cobalt (Yu and Yang, 1979). Feeding the system with labelled MVL in presence of cobalt should give some information as to the dependence - or independence - of ABA biosynthesis relative to ethylene production. There are no reports on the effect of nickel on ACC oxidase, but this metal ion has been included in the set of experiments because its physico-chemical characteristics might enable it to interfere with the cobalt binding site on the enzyme, and have an effect on ABA biosynthesis.

As shown in Table 3.5, cobalt and nickel were found to cause an increase in MVL incorporation into ABA. As the cobalt treatment is expected to have interrupted

Table 3.5. Effect of cobalt and nickel on [^{14}C]MVL incorporation into ABA in ripening avocado mesocarp. Substrate (5.5 MBq) was applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) with or without transition metal (0 or 5 μg) via the cut surface and incubated at 25^{o}C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

Transition metal (5µg)	Radioactivity in ABA (Bq)	% incorporation
None (Control)	315.40	5.73E-03
Cobalt	384.22	6.99E-03
Nickel	418.56	7.61E-03

ethylene biosynthesis, results show that ABA production does not appear to be halted by this event. The accepted link between the ethylene and ABA production might therefore be no more than a coincidence, ethylene production apparently not being a pre-requisite for ABA biosynthesis. On the contrary, results hint that ethylene might somehow downregulate ABA biosynthesis. The results obtained with nickel are more difficult to explain, as little literature is available on the role of this transition metal ion in plant biochemistry (Watt and Ludden, 1999). Iscan et al. (2000) have however reported that, in animal tissue, nickel decreased cytochrome P450 levels significantly. If nickel has the same effect on cytochrome P450 levels in avocado mesocarp, this might explain the significant increase in label incrporation into ABA, as ABA breakdown would be reduced by nickel.

3.3 SUMMARY OBSERVATIONS

The results presented in this chapter can be interpreted as follows:

-Naturally occurring XAN, ABA, PA and DPA have been unequivocally identified in ripening avocado fruit mesocarp.

-MVL is incorporated into ABA at significantly higher levels than acetate and glucose,
pyruvate being the precursor giving the lowest incorporation.

-It must however be kept in mind that pyruvate was labelled in position 1, this carbon being lost if pyruvate is converted to ABA *via* the triose phosphate pathway.

-Pyruvate is incorporated into ABA at low levels, but into XAN at high levels.

-Mevastatin did not significantly affect the incorporation of pyruvate, glucose or acetate into ABA, questioning the involvement of the mevalonate pathway in ABA biosynthesis from these precursors.

-Mevastatin did not increase incorporation of labelled MVL into ABA, although the dilution by *de novo* MVA was theoretically reduced.

-AMO 1618 and fluridone both reduced MVL incorporation into ABA, in accordance with the carotenoid route of ABA formation from MVL.

-However, the presence of substantial amounts of labelled ABA in both the AMO 1618- and the fluridone-treated tissue question the universality of this biosynthetic route.

-Ancymidol increased MVL incorporation into ABA, probably *via* cytochrome P450

inhibition of ABA catabolism, resulting in accumulation of ABA and label therein.

-Jasmonic acid increased MVL incorporation into ABA, probably by stimulating carbon flux through carotenoids, therefore making more labelled carotenoid precursors available for ABA biosynthesis.

-Tungstate had no significant effect of glucose and pyruvate incorporation into ABA, suggesting that no MoCo enzyme is involved in their conversion to ABA.

-Tungstate caused a 26% and 55% decrease respectively in acetate and MVL incorporation into ABA, suggesting that at least some of the acetate and MVL is converted to ABA *via* a pathway involving a MoCo enzyme.

-The noticeable amount of acetate and MVL incorporated into ABA in the presence of tungstate might indicate that some acetate and MVL is converted to ABA *via* a route not involving a MoCo enzyme.

-Tungstate causes an increase in MVL incorporation into XAN concomitant with a decrease in MVL incorporation in ABA.

-This could indicate that XAN is the substrate of the MoCo enzyme.

-This could also mean that XAN is synthesised from MVL *via* a pathway not involving a MoCo enzyme.

-Cobalt did not impair *de novo* ABA biosynthesis, on the contrary it increased it.

-Nickel also increased MVL incorporation into ABA.

CHAPTER 4

DIFFERENTIAL LABELLING OF *cis,trans*- AND *trans,trans*-ABSCISIC ACID FROM [^{13}C]ACETATE AND [^{13}C]GLUCOSE, AND EVIDENCE FOR GLUCOSE-INDUCED STIMULATION OF ABSCISIC ACID METABOLISM

4.1 INTRODUCTION

Genetic and biochemical studies using plant tissue systems have confirmed that ABA is an apocarotenoid derived from the mevalonate pathway *via* the metabolism of 9'-*cis*-xanthophylls (Parry, 1993; Cowan and Richardson, 1997; Schwartz *et al.* 1997a). Since xanthophyll formation is restricted to plastids it might be expected that ABA would arise *via* intraplastidic isoprenoid biosynthesis. Although several reports have suggested chloroplast autonomy for terpenoid synthesis in plants (Ramachandra Reddy and Das, 1987; Schulze-Siebert and Schultz, 1987*a*; 1987*b*; Schulze-Siebert *et al.* 1987) the pathway of formation of plastid isoprenoid compounds remains the subject of intense debate (Liedvogel, 1986; Bach, 1987; 1995; Gray, 1987; Kleinig, 1989; Chappell, 1995). A novel route leading to the formation of IDP was recently described for species of eubacteria that do not use the acetate/mevalonate pathway for isoprenoid biosynthesis, but instead uses precursors derived from triose phosphate metabolism (Rohmer *et al.* 1993). Similarly, McCaskill and Croteau (1995), and Loreto *et al.* (1996) obtained good evidence to support the biosynthesis of monoterpenes from plastid- and not cytosolic (MVA)-derived IDP. Confirmation of

operation of a non-mevalonate pathway of IDP synthesis in plant cells has recently been obtained (Schwender *et al.* 1996). Using ^{13}C-labelled glucose and acetate, these authors demonstrated that the ^{13}C-labelling pattern of all plastid isoprenoids in *Scenedesmus obliquus* proceeded *via* the glyceraldehyde 3-phosphate/pyruvate route. In this pathway, IDP formation involves condensation of a C_2 unit, derived from pyruvate decarboxylation, with glyceraldehyde-3-phosphate followed by a transposition to yield the C_5 isoprene skeleton.

