Flavonoid Occurrence, Regulation in Plant Tissues and Dietary Contribution to Health

A thesis submitted to the University of Glasgow for the degree of

Doctor of Philosophy

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Declaration

The composition of this thesis and the work described within it was carried out entirely by myself unless otherwise cited or acknowledged. Its contents have not previously been submitted for any other degree. The research for this thesis was carried out between October 1996 and September 1999.

Signed.....

A.J Stewart

February 2000

Abbreviations

ACC	acetyl-coA-carboxylase
APCI	atmospheric pressure chemical ionisation
au	aurea
bli	blue light intermediate
blu	blue light uninhibited
BSA	bovine serum albumin
CAB	chlorophyll a/b binding protein
CAS	cold acclimation specific
C4H	cinnamate-4´-hydroxylase
CHD	coronary heart disease
CHI	chalcone isomerase
4CL	4-coumarate: coA ligase
CHS	chalcone synthase
CPD	cyclobutane pyrimidine dimer
CRY	cryptochrome
DFR	dihydroflavonol reductase
E.coli	*Escherichia coli*
EVN	English Village Nurseries
F.A	fatty acid
FAH	ferulic acid hydroxylase
F3´H	flavonol 3´ hydroxylase
F5´H	flavonol 5´ hydroxylase
FL3´M	flavonol 3´ methylase
FLS	flavonol synthase

FNS	flavone synthase
GC	guanylyl cyclase
GUS	β-glucuronidase
HIR	high irradiance response
HPLC	high performance liquid chromatography
hy	long hypocotyl mutant
IFD	2-hydroxyisoflavonone dehydratase
I-4′-G	isorhamnetin-4′-glucoside
IFS	2-hydroxyisoflavanone synthase
KIN	cold induced
LAR	leucoanthocyanidin 4-reductase
LCMS	liquid chromatography-mass spectrometry
LDL	low density lipoprotein
LFR	low fluence response
lh	long hypocotyl mutant
MS	Murashige & Skoog
4-MU	4-methyl-umbelliferone
MUG	4-methyl-umbelliferyl-glucuronide
NM4	non-mutant 4
nph	non phototrophic hypocotyl
npq	non-photochemical quenching
O.D	optical density
PAL	phenylalanine ammonia lyase
PFD	photon flux density
Pfr	far-red absorbing form of phytochrome

PHY	phytochrome
ppm	parts per million
Pr	red light absorbing form of phytochrome
PSI	photosystem I
Q-3-G	quercetin 3 glucoside
Q-4'-G	quercetin 4' glucoside
R/FR	red/far-red
ROS	reactive oxygen species
SIM	selected ion monitoring
SOD	superoxide dismutase
tt	transparent testa
UVA	ultraviolet A light, wavelength 320-390 nm
UVB	ultraviolet B light, wavelength 280-320 nm
UVC	ultraviolet C light, wavelengths below 280 nm
VLFR	very low fluence response
v/v	volume/volume
WT	wild type
w/v	weight/volume

Abstract

Interest in the flavonol content of food products continues to be fuelled by reports of wide ranging health benefits, many dependent on the ability of flavonols to act as powerful antioxidants (Rice-Evans *et al.*, 1997). Recent work has identified tomato fruits as a rich source of flavonols (Hertog *et al.*, 1992, Crozier *et al.*, 1997).

In this study the flavonol content of tomato fruits was investigated in relation to variety, size, season and country of origin. The flavonol content of ten commonly consumed tomato based food products was also assessed.

Free and conjugated flavonols were identified and quantified using reversed phase HPLC with sensitive detection by UV and fluorescence detection.

The total flavonol content of tomato varieties analysed in this study varied from 0.9-22.2 μg/g fresh weight. Smaller cherry tomato fruits grown in warm sunny climates such as Spain and Israel were found to contain far higher concentrations of flavonols than British fruits. The adoption of 'high flavonol' tomato varieties and production methods allowing greater sun exposure of developing tomato fruits may allow for an increase in the flavonol content of British produce. Tomato flavonols were able to survive industrial processing methods and could be detected in a wide range of tomato-based food products. Tomato juice and tomato puree were found to be particularly rich in flavonols, 14-16 mg/L and 70 μg/gfwt respectively. This study has enabled the identification of tomato fruits and processed products rich in flavonols.

Identification of flavonol rich foods is clearly important with respect to their potential nutritional value. However, it is also necessary to determine whether these flavonols are absorbed by the human body during digestion. Following consumption

of Spanish cherry tomatoes or tomato juice, conjugated quercetin was detected unchanged in plasma and urine. This suggests that tomato flavonols are absorbable and bioavailable.

Flavonol synthesis in plants involves complex environmental regulation, the principal components of which include light, nutrition, disease and temperature. Previous studies indicated a link between plant nutrition and flavonoid accumulation but were unable to identify individual flavonoid compounds. In addition, although tomato was frequently used as a test system for the study of nutrient stress on flavonoid accumulation, the effects of nutrition on tomato fruit tissues were not assessed.

The effect of reduced nitrogen and phosphorus nutrition on the flavonol content of plant tissues was initially tested on seedlings of *Arabidopsis thaliana* and tomato. Conjugated quercetin, kaempferol and isorhamnetin were detected in both *Arabidopsis* and tomato seedling tissues. Exposure to nitrogen or phosphate stress demonstrated a clear inverse relationship between nitrogen and phosphate nutrition and flavonol content. On the basis of this observation, a trial was established under commercial conditions to determine the effect of nutrient stress on the flavonol content of tomato leaf and fruit tissue. In line with previous work (Carpena *et al.*, 1982; Bonguc-Bartelsman & Phillips, 1995) reduced nitrogen availability caused an increase in the flavonol content in the leaves of tomato plants, reduced phosphorus nutrition did not elicit this response. Low nitrogen or phosphate availability caused an increase in the flavonol content of tomato fruit skins early in the ripening process. Any effect of nutrient stress on the flavonol content of tomato fruit tissues was lost as ripening progressed. This study provides clear evidence that the flavonol content of plant tissues is influences by their nutritional status.

The effect of light quality and low temperature on flavonoid biosynthesis was investigated by studying chalcone synthase (*CHS*) promoter activity and transcript accumulation in *Arabidopsis thaliana*. *CHS* gene transcription is known to be regulated in *Arabidopsis* by UVB and UVA/blue light via separate but interacting phototransduction pathways (Fuglevand et al., 1996; Christie & Jenkins, 1996). In addition, *CHS* transcription is reported to increase in response to low temperature in the presence of light (Leyva *et al.*, 1995).

The effect of combining blue, UVA or UVB light with low temperature on *CHS* promoter activity was assessed using the transgenic *Arabidopsis* line NM4 containing the *Sinapis alba* chalcone synthase promoter linked to a β-glucuronidase (GUS) reporter gene. Combining blue/UVA light with low temperature (10 °C) induced a synergistic increase in *CHS-GUS* expression and *CHS* transcript accumulation. This response indicates that cold and blue/UVA light regulate *CHS* expression via separate but interacting signal transduction pathways. Exposing plants to UVB light and low temperature did not elicit this response.

The *Arabidopsis hy4* blue/UVA photoreceptor mutant (Cashmore, 1997) was employed to determine whether the synergistic interaction between low temperature and blue/UVA light involves a signal transduction mechanism originating from the CRY1 photoreceptor. *hy4* seedlings were exposed to blue/UVA light at 10 or 20 °C. The synergistic induction of *CHS* transcription observed in wild type *Arabidopsis* seedlings was not observed in the *hy4* mutant. It is therefore proposed that the signal transduction mechanism originating from cold perception is interacting with the blue/UVA phototransduction pathway originating from the CRY1 photoreceptor.

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1.1 Introduction

Recent work has shown that a wide range of fruit and vegetables contain potentially beneficial non-nutritive compounds, flavonoids (Hertog *et al.*, 1992). The production of these compounds in plant tissues is influenced by a wide range of environmental factors including light, nutrition, disease and low temperature (Landry *et al.*, 1995, Bongue-Bartelsman & Phillips, 1995, Dixon & Paiva, 1995, Christie *et al.*, 1994). Due to the effects of environmental regulation on flavonoid production, fruits and vegetables from different sources may vary significantly in flavonoid content (Hertog *et al.*, 1992, Crozier *et al.*, 1997).

The flavonoids are a diverse family of low molecular weight polyphenolic compounds found ubiquitously in plant tissues. These compounds are usually found conjugated to sugar molecules and are commonly found in the upper epidermal tissues of the plant.

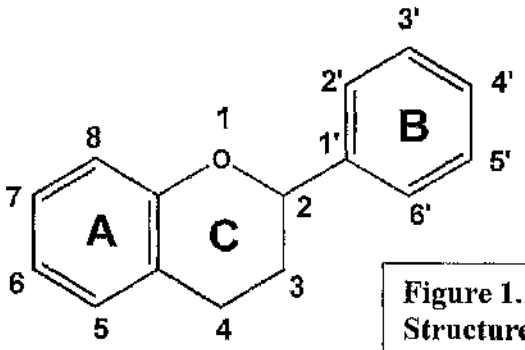


Figure 1.1 The Generic Structure of Flavonoids

Flavonoids are based on a three-ring structure (Figure 1.1), with two benzene rings (A & B) linked through a heterocyclic pyran ring (ring C).

Flavonoids can be separated into six main groups according to their chemical structure, flavones, isoflavones, flavonols, flavanones, flavan-3-ols and anthocyanins.

Anthocyanins- anthocyanins are responsible for the red/purple pigmentation of flowers, roots, stems and leaves. The vivid colours produced by anthocyanins are based on three structures, cyanidin (Magenta), peonidin (pink) and delphinidin (mauve, violet) (see Figure 1.2).