On the basis of this series of investigations, experiments were designed to investigate the source of carbon for ABA biosynthesis because of the large body of literature which, in contrast to the above, clearly support the incorporation of [^{14}C]- and [^{3}H]MVL into ABA (Milborrow and Robinson, 1973; Milborrow, 1976, 1978; Cowan and Railton, 1987 c; Lee and Milborrow, 1997a,b).

Formation of MVA is generally accepted to occur by the condensation of three acetyl-CoA units to HMG-CoA, which is then reduced to MVA by HMGR. Interestingly, treatment of developing avocado fruit with mevastatin, a specific inhibitor of HMGR, caused a dramatic increase in ABA concentration (Cowan *et al.* 1997). In an attempt to explain this observation the authors suggested that ABA might be derived by an alternative pathway not involving MVA. In addition, labelling studies using either [^{14}C]- or [^{3}H]MVL have revealed that incorporation of radioactivity into ABA by avocado mesocarp seldom exceeds 0.1% (Milborrow, 1976). While this might be attributed to the spectrum of isoprenoids produced by avocado it could also suggest that the bulk of carbon required for ABA biosynthesis is not derived *via* MVA. Results presented in Table 3.3 show a slight reduction in [^{14}C]MVL incorporation into ABA in the presence of mevastatin. This is in accordance with the previous hypothesis, as if MVA was the precursor for most of the ABA biosynthesised, inhibition of HMGR should have increased label incorporation into ABA by reducing dilution of the labelled precursor.

The effect of glucose on ABA metabolism was also investigated in light of the suggestion by Lichtenthaler and Rohmer (1996) that IDP, derived from the metabolism of glucose (i.e. triose phosphates) and not MVA, acted as the sole precursor for carotenoid biosynthesis in green algae and a range of higher plants including, *Lemna, Daucus* and *Hordeum*.

Sugars are an important source of energy and supply the carbon skeletons for growth and development of plants and plant parts. In addition, sugars act as signalling

molecules and the associated transduction pathways impact on both plant metabolism and plant development (Koch, 1996). While the importance of sugar-mediated development is gradually being realised (Jang and Sheen, 1994; Sheen, 1994; Jang *et al.* 1997; Smeekens and Rook, 1997), very little if anything is known about the effect of sugars on plant hormone metabolism. Thus, the link between alterations in plant hormone concentration, changes in sugar content and composition and plant growth and development remains poorly defined.

The present investigation describes experiments that were carried out to: i) determine whether ABA could be produced from either from acetate or glucose *via* a route other than that involving MVA; and ii) investigate the influence of glucose on ABA metabolism.

4.2 ABA BIOSYNTHESIS FROM ACETATE AND GLUCOSE

4.2.1 INCORPORATION OF [^{14}C]-LABELLED ACETATE, GLUCOSE AND MVL INTO ABA

Initially, the ability of acetate and glucose to act as precursors of ABA was investigated by comparing the amount of radioactivity in ABA from [^{14}C]-labelled MVL with that obtained using [^{14}C]-labelled acetate and glucose. Results in Table 4.1 show that glucose and acetate labelled ABA similarly based on amount of radioactivity supplied, and that the intensity of labelling was approximately 70% less than that from *R*-[2-^{14}C]MVL. However, [2-^{14}C]acetate labelled ABA more intensely than both MVL and glucose on a mass-to-mass basis. In addition, glucose suppressed the accumulation of endogenous ABA in mesocarp of ripening avocado by approximately 55% which might have accounted for the low incorporation observed. This preliminary observation suggested that acetate, and hence MVL, was a precursor to ABA in a pathway not originating from glucose and that the latter, was capable of interfering with ABA metabolism. To address these issues in more detail the incorporation of label from either [1-^{13}C]acetate or [1-^{13}C]glucose into ABA was studied by GC-PICI-MS, and the influence of glucose on ABA metabolism in avocado mesocarp determined.

Table 4.1 - Incorporation of radioactivity from [^{14}C]-labelled acetate, glucose, and MVL into ABA, and effect of substrate on endogenous ABA concentration, in ripening avocado mesocarp. Substrates (0.04 or 0.45 μmole) were applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) via the cut surface and incubated at 25°C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

Substrate	Mass (μmole)	Radioactivity in ABA (Bq)	% incorporation	ABA (nmol.g^{-1} DW)
[1-^{14}C]Acetate	0.04	139.49	0.0152	9.88±1.86
	0.45	1554.87	0.0172	9.65±2.01
[1-^{14}C]Glucose	0.45	135.62	0.0148	5.19±0.89
[2-^{14}C]MVL	0.45	498.57	0.0544	8.94±1.25

4.2.2 INCORPORATION OF [^{13}C]-LABELLED ACETATE, GLUCOSE AND MVL INTO ABA

Theoretical biochemistry dictates that the metabolism of [1-^{13}C]acetate *via* the classical acetate/mevalonate pathway would yield [1,3-^{13}C]IDP. By comparison, metabolism of acetate *via* the glyoxylate shunt followed by condensation of the products, pyruvate and glyceraldehyde phosphate, as suggested by Schwender *et al.* (1996) would result in the formation of IDP labelled with ^{13}C only at position 4. Since sesquiterpenoids comprise three isoprene units, incorporation of ^{13}C from acetate into ABA might be expected to give product ABA with either M^++ 6 amu (classical acetate/mevalonate pathway) or M^++ 3 amu (novel triose phosphate pathway) and the anticipated position of the ^{13}C label in ABA is shown in Figure 4.1A and B. From [1-^{13}C]glucose, [2-^{13}C]acetyl-CoA is formed *via* glycolysis and decarboxylation of pyruvate by pyruvate dehydrogenase. By the classical acetate/mevalonate pathway [2,4,5-^{13}C]IDP would be formed whereas the novel triose phosphate pathway of IDP synthesis yields [1,5-^{13}C]IDP (Schwender *et al.* 1996) and the expected pattern of ^{13}C-