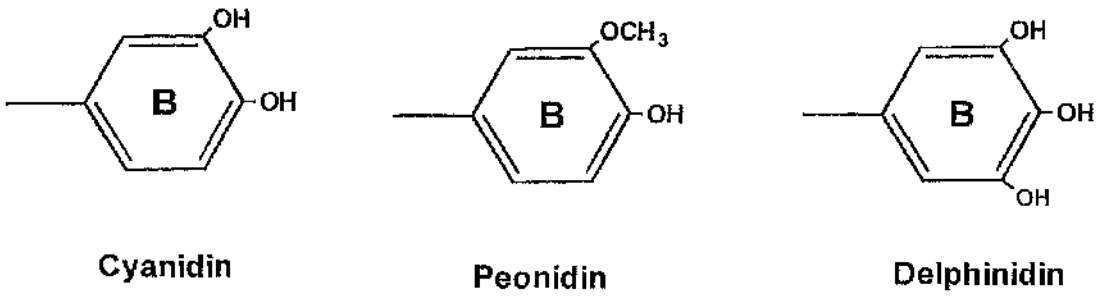


Figure 1.2 Basic Anthocyanin Structures Responsible for Plant Colour

These colours can be varied by co-pigmentation with other flavonoids and phenolics to produce a wide range of colours (Brouillard & Dangles, 1996). The anthocyanins play an important role in the attraction of pollinating insects and animals and in seed dispersal. Anthocyanin production can be induced by a variety of environmental factors including light (Li et al., 1993, Brandt et al., 1995), low temperature (Shvarts et al., 1997, Christie et al., 1994), pathogen attack (Dixon & Paiva., 1995) or nutritional stress (Bongue-Bartelsman & Phillips, 1995). The role of the anthocyanins in each of these situations is

not entirely clear, although evidence suggests that anthocyanins may filter out harmful wavelengths of light (Burger & Edwards, 1996) and may also protect plants against pathogen attack.

Isoflavonoids- The isoflavonoids are found almost exclusively in the family *Leguminosae*. More than 850 isoflavonoid aglycones have been reported of which the largest single group are the isoflavones which comprises more than 350 compounds. A range of functions have been proposed for the isoflavonoids. Some members of this family have been identified as phytoalexins with bactericidal and fungicidal activity, e.g the isoflavone glyceollin (Strack, 1997). Isoflavonoids with toxic activities against insect predators have been identified. The isoflavonoid licoisoflavone B is known to deter insects of the genera *Coleoptera* from feeding on *Lupinus augustifolius* of the *Leguminosae* (Harbourne & Grayer., 1996). Isoflavonoids are believed to act as signal molecules between the nitrogen-fixing bacteria *Rhizobium* and members of the *Leguminosae* in conditions of reduced nitrogen availability (Kosslak *et al.*, 1987).

Flavonols- Flavonols contribute to the white, cream or yellow colours of flower petals, with an important role in attracting pollinating insects (Harbourne & Grayer., 1996). Flavonols are induced by high light levels and in particular exposure to UV-B radiation. The flavonol structure absorbs radiation in the region 280-320 nm and therefore has the potential to protect against the penetration of damaging UV-B radiation (Sheahan, 1996). Some flavonols can act as feeding attractants for insects while other flavonols deter insect feeding. It was hypothesised by Harbourne & Grayer (1996) that at one time all flavonols

may have been inhibitory towards insects but that this inhibition has been overcome by some species of insect and henceforth these compounds function as attractants. Flavonols are induced by various environmental pressures such as pathogen attack (Dixon & Paiva., 1995), nutrient stress (Bongue-Bartelsman & Phillips., 1995) and low temperature (Leyva et al., 1995). Environmental regulation of flavonol production will be discussed in more detail in section 1.3.

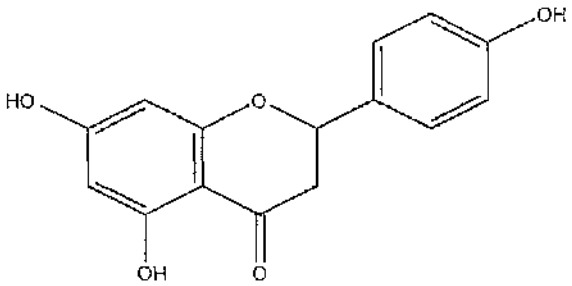
Flavan-3-ols- Flavan-3-ols (catechins) as with other flavonoids are induced by stresses such as pathogen and insect attack. Flavan-3-ols are believed to act as feeding deterrents due to their astringent taste and inhibition of digestion. (+)-Catechin is known to be inhibitory towards predatory insects such as *Heliothus zea (Lepidoptera)* and *Macrosiphum rosae (Homoptera)*. Conversely the catechin 7-O-xyloside attracts feeding insects of the order *Coleoptera* (Harbourne & Grayer, 1996). The polyphenolic structure of catechins may afford protection against damaging wavelengths of light. In addition, catechins form the structural elements of condensed tannins. Tannins cause the precipitation and cross linking of proteins; combination with these proteins produces complexes which play a structural role in plants and are used as physical barriers against pathogen attack (Haslam, 1998).

Flavones- Flavones in common with other members of the flavonoid family, are induced by many environmental stresses, including light and pathogen attack (Dixon & Paiva., 1995). Flavones such as luteolin contribute to the pigmentation of white flowers attracting pollinators by visual appearance and by absorption of UV radiation to which

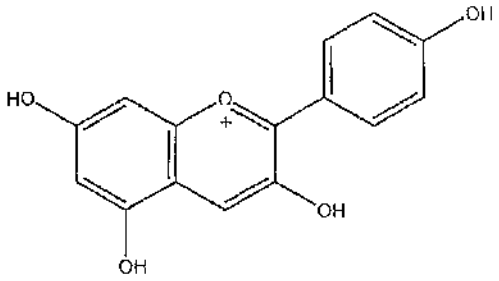
many species of insect are sensitive (Harbourne & Grayer., 1996). In addition a variety of flavones are known to be inhibitory towards feeding insects, luteolin and apigenin are inhibitory towards the growth of *Heliothus zea* larvae *(Lepidoptera)*.

Flavanone- Flavanones such as naringenin are precursors of all other flavonoid family members and as such are induced by a wide range of environmental factors. Flavonones themselves are likely to attenuate the absorption of UV-B, in addition there is some evidence that flavonones may protect against insect attack. Isolonocarpin was found to be inhibitory towards the larvae of army worms, *Spodoptera exempta, Lepidoptera*, (Harbourne & Grayer., 1996).

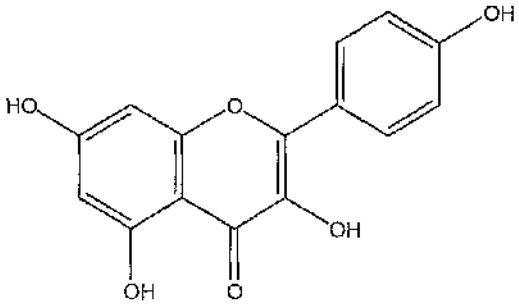
It is clear that the flavonoids have wide ranging functions enabling plants to respond to their environment. Some of these responses are better understood than others. The examples given above are likely to represent only a fraction of the possible functions of this complex family. Within each group significant structural variation is possible due to the large number of substitution patterns available. Such substitutions include hydrogenation, hydroxylation, methylation, sulphation and glycosylation. This variance may account for the large number of flavonoid structures presently characterised, over 4000 and this number is still increasing, (Cook & Samman., 1996).



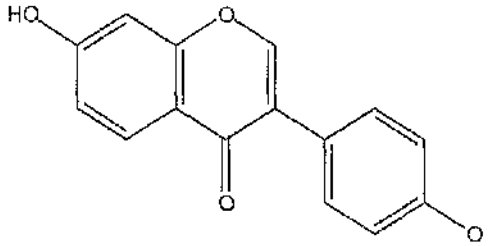
Flavonone



Anthocyanin

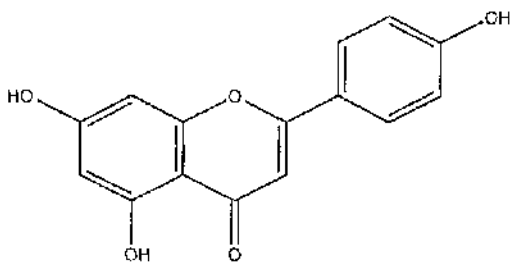


Flavonol

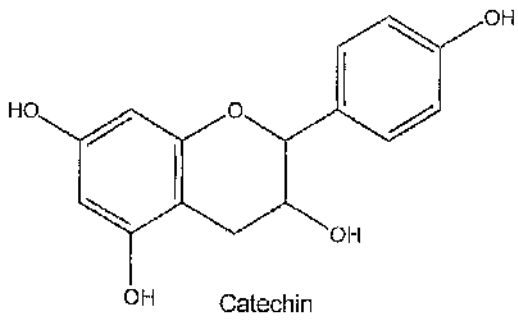


Isoflavone

Figure 1.3 Flavonoid Family Structures



Flavone



Catechin

1.2 Flavonoid Biosynthesis

Biosynthesis of flavonoids begins with phenylpropanoid units derived from the shikimate pathway (Figure 1.4). This pathway, found only in microorganisms and plants, is associated with the production of the aromatic amino-acids phenylalanine, tyrosine and tryptophan. Phenylalanine serves as a precursor for secondary metabolism (Herrmann, 1995).

General phenylpropanoid metabolism involves the deamination of phenylalanine by phenylalanine ammonia lyase (PAL), producing cinnamate. This is then acted upon by cinnamate-4-hydroxylase (C4H), in an NADPH and oxygen dependent reaction leading to the formation of 4-coumarate (Heller & Forkmann, 1994). The enzyme 4-coumarate: CoA ligase (4Cl) produces CoA esters from 4-coumarate and other cinnamic acids in a reaction requiring ATP, Mg^{2+} and CoASH. This enzyme is thought to control the flux of cinnamic acid esters into each of the specific pathways of phenylpropanoid biosynthesis.