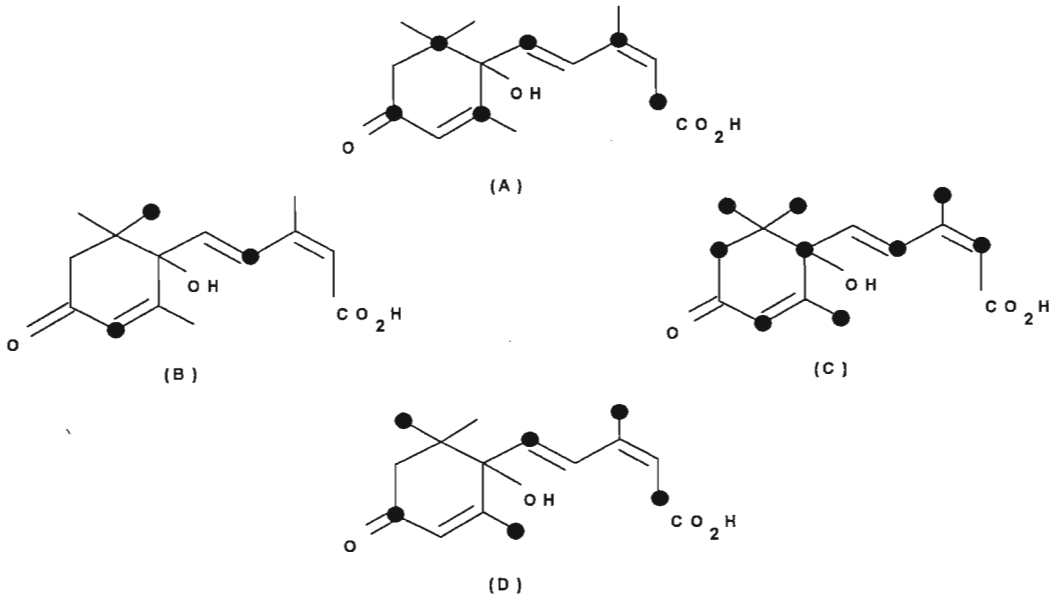

Figure 4.1 - Suggested position of ^{13}C label in ABA formed from IDP derived from [1-^{13}C]acetate by either the classical acetate/mevalonate (A) or the triose phosphate (B) pathway; and [1-^{13}C]glucose by either the acetate/mevalonate (C) or the triose phosphate (D) pathway (after Schwender et al., 1996).

labelling in ABA formed from each of these species of IDP is depicted in Figures 4.1 C and D. In a series of experiments designed to test the above hypothesis, [1-^{13}C]glucose and [1- ^{13}C]acetate were supplied to mesocarp of ripening avocado fruit and the incorporation of ^{13}C-label in ABA monitored over time using GC-PICI-MS. The results in Table 4.2 show that ^{13}C enrichment of the quasimolecular ion (*m/z* 279) and of the base peak (*m/z* 261) was consistent with acetate metabolism *via* the classical acetate/mevalonate pathway with a resultant labelling pattern in ABA of $[MH]^+$+ 6 atomic mass units. By comparison, labelling of ABA from [1-^{13}C]glucose was in contrast to formation of [2-^{13}C]acetyl-CoA *via* glycolysis and operation of a pyruvate dehydrogenase complex, indicating the possibility that glucose had been metabolised *via* the novel triose phosphate pathway and that the ABA thus formed was derived from [1,5-^{13}C]IDP. Analysis of both *cis,trans*- and *trans,trans*-ABA (Fig. 4.2), confirmed that [1-^{13}C]glucose and [1-^{13}C]acetate labelled ABA with an

intensity of $[MH]^+$ + 6 amu and that these substrates were therefore metabolised differently en route to ABA. Although produced in greater abundance, *cis,trans*-ABA was always less intensely labelled than its all-*trans* counterpart (Fig. 4.2). This result must suggest that *cis,trans*-ABA and *trans,trans*-ABA are derived independently.

Table 4.2 - Intensity of ^{13}C-labelling in the $[MH]^+$ quasimolecular ion and the $[MH - H_2O]^+$ ion of cis,trans-ABA determined by GC-PICI-MS analysis of the methyl ester derivative. [1-^{13}C]acetate or [1-^{13}C]glucose was supplied to mesocarp tissue of the same fruit in Tween 20:acetone:water (1:1:8, v/v) and incubated at 25°C for 0, 24 or 48 h. ABA was extracted, purified and enrichment with ^{13}C label was determined by methane PICI-MS as described in Materials and Methods. For convenience and ease of interpretation only data for the quasimolecular ion, m/z 279, and the base peak, m/z 261, are presented.

		[1-^{13}C]acetate		[1-^{13}C]glucose	
Ion	Time	+ 3 amu[A]	+ 6 amu	+ 6 amu	+ 9 amu
(*m/z*)	(h)	(% Relative Intensity)			
279, $[MH]^+$	0	0.08	ND[B]	ND	ND
	24	0.10	0.08	0.07	ND
	48	0.14	0.18	0.12	ND
261, $[MH - H_2O]^+$	0	0.42	ND	ND	ND
	24	0.55	0.12	0.14	ND
	48	0.50	0.50	0.29	ND

[A]amu = atomic mass unit.

[B]ND = not detected.

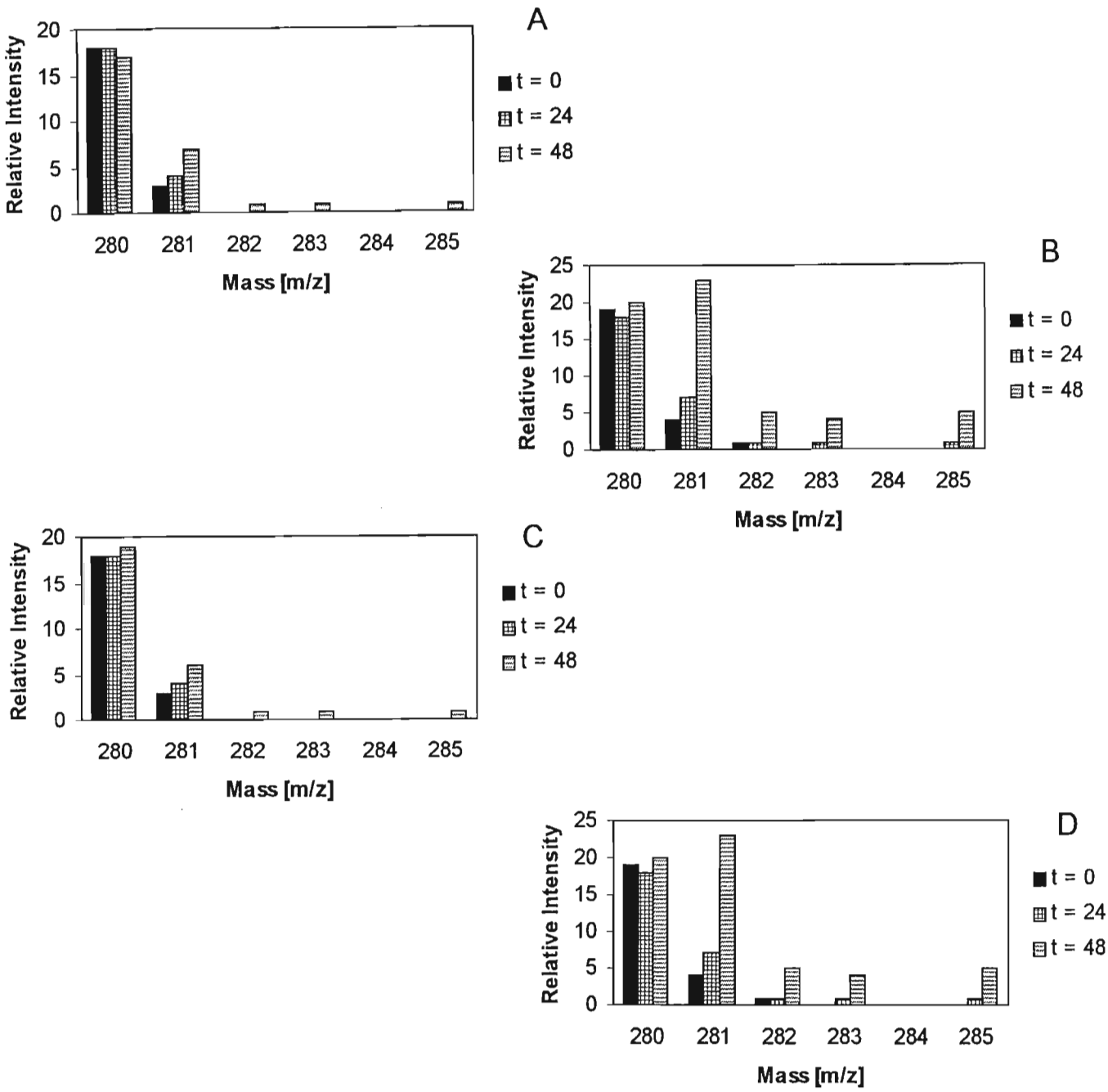

Figure 4.2 - Molecular ion peaks of ^{13}C-labelled cis,trans-ABA (A and C) and its all-trans-isomer (B and D) extracted from mesocarp of ripening avocado fruit supplied either [1-^{13}C]acetate (A and B) or [1-^{13}C]glucose (C and D). Substrate (100 mg) was supplied to mesocarp of one half of an avocado fruit (100 g fresh weight) in Tween20:acetone:water (1:1:8, v/v) and the tissue incubated in a water-saturated environment for 0, 24 or 48 h. ABA was extracted, purified and enrichment with ^{13}C label was determined by methane PICI-MS as described in Materials and Methods. Data are expressed relative to the intensity of the molecular ion of unlabelled ABA (m/z = 279).

4.3 GLUCOSE MODULATION OF ABA METABOLISM

In view of the data presented in Table 4.1 and 4.2 which indicated that glucose could cause up to a 55 percent reduction in mesocarp ABA content, and that glucose could be incorporated into ABA respectively, the effect of glucose on ABA biosynthesis in mesocarp of ripening avocado fruit was further investigated.

The results in Table 4.3 confirmed the observation that glucose caused a significant reduction in mesocarp ABA level. The data shown in Figure 4.3 further showed that this reduction in ABA level occured concomitantly with a decrease in PA and DPA, while the XAN level was not found to be markedly affected by glucose.

Studies using [^{14}C]-labeled MVL in conjunction with glucose showed that low amounts of glucose had little or no effect on the incorporation of [^{14}C] from *R*-[2-^{14}C]MVL into ABA, whereas at the higher concentration (i.e. 5.0 µmoles) this process was stimulated (Table 4.4).

Table 4.3 - Effect of D-glucose on net ABA accumulation in mesocarp of ripening 'Hass' avocado fruit. Mesocarp of ripening avocado fruit was supplied solutions of Tween 20:acetone:water (1:1:8, v/v) containing glucose (0, 0.5 or 5.0 µmoles) via the cut surface and incubated at 25°C for 24 h in a water-saturated environment. ABA was extracted, purified and quantified as described in Materials and Methods. Data are the average of two experiments and are expressed as the difference between final (t_{24}) and initial (t_0) values.

Mass of glucose (µmoles)	ABA content (µg.g^{-1} DW)	% of control
0 (Control)	5.76	(100)
0.5	2.88	50
5.0	0.72	13

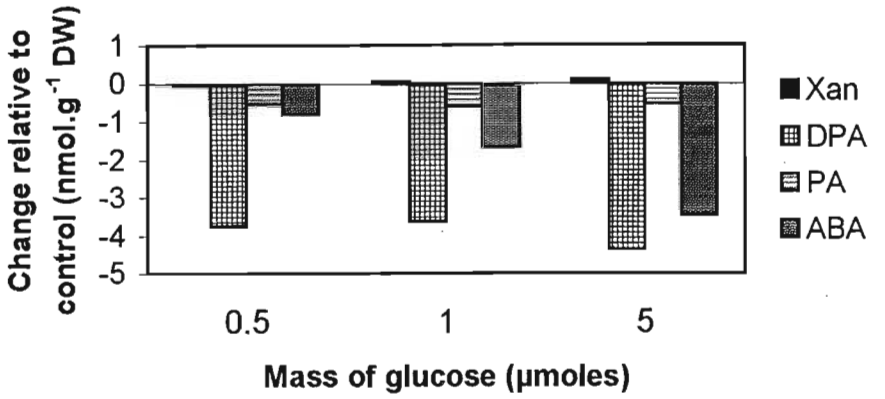

Figure 4.3 - Effect of increasing concentration of D-glucose on ABA metabolism in mesocarp of ripening avocado fruit. In each experiment glucose (0, 0.5, 1.0 or 5.0 µmoles) was applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) via the cut surface and incubated at 25°C for 24 h in a water-saturated environment. ABA, XAN, PA and DPA were extracted, purified and analysed as described in Materials and Methods. Data are the average of two experiments, and are expressed as the difference between final (t_{24}) and initial (t_0) values.