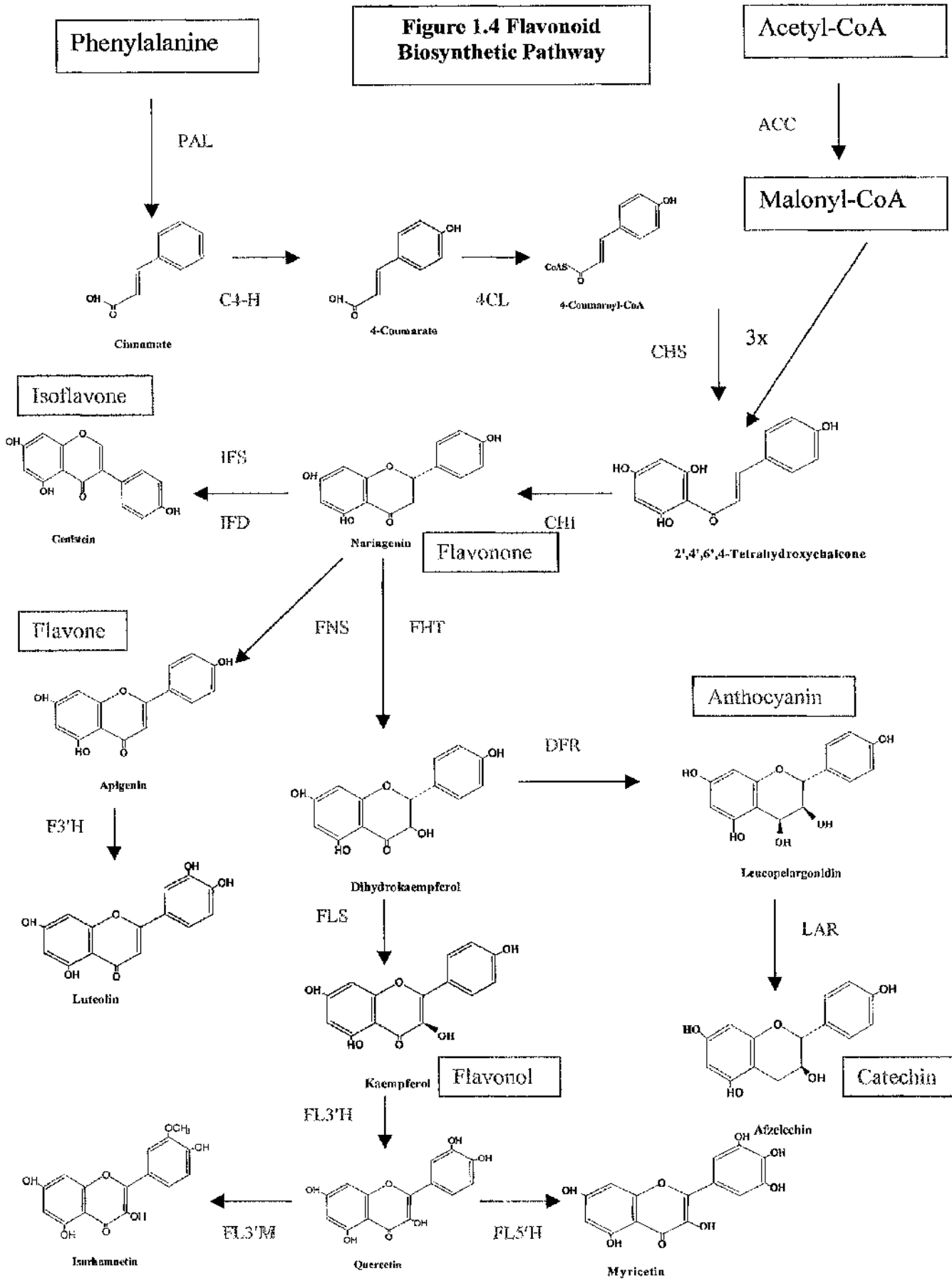
All flavonoids are derived from a chalcone intermediate. The formation of the chalcone is specific to flavonoid biosynthesis and is catalysed by the enzyme chalcone synthase (CHS). Chalcone synthase catalyses three successive condensation reactions with three acetate units derived from malonyl-CoA, producing the intermediate 2'4'6'-4-tetrahydroxychalcone (Heller & Forkman, 1994). Chalcones produced by CHS serve as a substrate for chalcone isomerase (CHI), involving cyclisation with the production of the flavonoid naringenin. From naringenin the pathway branches to form isoflavones, flavones, anthocyanins, flavan-3-ols and flavonols (Figure 1.4). In addition to the

substitution of methyl and hydroxyl groups, introduction of sugar moieties is common, forming conjugated flavonoids with increased solubility.

Table 1.1 Enzymes Involved in Flavonoid Biosynthesis

General metabolism	
PAL	Phenylalanine-ammonia lyase
ACC	Acetyl-coA Carboxylase
General Flavonoid Metabolism	
C4-H	Cinnamate 4-hydroxylase
4CL	4-Coumarate : coA Ligase
CHS	Chalcone Synthase
CHI	Chalcone isomerase
Isoflavone Synthesis	
IFS	2-hydroxyisoflavanone synthase
IFD	2-hydroxyisoflavonone dehydratase
Flavone Synthesis	
FNS	Flavone synthase
F3′H	Flavone 3′ hydroxylase
Flavonol Synthesis	
FHT	Flavonone 3′ hydroxylase
FLS	Flavonol synthase
FL3′H	Flavonol 3′ hydroxylase
FL3′M	Flavonol 3′ methylase
FL5′H	Flavonol 5′ hydroxylase
Anthocyanin Synthesis	
DFR	Dihydroflavonol reductase
Catechin Synthesis	
LAR	Leucoanthocyanidin 4-reductase

Figure 1.4 Flavonoid Biosynthetic Pathway



1.3 Environmental Regulation of Flavonoid Synthesis in Plant Tissues

1.3.1 Effect of Light on Flavonoid Synthesis

One of the putative functions of flavonoids in plants is the absorption and safe dispersal of harmful frequencies of radiation (Landry *et al.*, 1995). The synthesis of flavonoids is up-regulated in response to light stress, particularly UV-B (Fuglevand *et al.*, 1996). In addition the location of flavonoids in the upper epidermis of leaves and stems could reduce photo-induced damage to the photosynthetic mesophyll.

Light is constructed of a range of wavelengths from ultraviolet to infrared, photosynthetically active radiation lies in the region 400-700 nm, violet to red. In order to protect themselves against shorter wavelength, potentially damaging radiation, plants must be able to detect a wide range of light qualities. Known photoreceptors include phytochromes (red/far-red), cryptochromes (blue/UV-A), phototropism and a putative UV-B photoreceptor. Such receptors cannot work in isolation. Complex interactions in the transduction of information from the various photoreceptors must occur in order to construct a complete picture of the light environment (Fuglevand *et al.*, 1996). Such interactions are only now being elucidated.

1.3.1.1 Phytochrome

Phytochrome was the first photoreceptor to be isolated and characterised. It consists of a 116-127 kDa polypeptide chain attached to a tetrapyrole chromophore named phytochromobilin. Phytochromes occur in two photo-interconvertible forms, the red light

absorbing Pr (max 666 nm) and physiologically active Pfr absorbing far-red radiation (max 730 nm).

Two operationally distinct classes of phytochromes have been identified, type I and type II. Type I, light labile phytochrome, predominates in etiolated tissue. On exposure to red light type I phytochrome is rapidly converted into the Pfr form initiating inhibition of hypocotyl elongation and cotyledon expansion. Type I Pfr is then rapidly degraded and type II, light stable, phytochrome becomes the major phytochrome photoreceptor in green tissue.

Phytochrome responses can be categorised as low fluence responses (LFR's), very low fluence responses (VLFR's), and high irradiance responses (HIR's). Low fluence responses are induced at fluence 1-1000 $\mu mol/m^2$ of red light. Such responses show classical red/ far red reversion, with induction at 650-670 nm and reversion at 720-740 nm. Induction produces Pfr stimulating germination, photomorphogenesis and floral initiation. VLFR's do not show R/FR reversibility. Fluence rates of 10^{-4}-10^{-2} $\mu mol/m^2/s$ red light can induce phytochrome responses. Low fluence of any light can induce this response, including FR, as Pr absorbs FR radiation sufficiently to induce a response. HIR's require prolonged irradiation in order to elevate Pfr levels over several hours.

Phytochrome genes commonly occur in small families. Five phytochrome genes have been cloned from *Arabidopsis thaliana*, *PHYA-E* encoding phytochrome apoproteins (Sharrock & Quail, 1989). *PHYA* encodes type I phytochrome. Phytochrome A (PhyA) predominates in etiolated tissue and is down regulated in light. *phyA* mutants have been isolated and may help determine the functions of PhyA. *Arabidopsis* long hypocotyl mutants, *hy1*, *hy2* and *hy6* are believed to contain a lesion within the

chromophore biosynthesis pathway leading to a PhyA deficiency and loss of R/FR reversibility. However these mutants are likely to be deficient in all phytochromes (Chory *et al.,* 1989). *hy8* is selectively deficient in PhyA; other species of phytochrome appear unaffected. When grown in continuous white, red or end-of-day FR light, *hy8* plants display no obvious mutant phenotype. In constant FR however, *hy8* plants continue hypocotyl elongation and retain an etiolated appearance as if grown in darkness, (Parks & Quail, 1993). These mutants are characterised by a failure to perceive continuous far-red radiation, leading to an etiolated phenotype, i.e. increased hypocotyl length, reduced leaf and chloroplast development and reduced expression of photosynthetic genes, e.g. encoding chlorophyll a/b binding protein (*CAB*). A PhyA deficient mutant isolated from tomato (*aurea* mutant) showed a similar etiolated phenotype (Adamse *et al.*, 1988). PhyA over-expression in transgenic plants produces a phenotype opposite to that of the *hy* mutants. Over-expression of oat PhyA in transgenic *Arabidopsis* led to inhibition of hypocotyl elongation in light with a strong R/FR reversible response (Boylan *et al* .,1991). PhyA appears to mediate a FR-HIR. The role of such a response in seedling development may allow etiolated seedlings under a canopy to respond to continuous far-red radiation to allow photomorphogenesis to an auxotrophic growth habit. Subsequent light induced reduction in PhyA would allow phytochrome B (PhyB) mediated shade-avoidance strategies to take over. This would induce hypocotyl elongation allowing the seedling to penetrate the canopy in a photosynthetically competent state.

PHYB encodes a type II light stable photoreceptor. PhyB deficient mutants have been isolated, *hy3* (*Arabidopsis*) and *lh* (cucumber). *hy3* contains normal levels of PhyA and PhyC however PhyB content is reduced to ~2.5 % of wild type with reduced levels of

both PhyB mRNA and protein. The PhyB deficient phenotype in *Arabidopsis* was characterised by an elongated hypocotyl in the presence of light and a reduced shade avoidance response (Somers *et al.*, 1991). *hy3* was also found to be unresponsive to end-of-day far-red treatment (Nagatani *et al.*, 1991). The *lh* mutant was found to contain less than 1 % of wild type levels of PhyB, PhyA levels were unchanged but alterations in content of other phytochromes could not be excluded. The *lh* mutant also showed increased hypocotyl elongation in light and loss of shade avoidance strategy (Lopez-Juez *et al.*, 1992). Over-expression of PhyB in transgenic *Arabidopsis* using a constitutive cauliflower mosaic virus *35s* promoter led to a short hypocotyl phenotype in response to light, (Wagner *et al.*, 1991).

It appears that both PhyA and PhyB are involved in regulation of hypocotyl elongation. A full hypocotyl inhibition response to light may involve additional plant photoreceptors such as blue light receptors. PhyB also appears to play a role in the end-of-day far-red response and shade avoidance strategy affecting stem elongation, apical dominance and time of flower set and senescence.

PhyD shows ~ 80 % amino acid identity to PhyB and is believed to be involved in sensing R/FR ratio influencing shade avoidance. PhyD is produced mainly in cotyledons and leaves although both PhyD and PhyE are detected in the hook and hypocotyl of etiolated seedlings suggesting a role in de-etiolation, (Goosey *et al.*, 1997). PhyC shows greater similarity to PhyB and PhyD than to PhyA and PhyE.

1.3.1.2 Phytochrome Signal Transduction

Three signal transduction pathways mediating phytochrome responses have been identified. All pathways appear to originate from the activation of heterotrimeric G-proteins. These G-proteins consist of three sub-units, α,β and γ. Activation occurs when the α-moiety binds GTP and dissociates from the β and γ sub-units to bind a target enzyme affecting its activity. Hydrolysis of GTP to GDP deactivates the α-subunit which reassociates with the β and γ units, (Roux., 1994).

Injection of oat PhyA protein into cells of the phytochrome deficient tomato aurea mutant (*au*) restored phytochrome-mediated responses, e.g. anthocyanin accumulation, chloroplast development and photosynthetic gene transcription. When PhyA was injected along with an inhibitor of G-protein activation (GDPβS) phytochrome responses were not restored. In addition, injection of an inhibitor of heterotrimeric G-proteins (A-subunit of pertussis toxin) also prevented restoration of phytochrome responses. As all phytochrome responses were prevented by inhibition of heterotrimeric G-proteins it was concluded that these activated proteins must play an early role in phytochrome signal transduction (Neuhaus *et al.*, 1993).

Injection of calcium or activated calmodulin into tomato *au* cells stimulated transcription of some photosynthesis-related genes resulting in chlorophyll containing plastids. Photosystem I or cytochrome b6f complexes were not formed preventing the formation of fully mature chloroplasts via the Ca/Calmodulin pathway. Phytochrome induction of the flavonoid biosynthetic pathway characterised by increased *CHS* transcription and anthocyanin accumulation was found to require cGMP. A cGMP analogue given to plant cells in darkness was found to be able to induce *CHS*

transcription. It has been hypothesised that in response to light cGMP levels could be elevated by increased guanylyl cyclase (GC) activity imposing regulatory activity on genes of the flavonoid biosynthetic pathway (Bowler *et al.*, 1994). The third phytochrome signal transduction pathway leads to the production of PSI components and cytochrome b6f complexes and has a requirement for both cGMP and Ca/Calmodulin.

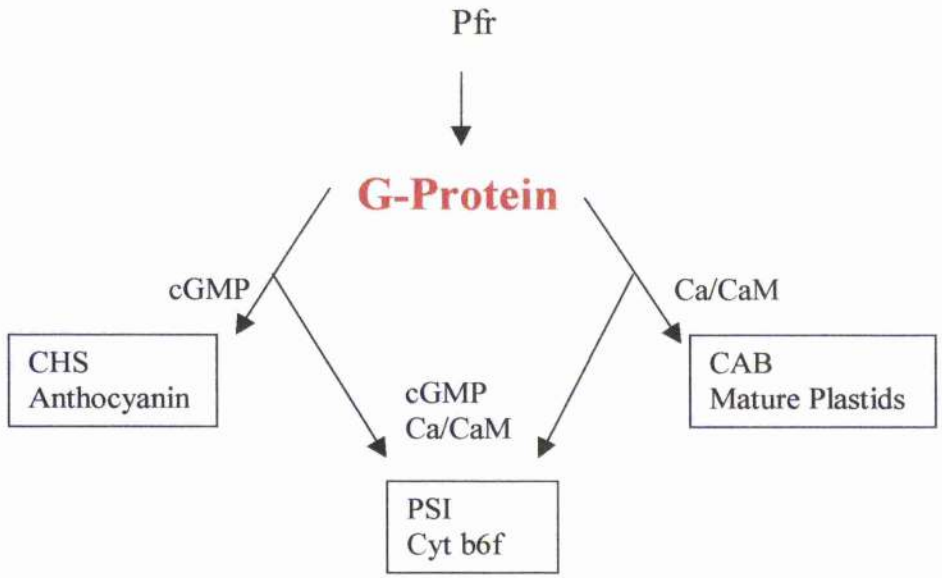


Figure 1.5 Phytochrome Signal Transduction. Modified from Jenkins (1999).

Increased activity of the cGMP pathway causes down regulation of both the Ca/Calmodulin and cGMP-Ca/Calmodulin pathways. It has been suggested that initial activation of the cGMP pathway would allow accumulation of photoprotectants before photosynthetic apparatus sensitive to the damaging components of sunlight is synthesised. Priority is then transferred to the other two pathways. A rise in Ca^{2+} negatively regulates the cGMP pathway but not the cGMP-Ca/Calmodulin pathway allowing attainment of photosynthetic capacity (Bowler *et al.*, 1994).

1.3.1.3 Cryptochrome

Recent advances in molecular genetic techniques have enabled the identification of a blue light photoreceptor. Due to the elusive nature of this photoreceptor, it was named cryptochrome (CRY1). CRY1 is known to act in both seedlings and mature plants influencing developmental processes such as stem elongation, leaf expansion and gene expression (Jackson & Jenkins, 1995).

An important step in the discovery of cryptochrome was the identification of the *hy4* mutant of *Arabidopsis thaliana*. This mutant displayed a long hypocotyl phenotype under blue light conditions but showed a normal phytochrome response (Koornneef *et al.*, 1980).

The hy4 gene product was found to show significant homology to microbial DNA photolyases, such a structure would allow blue light dependent electron transfer to occur. Cry1 protein is known to bind FAD non-covalently. Blue light absorption characteristics may require a further chromophore as was detected in a Cry1 fusion protein containing a pterin group with peak absorption characteristics in the blue region of the spectrum (Malhotra *et al.*, 1994). The pterin chromophore may facilitate primary light harvesting, contributing to the known action spectra of Cry1. This energy may then be transferred to the flavin moiety bound at the C-terminus. A redox mechanism of Cry1 activity at the plasma membrane has been postulated (Spalding & Cosgrove, 1989, Long & Jenkins, 1998).

Control of gene expression concerning *CHS*, *CHI*, and *DFR* is known to be impaired in the *hy4* mutant resulting in e.g. reduced anthocyanin accumulation (Jackson & Jenkins, 1995).

HY4 is known to belong to a small multigene family; other members of this family may encode further UVA/Blue photoreceptors. Blue light mutants were also isolated by Liscum and Hangarter, 1991, (*blu* mutants). These mutants also showed increased hypocotyl extension in response to blue light. The *blu* mutants represent alleles of *hy4*. Blue light intermediate (*bli*) mutants were also isolated; these may represent novel or weak alleles of *hy4*.

Screening for further blue light receptors on the basis of homology to *CRY1* revealed only one related sequence in the *Arabidopsis* genome (*CRY2*). Cry2 was found to have high sequence homology to Cry1 in the amino/photolyase domain (58 % homology) with only 15 % homology to the C-terminal region (Ahmad & Cashmore., 1996). *Arabidopsis* plants with a mutation in the *CRY2* gene displayed a long hypocotyl phenotype under low intensity blue light. Those plants overexpressing *CRY2* showed heightened sensitivity to blue light producing very short hypocotyls (Lin *et al.*, 1998). *cry2 Arabidopsis* mutants displayed delayed flowering in long day conditions as compared to wild type. Therefore, in addition to the regulation of hypocotyl elongation, *CRY2* is believed to be involved in the regulation of floral induction according to daylength (Guo *et al.*, 1999).

Cry2 protein is known to be located in the nucleus. This localisation is achieved by a nuclear localisation signal contained within the carboxyl terminus (Kleiner *et al.*, 1999). Protein localisation is not regulated by light quality. However, exposure to blue light for 30 min or longer causes a decrease in protein levels believed to be mediated by protein degradation.

Sequences with homology to *CRY1* have been identified in pea, tomato and rice (Ahmad & Cashmore, 1996). In addition a photolyase/cryptochrome sequence lacking the identifying characteristics of either *CRY1* or *CRY2* has been identified in mustard (*Sinapis alba).* This may represent a distinct photolyase/cryptochrome gene. Alternatively errors in cloning may have caused the misidentification of a *CRY1/CRY2* homologue (Cashmore, 1997).

Two further blue light photoreceptors unrelated to *CRY1* have been identified. *NPH1* (non-phototropic-hypocotyl) is believed to encode the apoprotein of a blue light receptor involved in phototropism. This protein is known to be associated with the plasma-membrane and becomes rapidly phosphorylated in response to blue light. Four *NPH* mutant loci have been identified in *Arabidopsis* all impaired in phototropic responses (Briggs & Liscum., 1997).

The *Arabidopsis* mutant *npq1,* deficient in xeaxanthin , was found to be unable to induce stomatal opening in response to blue light. It is postulated that xeaxanthin may serve as the chromophore for a blue light receptor involved in regulation of stomatal movements (Zeiger & Zhu., 1998).

1.3.1.4 Cryptochrome/Phytochrome Interactions

Phytochrome deficient long hypocotyl mutants are known to retain hypocotyl responsivity to blue light. It was therefore concluded that the photoreceptors involved in these reactions act independently. However a study of severely phytochrome deficient *Arabidopsis thaliana* mutants *hy1phy1* and *phyAphyB* (Ahmad & Cashmore, 1997)

revealed that both mutants displayed a large reduction in hypocotyl inhibition in blue light. It was concluded that either phyA or phyB fulfils the requirement for co-action between cryptochrome and phytochrome. Phytochrome deficient mutants *hy1phyB* and *phyAphyB* also showed reduced anthocyanin accumulation in blue light. This indicates a requirement for phytochrome/cryptochrome co-action in *CRY1* induced anthocyanin accumulation. A study by Casal & Boccalandro (1995) demonstrated that hypocotyl inhibition and cotyledon expansion was greater when etiolated wild type *Arabidopsis thaliana* seedlings received a pulse of red light directly after a blue light treatment. Using photomorphogenic mutants, *phyB*, *phyA* and *hy4*, functional dependence of *CRY1* on phytochrome was again demonstrated. More recently it was demonstrated that the functional dependance of *CRY1* on phytochrome relates only to conditions of limited light. Where blue light was supplied for a short period (3h/d) or active levels of phyB were reduced using a high imput of far-red light, synergism between *CRY1* and phytochrome was observed (Casal & Mazzella, 1998).