Table 4.4 - Effect of glucose on the incorporation of [2-^{14}C]MVL into ABA by mesocarp of ripening avocado fruit. In each experiment [2-^{14}C]MVL (140 kBq) and glucose (0, 0.5, 1.0 or 5.0 µmoles) were applied to mesocarp of the same fruit in solutions of Tween20:acetone:water (1:1:8, v/v) via the cut surface and incubated at 25^{o}C for 24 h in a water-saturated environment. ABA was extracted, purified and analysed as described in Materials and Methods.

	Mass of glucose (µmoles)	Radioactivity in ABA (Bq)	ABA specific activity (Bq/nmol)
Experiment 1	0	24.40	0.42
	0.5	23.43	1.33
	1.0	23.31	1.35
	5.0	78.01	6.98
Experiment 2	0	21.32	0.80
	0.5	22.49	1.02
	1.0	26.22	1.26
	5.0	58.42	2.61

4.4 SUMMARY OBSERVATIONS

The results presented in this chapter can be interpreted as follows:

-Acetate and glucose label ABA similarly in terms of percent incorporation into ABA.

-MVL labels ABA 70% more effectively than both acetate and glucose.

-Acetate labels ABA more intensely than both MVL and glucose on a mass-to-mass basis.

-glucose suppresses accumulation of endogenous ABA in ripening avocado mesocarp, which might account for the low incorporation from glucose.

-^{13}C enrichment studies indicate that ABA is formed from acetate *via* the acetate / mevalonate pathway.

-similar studies with glucose disagree with the conversion of the precursor to ABA *via* formation of [2-^{13}C]acetyl CoA by glycolysis

-^{13}C labelling of *cis,trans* and *trans,trans* ABA from acetate and glucose indicate that these substrates were metabolised following different routes, suggesting that the isomers are not formed by the same pathway.

CHAPTER 5

DISCUSSION AND CONCLUSION

The ABA biosynthetic pathway in plants is still a contentious issue, and very little is known about the enzymes involved, especially between the proposed precursor XAN and ABA. The origin of carbon for ABA production also remains unknown. The aim of this research was therefore to investigate the origin of ABA in ripening avocado mesocarp, and attempt to elucidate the steps between XAN and product ABA. The identity of ABA, XAN, PA and DPA as endogenous compounds in ripening avocado mesocarp and unequivocally established by GC-MS. Labelling studies revealed that radioactivity from exogenous acetate, mevalonate, pyruvate and glucose could be detected in ABA. However, [2-^{14}C]MVL was better incorporated into ABA than [1-^{14}C]glucose, [1-^{14}C]pyruvate and [1-^{14}C]acetate. Further studies using higher doses of [1-^{14}C]acetate, [1-^{14}C]glucose and [2-^{14}C]MVL confirmed this finding, although on a mass-to-mass basis [1-^{14}C]acetate labelled ABA more intensely than the other substrates. To investigate the biosynthetic origin of the carbon used to produce ABA, the relative contribution of the classical acetate / mevalonate and non-mevalonate pathways was evaluated using ^{13}C-labelled acetate and glucose followed by physico-chemical analysis of the purified ABA. The results indicate, tentatively, that enrichment of ABA with ^{13}C is in accordance with acetate being metabolised to ABA *via* the mevalonate pathway, while glucose was converted to ABA *via* the non-mevalonate pathway. In addition, differential labelling of the *cis,trans-* and *trans,trans-* isomers of ABA (each isomer similarly labelled whether from acetate or from glucose) indicated that these isomers were not synthesised by the same pathway. Together, the results of the biochemical studies indicated that both the mevalonate and non-mevalonate pathways of IDP synthesis contributed carbon to ABA production in ripening avocado. To further elucidate this aspect, chemical inhibitors (and modifiers) were used in an attempt to manipulate carbon flux into ABA. Thus, mevastatin (which inhibits MVA formation and bulk cytosolic isoprenoid synthesis) had no significant effect on incorporation of

radioactivity from acetate, glucose and pyruvate into ABA. AMO 1618 and fluridone, two inhibitors of carotenoid biosynthesis, reduced incorporation of MVL into ABA. Jasmonic acid, a reported stimulator of carbon flux through carotenoids, increased the incorporation of MVL into ABA. Tungstate had no significant effect on glucose and pyruvate incorporation into ABA, while it markedly reduced acetate and MVL incorporation into ABA. However, the decrease in MVL incorporation into ABA was concomitant with increased incorporation into XAN. Cobalt, an inhibitor of the climacteric-induced formation of ethylene, was found to increase incorporation of MVL into ABA. Nickel, a reducer of cytochrome P450 levels in mice liver tissue, also increased incorporation of MVL into ABA.

5.1 RIPENING AVOCADO FRUIT AS A SYSTEM FOR ABA BIOSYNTHETIC STUDIES

Ripening avocado fruit is a biological system that has been used over the last three decades to investigate aspects of the ABA biosynthetic pathway. This fruit is the richest source of ABA, and it converts labelled MVL into ABA more efficiently than any other tissue. In addition, *de novo* ABA synthesis from applied precursors is very rapid despite the already high endogenous ABA concentration. (Milborrow, 1974). Unlike other tissues, which require some form of stress to trigger ABA biosynthesis (Wright, 1969; Milborrow and Noddle, 1970; Wright and Hiron, 1969, 1972), ABA biosynthesis in avocado mesocarp is unaffected by wilting (Milborrow and Robinson, 1973). The incubation conditions used in this research (water-saturated environment) therefore did not affect the tissue's ability to synthesise ABA, but were aimed at retarding the dehydration of the tissue, allowing for cellular metabolic activity to proceed for as long as possible during incubation. It is also important to note that although some experiments in this study were carried out only once, this was largely due to the cost of radioactive chemicals and labelled substrates, and the fact that the amount of tissue is limited in one fruit. The effect of this reduced number of replicates on the value of observed results was counterbalanced by attentive extraction and purification of compounds of interest and the knowledgeable use of high precision scientific instruments to measure their levels.

5.2 ORIGIN OF CARBON FOR ABA BIOSYNTHESIS IN AVOCADO

It has been generally accepted that the carbon source for ABA biosynthesis in all plant tissues is provided by the mevalonate pathway, in the form of isopentenyl diphosphate (IDP). IDP is the universal building block for all isoprenoids (Cvejic and Rohmer, 2000). The acetate / mevalonate pathway has been regarded as the sole biosynthetic route to IDP (Gray, 1987). However, recent evidence has indicated the existence of a mevalonate-independent pathway (Rohmer *et al.,* 1993). Schwender *et al.* (1996) established the existence of such a pathway in the unicellular alga *Scenedesmus,* where it was responsible for carotenoid production. Lichtenthaler *et al.* (1997) showed that monocotyledonous and dicotyledonous plants formed sterols from mevalonate in the cytoplasm, while in chloroplasts carotenoids and other isoprenoids were derived from IDP formed *via* the triose / pyruvate pathway. Abundant literature is now available, reporting on the occurrence of this pathway in various plant tissues / systems (Milborrow and Lee, 1998a; Schwender *et al.*, 1997; Lange and Croteau, 1999; Adam and Zapp, 1998; Adam *et al.*, 1999; Eichinger *et al.*, 1999; Araaki *et al.*, 2000). The triose / pyruvate pathway involves the formation of 1-deoxy-D-xylulose-5-phosphate (DOXP) from glyceraldehyde-3-phosphate and pyruvate catalysed by the enzyme DOXP synthase (Lichtenthaler, 1998). DOXP is subsequently transformed to 2-C-methyl -D-erythritol 4-phosphate by intramolecular C-C skeleton rearrangement (Zeidler *et al.*, 1997; Schwender *et al.*, 1997; Arigoni *et al.*, 1997). The latter compound then gives rise to IDP (Lichtenthaler, 1998). The IDP pool, which is the source of carbon for ABA biosynthesis (and isoprenoids in general), is therefore fed by both the DOXP and the mevalonate pathways. The IDP biosynthetic pathway used in the formation of a terpenoid can be identified by analysing the ^{13}C-atoms incorporated from [1-^{13}C]glucose or [1-^{13}C]acetate into the compound of interest (Adam and Zapp, 1998). Depending on the pathway followed, glucose and / or acetate are metabolised differently, resulting in a specific ^{13}C labelling pattern of the isoprene unit (for details, see Section 4.2.2). Since the IDP molecule is differently labelled, so are the compounds arising from it, including ABA.

In this study, the PICI mass spectra of *cis,trans* and *trans,trans* ^{13}C-ABA showed that the enrichment patterns were in accordance with ABA being formed from acetate *via*

the acetate / mevalonate pathway and glucose *via* the DOXP pathway (see Table 4.2). These results were unfortunately not confirmed by NMR analysis due to the lack of sufficient mass of labelled compound, but the mass spectra clearly indicate enrichment of the ABA molecule with ^{13}C by 6 atomic mass units. The glucose data is therefore in accordance with the reported occurrence of the DOXP pathway in chloroplasts derived from avocado fruit tissue (Milborrow and Lee, 1998a). Glucose is converted to pyruvate in glycolysis and it is the latter compound which is metabolised to IDP *via* the DOXP pathway. On the other hand, the ABA spectra obtained after feeding [1-^{13}C]acetate revealed that ABA was formed from IDP which had been metabolised *via* the classical acetate / mevalonate pathway. The two known pathways of IDP synthesis therefore seem to both contribute to ABA biosynthesis in ripening avocado fruit.

Another interesting aspect of the ABA spectra is that the *cis,trans* and *all-trans* isomers of ABA were found to be differently labelled (see Figure 4.2). This observation must suggest that they are synthesised by two different pathways, and occur not as a result of *in vivo* isomerisation as has previously been postulated. This possibility cannot be excluded, but it would be a very unusual occurrence for a tissue to have two different pathways for the biosynthesis of isomers of the same compound. Chemical synthesis of XAN was achieved in our laboratory using 4-keto-XAN-acid as an intermediate (Walljee, 1999), and this compound was shown to have the identical EI mass spectrum to that of ABAMe (same molecular mass, same base peak, same major ion peaks, see Figure 5.1). It is therefore possible that the compound referred to as *cis,trans* ABA (see Chapter 4) might in fact be 4-keto-XAN-acid, which would explain the differential labelling between the two supposed isomers of ABA. These compounds are very similar chemically and they cannot be separated by the commonly used TLC and HPLC purification procedures described in Materials and Methods. Only NMR analysis and rigorous chemical studies would allow for their characterisation.

Studies using [2-^{14}C]MVL, [1-^{14}C]acetate, [1-^{14}C]glucose and [1-^{14}C]pyruvate clearly show that MVL is markedly better incorporated into ABA than the other three substrates. MVL is hydrolysed *in vivo* to MVA, which is converted to IDP *via* MVA-5-phosphate and MVA-5-pyrophosphate. Acetate, as discussed above, is metabolised to IDP *via* the mevalonate pathway. The fact that it is less strongly incorporated into ABA than MVL is expected, as acetate first has to be metabolised to MVA, *via*