1.3.1.5 UV-B Photoreceptor

Studies into UV-B protection of plants have become more pertinent in recent years due to the depletion of the stratospheric ozone layer, which absorbs radiation in the UV-B band (280-320 nm). This depletion is expected to cause an increase in UV-B exposure at ground level. Exposure to UV radiation can have various damaging effects on plants. DNA has an absorption maximum at 260 nm and can therefore be damaged directly by

absorption of UV-B. In addition UV-B exposure can induce the formation of reactive oxygen species (ROS) causing oxidative stress.

DNA damage leads principally to the formation of toxic and mutagenic compounds such as cyclobutane pyrimidine dimers (CPD's) and pyrimidine (6-4) pyrimidone photoproducts. Absorption of UV-B can also lead to DNA and chromosome breakage.

Plants have evolved a variety of mechanisms to protect themselves from the damaging effects of sunlight. These mechanisms involve screening out UV-B by the production of UV absorbing photoprotectants, increasing UV transmission or increasing leaf mesophyll thickness to prevent damage (Li *et al.*, 1993, Day *et al.*, 1995) or repairing damaged DNA. Plants have been shown to induce synthesis of phenolic compounds such as flavonoids and hydroxycinnamates in response to UV-B exposure (Chappel & Hahlbrock, 1984). Flavonoid synthesis occurs locally only in plant tissues directly exposed to UV-B, also induction of these photoprotective compounds is dependent on the developmental state of the tissue. Young leaves contain higher levels of photoprotectants and these compounds are preferentially increased in early stages of development during UV-B stress as compared to older leaves (Lois *et al.*, 1994). These phenolic compounds are present in the upper epidermal tissues and are believed to reduce penetration of UV-B in to the photosynthetic mesophyll. Flavonoids in methanol yield two absorbance peaks, the first at 240-285 nm results from the benzoyl ring of the flavonoid structure, the second at 300-400 nm from the cinnamoyl ring (Sheahan, 1996). Such compounds would therefore be expected to give protection against UV-B.

The study of *Arabidopsis* mutants deficient in aspects of flavonoid synthesis may illustrate the importance of flavonoids in UV-B attenuation. 11 transparent testa mutants (*tt*) deficient in aspects of flavonoid biosynthesis or regulation of transcription have been identified, (Shirley *et al.*, 1995). The flavonoid genes *CHS*, *CHI* and *DFR* have been cloned from *Arabidopsis* corresponding to *tt4*, *tt5* and *tt3* respectively.

tt4 contains a mutation in the *CHS* structural gene and consequently accumulates fewer leaf flavonoids, particularly kaempferol derivatives. *tt5* is severely deficient not only in flavonoids but also sinapate esters. These mutants were found to be hypersensitive to UV-B compared to wild type *Arabidopsis*, with *tt5* showing greatest sensitivity. This demonstrates the importance of synthesis of flavonoids and hydroxycinnamates in UV-B protection (Li *et al.*, 1993). Further studies by Landry *et al*, (1995) compared *tt5* with an *Arabidopsis* ferulic acid hydroxylase mutant (*fah1*) deficient in sinapate esters. *fah1* was found to have greater sensitivity to UVB than *tt5*, with increased protein and lipid damage indicating penetration of UV-B. Sheahan (1996) compared UV-B attenuation by flavonoids and sinapate esters using *tt4* and *fah1* mutants. Analysis of *tt4* indicated a UV-B screening function and protection of the photosynthetic apparatus against oxidative damage by flavonoids. However fluorescence analysis of UV-B attenuation suggested that sinapate esters may absorb UV-B more effectively than flavonoids.

It is clear that induction of so called 'sunscreen' compounds such as flavonoids and hydroxycinnamates in *Arabidopsis* are vital for protection against the damaging effects of UV-B in sunlight.

1.3.1.6 UV-B Perception

Little is known about UV-B perception via a putative photoreceptor. In practice such a receptor may be expected to involve a protein with a flavin and/or pterin chromophore which would be expected to absorb radiation in the UV-B range. Direct absorption of UV-B by DNA or UV-B generated reactive oxygen species may generate a response to UV-B directly (Jenkins, 1997). Absorption of UV-B at high fluence rates induces defensive responses and causes damage. However, at low fluence rates there is evidence that UV-B reception may play a role in photomorphogenesis influencing inhibition of hypocotyl elongation and cotyledon expansion. In addition, interactions between a UV-B photoreceptor and phytochrome may be required for UV-induced photomorphogenesis (Kim *et al.*, 1998).

Identification of a UV-B perception mutant may aid the search for a specific UV-B photoreceptor and understanding of signal transduction mechanisms.

1.3.1.7 Blue, UVA and UVB Interactions

Interactions between signal transduction pathways mediating light perception have been studied by examining *CHS* promoter function and gene transcription. Additive effects on *CHS* promoter activity are observed when plants receive blue and UVA radiation simultaneously, however both blue and UVA radiation produce synergistic increases in *CHS* promoter activity when combined with UVB treatment (Fuglevand *et al.*, 1996). Furthermore, pathways mediating blue and UVB, or UVA and UVB synergism were

found to be distinct, thus illustrating the complexity of the mechanisms mediating signal transduction. The *hy4* blue light receptor mutant was found to retain synergistic responses between blue/UVA and UVB but demonstrated a limited response to blue and UVA radiation (Fuglevand *et al.*, 1996).

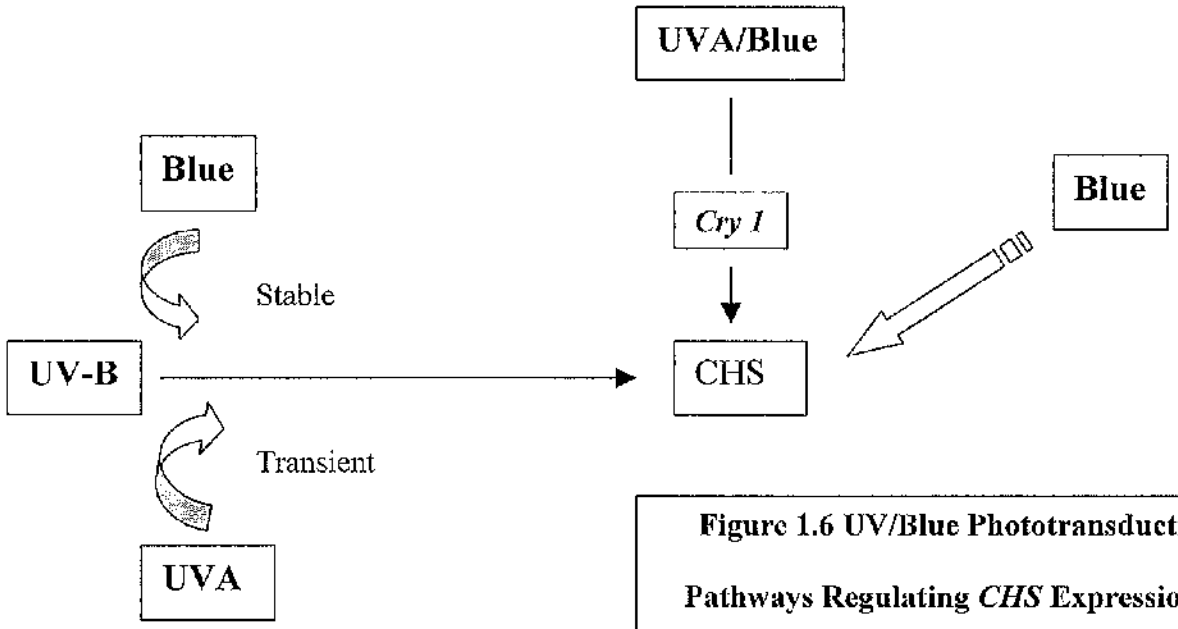


Figure 1.6 UV/Blue Phototransduction Pathways Regulating *CHS* Expression in *Arabidopsis thaliana*

Taken from Fuglevand *et al*, 1996

1.3.1.8 UVA/Blue and UV-B Signal Transduction

Pharmacological studies using inhibitors and antagonists of known signal transduction mechanisms have begun to unravel the events of UVA/blue and UV-B signal transduction (Christie & Jenkins, 1996, Long & Jenkins, 1998).

Using a photomixotrophic *Arabidopsis* cell culture, signal transduction was assessed by analysing the effect of a variety of inhibitors on *CHS* gene transcription. Using calcium channel blockers it was determined that an increase in cytosolic calcium was necessary but not sufficient for induction of *CHS* transcription in both UVA/blue and UV-B induction. This calcium is believed to be released from an internal store. A requirement for protein phosphatase and kinase activity and cytosolic protein synthesis was also demonstrated. Activity of calmodulin was found to be necessary for UV-B but not UVA/blue signal transduction. Such studies show that both UVA/blue and UV-B pathways are distinct from phytochrome signal transduction.

1.3.2 Low Temperature

Due to the sedentary nature of plants, the ability to adapt to stresses such as low temperature is necessary for survival. The effects of low temperature on plants are many-fold affecting many aspects of plant development and metabolism. Cold stress is known to cause an upregulation of the phenylpropanoid pathway usually observed as increased deposition of anthocyanins on leaves and stems (Gilmour & Hajela, 1988, Leyva *et al.*, 1995).

Cold perception by plant cells begins with an influx of mostly extracellular calcium, with some calcium believed to be released by the vacuole. This creates a transient calcium peak within the cytosol (Knight *et al.*, 1996). In plants with the ability to acclimate to low temperature, this calcium transient will be linked to a protein phosphorylation cascade either direcly via calcium dependent protein kinases or

indirectly via calmodulin. This signal transduction event ultimately leads to changes in gene transcription. A transient increase in cytosolic calcium is required for activation of cold acclimation specific (*CAS*) and cold-induced (*KIN*) gene transcription (Monroy & Dhindsa, 1995, Knight *et al.*, 1996). Cold acclimated *Arabidopsis thaliana* are known to have an altered calcium signature in conditions of low temperature, which may indicate that the system is 'primed' to respond to the next period of cold, (Knight *et al.*, 1996).

Many plant responses are designed to prevent or reduce tissue damage due to cold. Membrane lipid composition can be altered to maintain membrane fluidity and absisic acid accumulates, which is believed to prevent ion-leakage (Graham & Patterson, 1982).