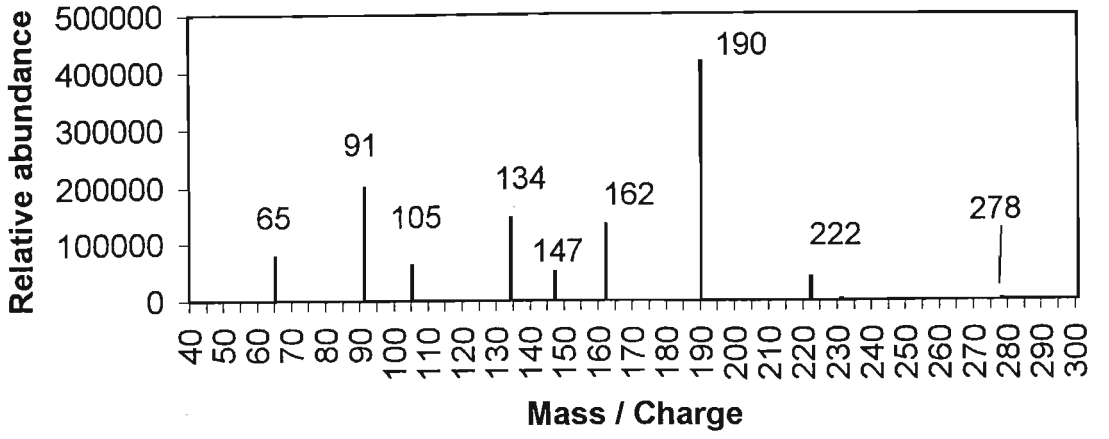

Figure 5.1 Electron impact mass spectrum of the methyl ester derivative of 4-keto-XAN-acid, and chemical structure of 4-keto-XAN-acid. The m/z 125 ion associated with the side chain of dienoic acids was apparently omitted in the mass spectrum presented (after Walljee, 1999).

acetyl-CoA, acetoacetyl-CoA and 3S-hydroxymethylglutaryl-CoA (HMGCoA), before being converted to IDP. Being further away from IDP in the pathway, it is expected that incorporation of this precursor will be lower than that of MVL, as it can be used *in vivo* by various pathways other than the mevalonate pathway. The very low label incorporation from pyruvate is also expected. The pyruvate used was labelled at position 1. Whether pyruvate is converted to hydroxyethyl thiamine and then metabolised *via* the DOXP pathway, or converted to acetyl-CoA and then metabolised *via* the mevalonate pathway, the first transformation results in the loss of the carbon 1 as CO2. No labelling of ABA was therefore expected with this precursor. Labelling from glucose was also expected to be rather low. Sucrose is the main form in which carbon and energy are translocated in plants, and is hydrolysed to produce glucose and fructose, which serve as carbon sources for a range of pathways. Glucose is utilised by pathways other than those leading to IDP formation, and the sugar must enter the glycolytic pathway to be converted to pyruvate and subsequently to IDP and ABA. Loss of label along this route is therefore to be expected. The poor incorporation obtained with glucose may also be due to the observed glucose-induced decrease in endogenous ABA levels (Table 4.3). This decrease in ABA levels, coupled with the observed glucose-induced increase in MVL incorporation into ABA, suggests that glucose may have some inhibiting effect on the mevalonate pathway,

reducing *de novo* MVA production and subsequent unlabeled-IDP formation. In fact this aspect has recently been demonstrated. Glucose applied to avocado fruit was shown to reduce HMGR activity and hence MVA production (Richings *et al.*, 2000). This would result in reduced total cytoplasmic IDP production, most of the IDP produced being labelled because derived from labelled MVL. The resulting ABA production would thus be expected to be lower in terms of mass, but showing stronger labelling.

5.3 CHEMICAL MODIFICATION OF THE ABA BIOSYNTHETIC PATHWAY

After establishing that the ABA biosynthetic pathway in avocado may proceed from IDP formed (1) from glucose *via* pyruvate and the DOXP pathway, (2) from acetate *via* the mevalonate pathway and (3) from MVL *via* MVA and subsequent phosphorylation and decarboxylation, various chemicals were used to investigate some of the commonly accepted characteristics and intermediates in the pathway. Mevastatin was used to block HMGR, the enzyme responsible for the conversion of HMGCoA to MVA. With the mevalonate pathway inhibited, in theory acetate incorporation into ABA is impaired, as this substrate is converted to IDP *via* the mevalonate pathway. The ca. 20% reduction in acetate incorporation into ABA in presence of mevastatin (Table 3.2) therefore confirms that this substrate is metabolised to ABA predominantly *via* the mevalonate pathway. The large percentage of labelled ABA found even in the presence of mevastatin indicates that the mevastatin treatment did not completely block all the sites of mevalonate pathway activity, either by not being able to reach these sites (compartmentation) or the amount applied being insufficient. Another explanation might have been that acetate is converted to pyruvate and then to IDP *via* the DOXP pathway, but the ^{13}C labelling data presented above seems to argue against this possibility.

The inhibition of the mevalonate pathway also caused a very slight increase in glucose and pyruvate incorporation into ABA. This is in accordance with the results discussed above, according to which glucose and pyruvate are metabolised to ABA *via* the DOXP pathway. MVL incorporation into ABA was decreased in the presence of mevastatin. The inhibition of the mevalonate pathway should, in theory, reduce

endogenous MVA production, resulting in higher label incorporation from MVL into ABA. That this was not observed might indicate (1) that the MVA pool in avocado mesocarp is naturally small, MVA (formed by *in vivo* hydrolysis of supplied MVL or by the mevalonate pathway) having a feedback inhibition effect on the mevalonate pathway, keeping endogenous production of unlabelled MVA to low levels even with no mevastatin present, or (2) that it was used for other purposes.

The involvement of carotenoids in the ABA pathway was also investigated indirectly, using carotenoid biosynthesis inhibitors. AMO 1618, an inhibitor of lycopene cyclase and GA synthesis, as well as fluridone a specific inhibitor of phytoene desaturase, both caused a significant decrease in MVL incorporation into ABA. These results are in accordance with ABA being synthesised from MVL *via* carotenoids in the chloroplast, as inhibition of *de novo* carotenoid synthesis significantly affected labelling of ABA. The role of carotenoids as precursors to ABA was further supported by the jasmonic acid-induced increase in MVL incorporation into ABA. Jasmonic acid is a plant growth regulator known to stimulate senescence and carbon flux through carotenoids, thus increasing label incorporation into carotenoids. The fact that ABA is more strongly labelled when the carotenoid pool is itself more intensely labelled suggests that ABA is derived from carotenoids. These results therefore indicate that most of the IDP produced via the mevalonate pathway in the cytoplasm was further metabolised to ABA in the chloroplast *via* carotenoids.