Studies of phenylpropanoid gene regulation in wheat transferred from 25 °C to 10 °C revealed little change in *PAL* or *CHS* transcription over the first twelve hours followed by a profound increase in transcription over the following twelve hours, 8 and 50 fold increases in transcription respectively (Christie *et al.*, 1994). Slight increases in transcription were also observed for *4Cl* and *CHI*. To determine the functional significance of this response, anthocyanin levels were monitored. At 5 °C transcript and anthocyanin levels remained unchanged, at 10 °C transcript levels were increased but anthocyanin levels showed little change until plants were returned to a 25 °C environment. At 15 °C transcript and anthocyanin accumulation was observed. In wheat, temperatures below 15 °C appeared to inhibit post-transcriptional processes required for anthocyanin biosynthesis (Christie *et al.*, 1994).

Increased transcription of *PAL*, *CHS* and accumulation of flavonoids during cold stress is reported to occur only in the presence of light, which may indicate a

photoprotective role (Leyva *et al.*, 1995). Simultaneous exposure to low temperatures and moderate-high PFD can cause severe inhibition of photosynthesis e.g. cucumber, a chilling sensitive plant, shows a 60 % inhibition of photosynthesis after 2.5 h at 4 °C in moderate light conditions (Hodgson & Raison, 1989). Reduced efficiency of the photosynthetic apparatus at low temperatures due to factors such as membrane disruption and reduced enzyme activity can rapidly lead to a build up of highly oxidative photosynthetic by-products in the presence of light. Oxidative products of photosynthesis would normally be eliminated by superoxide dismutase (SOD) and catalase, but at low temperatures the activity of these enzymes is inhibited (Graham & Patterson, 1982). Products of the phenylpropanoid pathway such as flavonols and anthocyanins may act as photoprotectants and possibly as free radical scavengers.

A gradual reduction in temperature would increase production of anthocyanins and other photoprotective pigments such as flavonols, filtering out harmful levels of light thereby affording protection against some of the deleterious effects of cold on photosynthesis. Although this process may occur during cold acclimation, it is not required for acclimation. *Arabidopsis* mutants, *ttg* and *tt4*, defective in anthocyanin accumulation retain the ability to acclimate to low temperatures (Graham & Patterson, 1982).

1.3.3 Nutritional Status

A variety of nutrient deficiencies are characterised in plants by an accumulation of flavonoids, notably the red/purple pigments identified as anthocyanins (Ulrychova &

Sosnova, 1970). Indeed, early studies attempted to assess leaf flavonoid content as an indication of altered plant metabolism due to nutritional deficiency using tomato as a model system (Carpena *et al.*, 1982, Zornoza & Esteban, 1984). A study of the flavonoid content of roots and fruits of tomato plants grown in normal versus P, Mn or B deficient media was undertaken (Zornoza & Esteban, 1984). Levels of flavonoids were found to be very low in roots as compared to fruits. No flavonoids were detected in roots where nutrients were limited. Conversely, total flavonoid content increased in fruits in response to P, Mn or B deficiency. No attempt was made in this study to identify individual compounds, instead flavonols, flavones and flavonones were quantified from crude extracts using UV absorption.

HPLC analysis of tomato leaf extracts from control and N-starved plants showed an increase in anthocyanin content, particularly petunidin and an increase in the flavonol glucoside quercetin-3-*O*-glucoside (Bongue-Bartelsman & Phillips, 1995). In addition it was found that nitrogen deprivation greatly increased the levels of *CHS* and *DFR* mRNA. An earlier study (Tan, 1980) demonstrated increased accumulation of PAL protein in apples following reduced availability of nitrogen and potassium.

One explanation for increased flavonoid synthesis under nitrogen stress suggests that enhanced PAL activity will release ammonia for amino-acid metabolism whilst carbon products released are shunted into the flavonoid biosynthetic pathway (Margna, 1977). Alternatively, nitrogen limitation will affect photosynthesis for example by decreasing available chlorophyll and disrupting photosynthetic membranes due to starch accumulation. This may lead to increased sensitivity to high light levels. Production of

photoprotective pigments such as anthocyanins and flavonols may afford protection against light induced oxidative damage (Guidi *et al*, 1998).

1.4 Flavonoids as Antioxidants

Free radicals can be defined as molecules or molecular fragments with an unpaired electron in the outer orbit producing high chemical reactivity (Stohs, 1995).

Such radicals can be produced by exposure to ionising radiation, disease, toxins or a variety of other stresses. Some of the most reactive compounds are known as reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical (OH^-) and singlet oxygen radical (O^-). Production of such compounds is tolerated due to effective scavenging mechanisms of enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Halliwell *et al.*, 1992). In addition, dietary antioxidants such as ascorbate (vitamin C), tocopherols (vitamin E) or carotenoids play a role in biological systems to prevent oxidative damage by radicals.

Recent studies have identified flavonoids as potentially important dietary antioxidants (Robak & Gryglewski, 1988, Salah *et al.*, 1995, Rice-Evans *et al.*, 1997). Daily flavonoid intake is estimated at 23 mg/day with major sources identified as tea, onions and apples (Hertog *et al.*, 1993). The antioxidant capacity of flavonoids depends on their ability to donate hydrogen forming a flavonoid radical, which can stabilise and delocalise the unpaired electron. The presence of transition metals promotes the generation of free radicals by reacting with hydrogen peroxide.

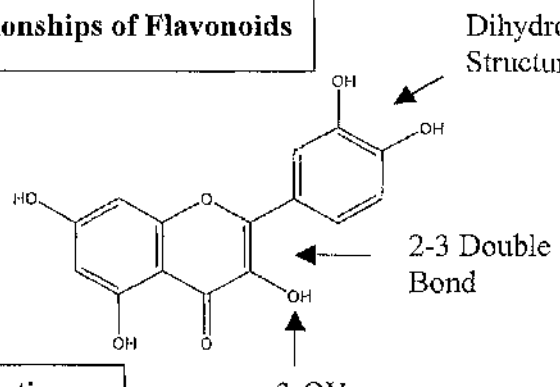
$Mn^{+} + H_2O_2 \longrightarrow M^{(n+1)+} + OH^{\cdot} + OH^{-}$

$Fe\ (II) + H_2O_2 \longrightarrow OH^{\cdot} + OH^{-} + Fe\ (III)$ -Fenton Reaction

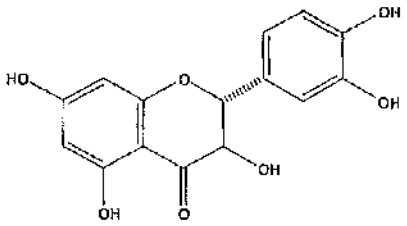
Some flavonoids can chelate metal ions making them unavailable for reaction (Halliwell, 1995).

Flavonoid structure is closely related to antioxidant activity, (Rice-Evans *et al.*, 1996). Structural requirements for antioxidant activity of flavonoids include the 3-OH on unsaturated ring C, 2-3 double bond on ring C and the dihydroxy structure on the B ring, e.g. quercetin. The dihydroxy substitutions of ring B appear to have the greatest effect on antioxidant activity. In the presence of this structure, activity can be increased further by the addition of 3-OH and 2-3 double bond. Blocking the 3 site on the C ring e.g. by conjugation with a sugar moiety prevents the involvement of the –OH group in electron delocalisation reducing the antioxidant potential (see Figure 1.7).

Figure 1.7 Structure-Antioxidant Activity Relationships of Flavonoids

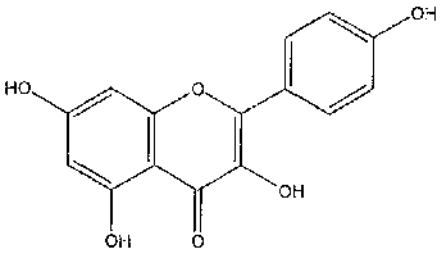


Quercetin
4.7



Taxifolin

1.9



Kaempferol

1.3

Values shown are trolox equivalent antioxidant activities, (Rice-Evans *et al.*, 1996).

1.5 Free Radicals and Disease

1.5.1 Atherosclerosis

Endothelial cells of the coronary arteries can be damaged directly by oxidative stress. Alternatively free radical damage to polyunsaturated fatty acids of low-density lipoprotein (LDL) can initiate lipid peroxidation chain reactions (see Figure 1.8).

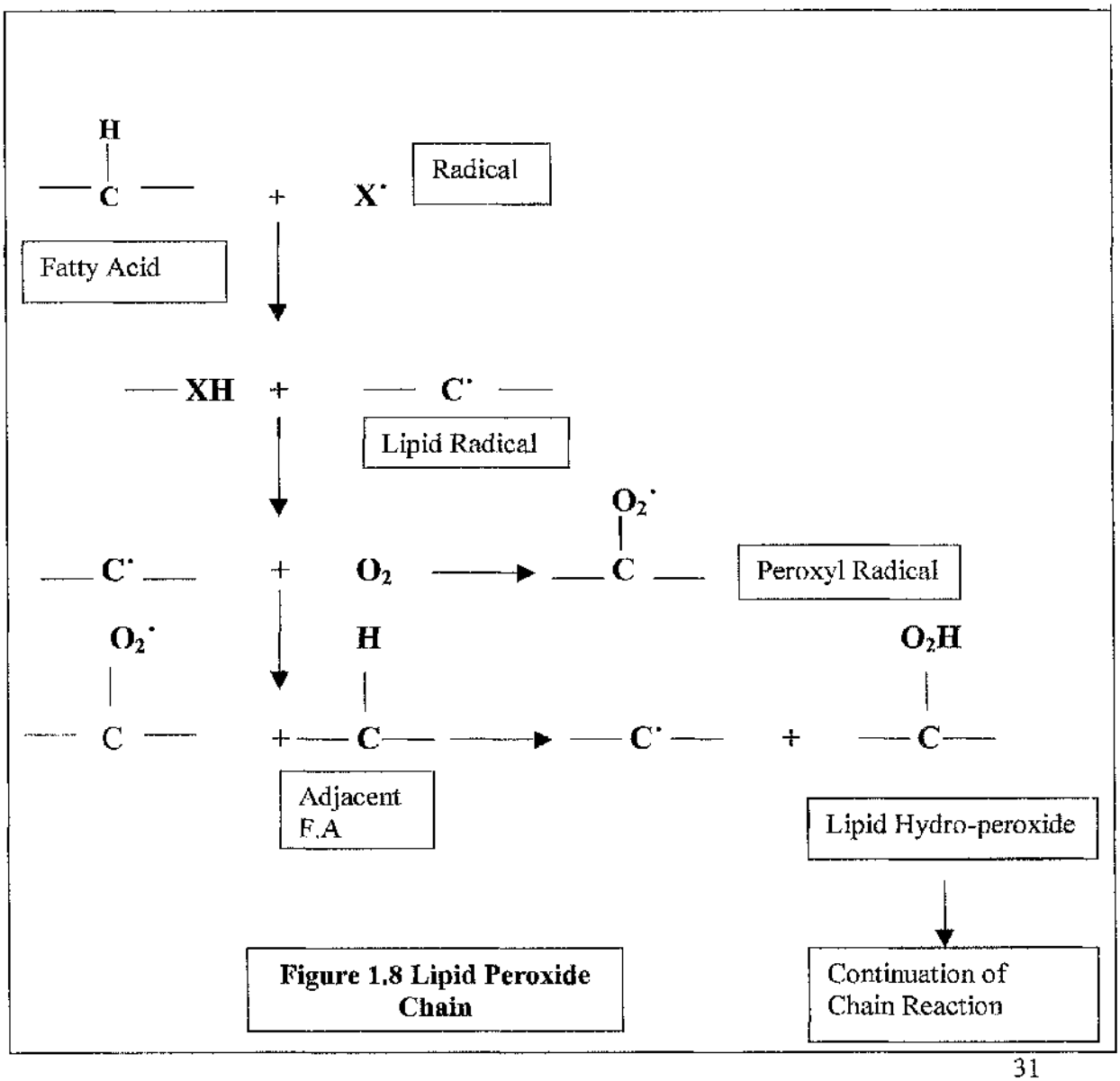


Figure 1.8 Lipid Peroxide Chain

Abstraction of a hydrogen atom from LDL fatty acids creates a lipid radical which reacts with molecular oxygen to form a lipid peroxyl radical. This lipid peroxyl radical can then remove a hydrogen atom from another polyunsaturated fatty acid in LDL or cell membranes thus perpetuating the chain reaction. In addition to the production of further lipid radicals, lipid hydroperoxide is formed, this contributes to LDL modification encouraging endocytosis by macrophages. LDL commonly transports cholesterol around the body, internalisation of LDL causes cholesterol accumulation within arterial macrophages converting them into 'foam-cells'. Macrophage foam cells associate with T-Lymphocytes and free radical producing smooth muscle cells forming an atherosclerotic plaque, resulting in narrowing of the arteries. In addition, bleeding within the plaque can allow the release of blood clots into the arteries, which if large enough, can cause blockage and myocardial infarction, see Figure 1.9. (Cook & Samman, 1996, Leake, 1995).

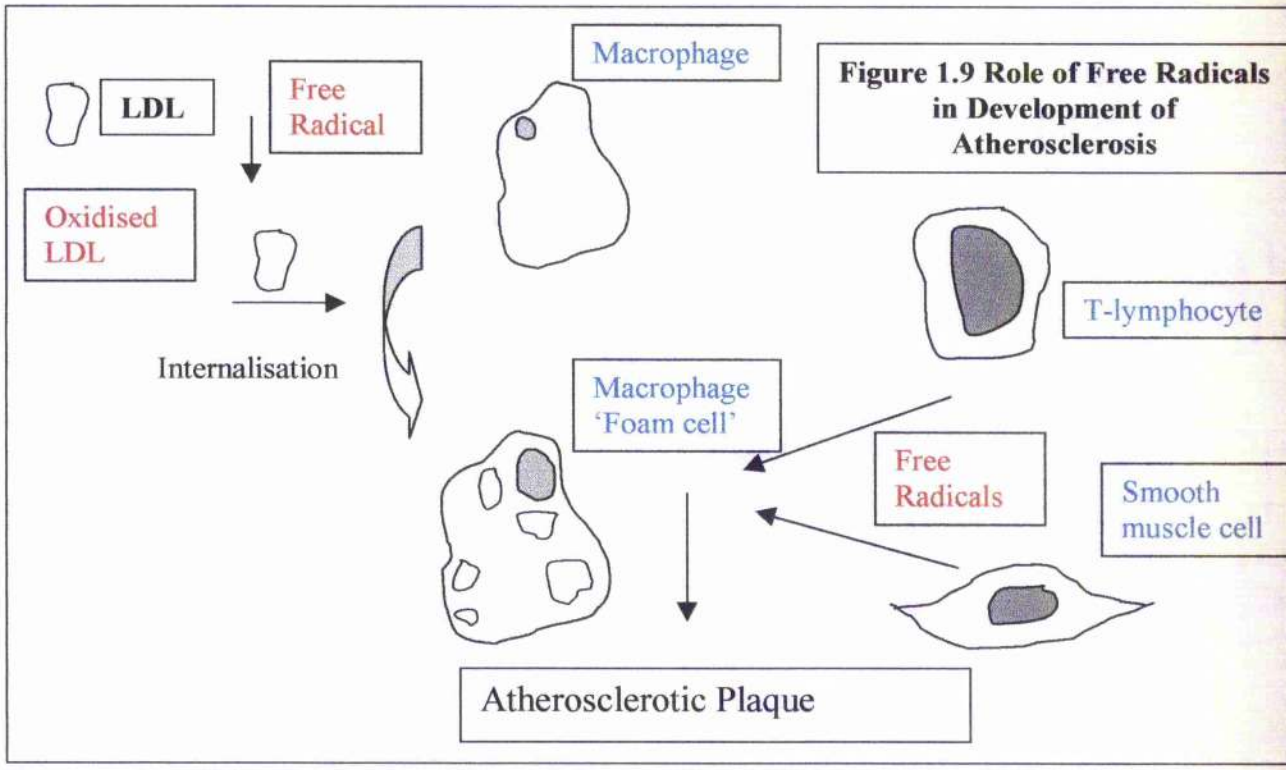


Figure 1.9 Role of Free Radicals in Development of Atherosclerosis

Modification of LDL by free radicals occurs after a lag period during which endogenous lipophilic antioxidants such as α and β-tocopherols are preferentially oxidised. It has been demonstrated that the presence of flavonol aglycones inhibits LDL peroxidation by macrophages and transition metals *in-vitro* (DeWhalley *et al.*, 1990).

The mechanism for this protective effect is not yet known although possibilities include preferential oxidation of flavonols conserving LDL antioxidants. Alternatively flavonols may prevent free radical release from macrophages, regenerate α-tocopherol by donating a hydrogen atom or bind transition metals (DeWhalley *et al.*, 1990). Ratty & Das (1988) also demonstrated the protective effect of flavonoids against LDL modification *in vitro*. The addition of a variety of flavonoids was found to inhibit oxidation of rat brain mitochondrial lipids by ascorbic acid or ferrous sulphate. Flavonoid aglycones were found to be more potent than conjugates, with quercetin, myricitrin, morin and fisetin found to be particularly active. Structure-activity relationships were found to be in-keeping with those determined by Rice-Evans *et al.*, (1996).

Flavonoids such as quercetin, epicatechin and epigallocatechin gallate have been shown to bind LDL *in vitro* and therefore would be available to protect against oxidation (Vinson *et al.*, 1995).

Flavonoids have been identified as antioxidants with sufficient activity and proximity to prevent LDL modification, a crucial step in the pathogenesis of atherosclerosis.

1.5.2 Coronary Heart Disease and Flavonoids

Indications of a protective effect of dietary flavonoids against development of free radical derived damage to endothelial cells of the coronary arteries and development of atherosclerosis have led to epidemiological studies of the relation between dietary flavonol intake and incidence of coronary heart disease (CHD).

The seven countries study was a cross-cultural analysis of dietary flavonoid intake and mortality rates from chronic diseases, in particular CHD and cancer (Hertog *et al.*, 1995). This study involved 16 cohorts in seven countries encompassing Europe, United States of America and Asia. Flavonoid intake was found to be inversely associated with incidence of CHD although intake of saturated fat was found to be the major determinant.

The Dutch contribution to the seven countries study was extended to determine the contribution of flavonoids in the Netherlands diet to the incidence of chronic disease in elderly men. This study, based in Zutphen, eastern Netherlands, found a significant negative relationship between flavonoid intake and mortality from CHD. A weaker negative association was determined for the incidence of fatal or non-fatal myocardial infarction (Hertog *et al.*, 1993).

A cohort study on coronary heart disease incidence and dietary flavonoid consumption based in Finland (Knekt *et al.*, 1996) also determined an effect of flavonoid intake in line with the Zutphen study. Flavonoid intake was found to be lower in the Finnish diet with primary dietary sources identified as apples and onions.

Contrasting results on prevention of CHD by dietary flavonoids were found in a study of male health professionals based in the United States. This study did not support a protective effect of high flavonoid intake on total incidence of CHD but could not rule out any protective effect on men with established coronary heart disease (Rimm *et al.*, 1996).

Such studies appear to indicate a protective effect of increased flavonoid consumption against coronary heart disease. However such studies cannot distinguish between total flavonoid intake from flavonoid absorption into the body.

1.5.2.1The French Paradox

Despite high intake of saturated fats and a high percentage of smokers in the population, France has a paradoxically low incidence of heart disease (Renaud & DeLorgeril, 1992). This contrasts with statistics for the U.K. where heart disease is far higher than expected. In France, death from coronary heart disease per 100,000 people is 101 for men and 32 for women. Corresponding figures for the U.K. are 448 (men) and 167 (women) (Leake., 1995). Higher intake of red wine is believed to account for the lower incidence of CHD in France with active components identified as phenolics (Frankel *et al.*, 1993).

Heart disease in general is lower in the Mediterranean where greater quantities of fresh fruit and vegetables are consumed and where fat intake is from mono-unsaturated fatty acids mostly in the form of olive oil. Such a diet would also be expected to be rich in flavonoids amongst other antioxidants.

1.5.3 Cancer Incidence

High dietary intake of fruits and vegetables is known to reduce the incidence of some cancers. The factors present within fruits and vegetables responsible for this protection have yet to be identified. Non-nutritive compounds such as flavonoids may contribute to the overall chemoprotective effect of fruit and vegetables.

Recent in vitro studies have shown a protective role of flavonoids against the growth of human tumor cells, with flavonoid potency affected by the number of hydroxyl groups and the type of sugar moiety attached (Kamei *et al.*, 1996). Animal studies, in which tumor production is artificially induced, have demonstrated reduced tumor growth with dietary supplementation of flavonoids. However in such studies flavonoids are commonly supplied in concentrations far higher than those found physiologically (Kuo., 1997).