The final aspect of the pathway that was investigated was the involvement of a MoCo enzyme (aldehyde oxidase) commonly believed to be involved in the conversion of XAN to ABA (Lee and Milborrow, 1997b). Tungstate is known to inhibit this type of enzyme, and the presence of tungstate caused a significant decrease in MVL and acetate incorporation into ABA, while incorporation of glucose and pyruvate was virtually unchanged. This can indicate that tungstate cannot enter the chloroplast and therefore cannot inhibit further metabolism of XAN in the plastid. If glucose and pyruvate are metabolised to IDP, and converted to XAN *via* carotenoids, biochemical theory dictates that further conversion to ABA would involve an aldehyde oxidase enzyme, converting XAN to XAN-acid which in turn is converted to ABA. Another possibility is that tungstate can enter the plastid, where glucose and pyruvate are converted to ABA *via* a pathway not involving a MoCo enzyme. On the other hand, the fact that MVL and acetate incorporation into ABA is reduced by tungstate would appear to indicate that these precursors are metabolised to ABA in the cytoplasm, and

that the cytoplasmic pathway also involves a MoCo enzyme. However, the data obtained from the manipulation of carotenoid biosynthesis discussed above indicated that these precursors were being incorporated into carotenoids *en route* to ABA. This is therefore in contradiction with a pathway of ABA biosynthesis occurring from precursor to end product in the cytoplasm. It is, however, possible that IDP from the mevalonate pathway is metabolised to XAN *via* carotenoids in the chloroplast, XAN then being exported back to the cytoplasm where it is converted by a MoCo enzyme to ABA. If tungstate can enter the cytoplasm but not the chloroplast, the pathway proposed above would be in accordance with the results obtained in this study regarding ABA biosynthesis. Although the data gathered in this study do not provide unequivocal confirmation of this proposed pathway in ripening avocado fruit, they support the theory behind it. ABA being produced in large amounts during ripening in avocado fruit, and carotenoids being the basis of photoprotection in plants, it would be surprising that the plant would sacrifice its much needed carotenoids to form ABA at such a critical stage. Similarly, when tissues are stressed, the resulting increase in ABA levels would affect carotenoid content - and thus photoprotection ability - if ABA was derived from carotenoids and XAN. The importance of keeping a good carotenoid pool for photoprotection in times of stress may be what was illustrated by the incorporation of mevalonate-derived IDP into carotenoids, the cytoplastic IDP pool also being used to complement the DOXP pathway supply, probably to increase carotenoid production. It would appear more logical that the chloroplastic pathway produces 4-keto-XAN-acid as its end product, while the cytoplastic pathway makes ABA from mevalonate pathway-derived IDP.

The reported involvement of cytochrome P450 in ABA catabolism to PA was also investigated. The plant growth regulator ancymidol, which reportedly inhibits cytochrome P450, caused an increase in MVL incorporation into ABA. Even though the data do not represent unequivocal evidence of the involvement of this enzyme in ABA catabolism, it certainly is indicative.

A link between ethylene and ABA production during the climacteric was also investigated. It is generally accepted that ethylene production precedes or occurs concomitantly with the rise in ABA levels in ripening fruits (Adato *et al.*, 1979). To determine this interaction, the reported inhibition of ACC oxidase (the enzyme responsible for ethylene production in plants) by cobalt was used. The fact that cobalt and nickel are very similar ions, both in terms of size (cobalt : 0.74 Å, nickel : 0.72 Å

radius) and natural valency (they occur as Ni^{2+} and Co^{2+} in most biological systems), suggests that these ions could have the same effect on enzyme inhibition / activation, if the valency required by the enzyme is 2+ (Field, personal communication)[4]. The observed cobalt-induced increase in MVL incorporation into ABA, when ethylene production was inhibited, provided evidence that endogenous ABA production is independent of ethylene production. This evidence is of tremendous interest in the field of post-harvest treatments. These treatments tend to be directed at reducing ethylene production, based on the assumption that by reducing ethylene production ABA production would also be retarded. It however appears that reduction of ethylene production does not in fact reduce ABA production.

5.4 CONCLUSION AND FUTURE PROSPECTS

The formation of ABA from acetate, glucose, MVL and pyruvate is in accordance with the metabolism of these substrates *via* two different pathways in ripening avocado fruit. In addition, these pathways may give rise to two chemically similar compounds, both identifiable by TLC, HPLC and GC-MS as ABA. One pathway is apparently confined to the chloroplast (carbon source = DOXP pathway) and one either taking place entirely in the cytoplasm or starting in the cytoplasm and thereafter continuing in the chloroplast (carbon source = mevalonate pathway).

The pathway originating from glucose / pyruvate in the chloroplast seems to take place in the plastid from precursor to end product, inhibitors like tungstate which are active in the cytoplasm seemingly not affecting intra-chloroplastic metabolism.

Although IDP produced *via* the DOXP pathway is known to be incorporated into XAN *via* the carotenoid pathway, the further conversion of XAN to ABA in the chloroplast is still an unresolved issue. The contemporary belief is that XAN is converted to AB-aldehyde which is oxidised by an aldehyde oxidase enzyme to ABA. These studies however furnished evidence supporting the existence of a cytoplasm-localised pathway that converts IDP from the mevalonate pathway into ABA, a MoCo

[4]: *Professor J.S. Field, School of Chemical and Physical Sciences, University of Natal, Pietermaritzburg, South Africa.*

enzyme seemingly involved at some stage. It is possible therefore possible that the difficulty experienced in trying to demonstrate the incorporation of XAN into ABA is due to the fact that XAN is not metabolised to ABA, but to 4-keto-XAN-acid.

As mentioned earlier, the realisation that 4-keto-XAN-acid and ABA share the same EI-MS spectrum makes it very likely that the compound extracted from plants and commonly identified as ABA could in fact be a mixture of these two compounds. It is therefore possible that the explanation for the lack of real progress in the unravelling of the ABA biosynthetic pathway in plants *in vivo* for the last 40 years lies in the inability to differentiate between these two compounds, making the interpretation of observed results far more complex and, in all probability, inappropriate.

The prime concern for future research is therefore to investigate the natural occurrence of 4-keto-XAN-acid in plants, and to make sure that the compound identified as ABA is indeed ABA, not 4-keto-XAN or a mixture of both. Settling this issue would enable accurate measurement of ABA levels and the effect of various treatments on ABA production could be monitored precisely.

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