Recent publication of the flavonoid content of a wide range of fruits vegetables and beverages in the Netherlands diet (Hertog., 1992) have enabled analysis of dietary flavonoid intake and cancer incidence.

The Zutphen epidemiological study analysed cancer incidence in relation to flavonoid intake. No correlation was found between flavonoid intake and lung, colorectal or all-cause cancer (Hertog *et al.*, 1995). Data from a cohort study of diet and disease carried out in Finland from 1966- 1972 was re-analysed by Knekt *et al* (1997) from the perspective of flavonoid intake and lung cancer incidence. A clear inverse association between flavonoid intake and incidence of lung cancer was observed. This association was most noticeable in the younger participants of the study. Further studies on flavonoid

intake and lung cancer incidence have been carried out in Spain where lung cancer is the primary cause of cancer death in men. No protective effect was determined for intake of quercetin, luteolin or total flavonoid intake and a non-significant negative relationship was determined for kaempferol intake. However, dietary questionnaires used in this study to evaluate flavonoid intake failed to include important flavonoid sources such as onions and red wine (Garcia-Closas *et al.*, 1998). A case control study on flavonoid intake and incidence of gastric cancer was carried out in Spain from 1987-1989 involving patients with gastric adenocarcinoma (Garcia-Closas *et al.*, 1999). In this study a protective effect of quercetin, kaempferol and total flavonoid intake was determined.

Results on the potency of flavonoids as anticarcinogens are at present equivocal, further studies are required before any conclusions can be drawn.

1.6 Flavonoid Absorption

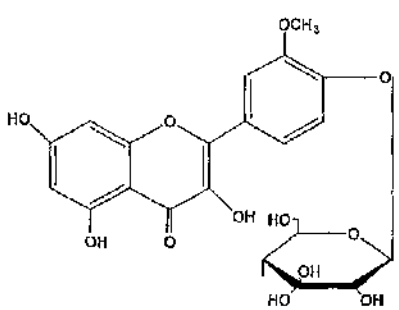
Early studies on flavonoid absorption focused on the absorption of the aglycone form of the flavonoid. Conjugated flavonoids commonly contain β-glycosidic bonds. No intestinal enzymes with the ability to break these bonds have been identified. Consequently it was believed that the release of free flavonoids from their sugar moicty would require the activity of colonic bacteria. Such bacterial breakdown was known to cause further damage to the flavonoid ring system (Kuhnau,1976).

Animal studies indicated that the flavonol quercetin was readily absorbable and could be detected in urine and bile following quercetin supplementation with ~20 % absorption (Ueno *et al.*, 1983, Das *et al.*, 1971, Manach *et al.*, 1997).

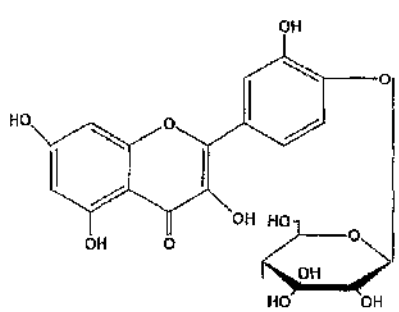
It was found that oral or intravenous supplementation of quercetin aglycone to human subjects did not lead to substantial accumulation in plasma or urine (Gugler *et al.*, 1975). Recent studies indicate that absorption of flavonoids may occur in the small intestine, thus avoiding degradation by colonic microflora. In addition, flavonoid sugar conjugates may be more effectively absorbed than aglycones.

Studies by Hollman *et al* (1995) investigated flavonoid absorption in healthy ileostomy patients, allowing analysis of absorption by the small intestine. Subjects followed a quercetin free diet for the duration of the study with the exception of quercetin rich food supplements supplied on days 4, 8 and 12. The extent of absorption was calculated from analysis of urine and illeostomy effluent. Absorption of quercetin was found to be 52 % (onion), 17 % (rutin) and 24 % (aglycone). Quercetin from onion is mainly present in the form of flavonol glucosides, results therefore indicate increased absorption of glucose conjugates as compared to the free flavonol. Intervention studies with onion feeds to human volunteers have demonstrated rapid absorption of quercetin glucosides (Hollman *et al.*., 1996, Janssen *et al.*, 1998, McAnlis *et al.*, 1999).

Aziz *et al.* (1998) demonstrated the accumulation of flavonol glycosides in human plasma and urine following consumption of onions.



Isorhamnetin-4'-glucoside



Quercetin-4'-glucoside

Figure 1.10 Structures of Primary Glucosides Present in Onion

Furthermore, isorhamnetin-4′-glucoside (I-4′-G) was found to accumulate in greater concentrations in plasma and urine than quercetin-4′-glucoside (Q-4′G) or other quercetin conjugates. Further studies are required to determine whether this indicates more effective absorption of I-4′-G, increased turnover of other quercetin conjugates or methylation of Q-4′-G to form I-4′-G (see Figure 1.10).

Following consumption of onions, flavonols rapidly accumulate in plasma with peak levels reached at 1-2 h. This implies absorption of flavonol conjugates from the small intestine. This study further implies that flavonol glucosides are absorbable and indicates that the sugar group present may influence uptake.

Many intervention studies have supplied flavonol supplements in quantities far exceeding dietary levels. Young *et al* (1999) investigated flavonol accumulation in human plasma and urine following a low daily dose of quercetin supplied by fruit juices. The concentration of quercetin in urine was found to increase with time reaching a steady level at 3-4 days. No significant increase in the quercetin content of plasma was observed.

Little information is available on metabolism of flavonoids following absorption in humans. Animal studies show that flavonoids can be metabolised in the liver to form glucuronide and sulphate conjugates. Flavonoid conjugates can then be excreted into urine or into bile allowing further modification and absorption in the large intestine, (Kuhnau, 1976).

1.7 Project Aims

Flavonoids have been identified as potentially important dietary antioxidants (Rice-Evans *et al.*, 1997). Within the flavonoid family, flavonol levels have been analysed and quantified in a variety of fruits, vegetables and beverages. Such studies indicated vast differences in flavonol content between different produce. One of the aims of this project was to identify factors determining the flavonol content of fruit of commercially grown tomatoes (*Lycopersicon esculentum).* Flavonol levels were studied in relation to variety, season and country of origin. In addition, the flavonol content of commonly consumed processed tomato products was assessed. It was hoped that this study would identify varieties or conditions of cultivation that promote the accumulation of high levels of flavonols within the fruits.

Although studies of the flavonol content of tomato fruits and processed products are pertinent to determination of their nutritional value, it is necessary to identify the extent of flavonol absorption into the human body during digestion. A small intervention study was carried out to establish the bioavailability of flavonols from tomato fruits and tomato juice.

Flavonoids are only synthesised by micro-organisms and plants (Hermann, 1995). Flavonol synthesis involves complex environmental regulation, the principal components of which include light, nutrition, disease and temperature. *Arabidopsis thaliana* was employed as a model system in which to study the effects of nutritional status, nitrogen or phosphorus availability, on flavonol content. Information from this study was used to establish a larger study of the effects of nitrogen and phosphorus nutrition on the flavonol

content of leaves and fruits of mature tomato plants grown in a commercial situation. The aim of this study was to determine whether nutritional deficiency could be employed to induce flavonol accumulation in tomato fruits.

Arabidopsis thaliana was again used as a model system in which to study the effects of light quality and low temperature on flavonoid biosynthesis. The action of the enzyme chalcone synthase (CHS) is the first committed step in the biosynthesis of flavonoids (Heller & Forkmann, 1994). The activity of the *CHS* promoter was studied via a β-glucuronidase (GUS) reporter gene system and also by determination of *CHS* transcript levels following exposure to various light qualities either alone or in combination with low temperature. This study had two main aims. The first was to determine the effect of light quality and low temperature on *CHS* transcript accumulation. The second aim was the determination of the effects of cold on the UVA/blue phototransduction pathway.

Summary of Aims

1. Identification of tomato fruit varieties or methods of cultivation leading to high flavonol fruits.
2. Study of the bioavailability of the flavonols in tomato fruits.
3. Study of how nutritional status may be manipulated to influence the flavonol content of plant tissues.
4. Study of the effect of light quality and low temperature on the regulation of a gene involved in flavonol biosynthesis.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

The chemicals used in this study were obtained from Sigma-Aldrich Co. (Poole, Dorset) unless otherwise stated.

2.1.2 Radiochemicals

[α-^{32}P] dCTP was supplied by Amersham International (U.K.).

2.1.3 DNA Modifying Enzymes

Restriction enzymes were obtained from Life Technologies, Paisley, Strathclyde together with their reaction buffers, which were provided at a 10x concentration.

2.1.4 Soil

The soil and sand used to grow plants were purchased from William Sinclair Horticulture Ltd. (Lincoln, U.K.). 1 part Silvaperl sand was mixed with 4 parts soil and autoclaved for 1 hour.

2.2 General Laboratory Procedures

2.2.1 pH Measurement

The pH of solutions was measured using a Jenway pH meter 3310 and combination electrode.

2.2.2 Autoclaving

Equipment and solutions were sterilised at 15 psi for 20 minutes in a Laboratory Thermal Equipment Autoclave 225E.

2.2.3 Solutions and Equipment for RNA Work

Solutions used in RNA work were treated with 0.1 % (v/v) diethylpyrocarbonate (DEPC, Sigma) overnight and then autoclaved. Glassware was sterilised by baking in an oven at 180 °C for four hours.

2.3 Analysis of the Flavonol Content of Plant Products

2.3.1 Tomato Suppliers

Scottish tomatoes were provided by Scotland's Tomatoes plc, (Clyde Valley, Lanarkshire, UK) while English tomatoes were obtained from English Village Salads plc (Banks, Southport, Lancs, UK). Seeds of tomato fruits with various skin colours were obtained from the C.M. Rick Stock Centre (University of California, Los Angeles, CA, USA) with the exception of Noire Charbonneuse, which was purchased from Simpson's Seeds (Surrey, UK). Spanish tomato fruits cv Bodar, Bond, Royesta and Havanera were kindly donated by Dr Jesus Chammaro, Institute of Celluology, CSIC, Valencia, Spain. All other tomato fruits were purchased from Safeway Stores plc (Byres Rd, Glasgow, UK).

2.3.2 Sample Preparation

Tomato fruits and processed products excluding tomato juice and tomato soup, were snap frozen in liquid nitrogen, lyophilised and ground to a fine powder prior to acid hydrolysis. Samples of tomato juice and tomato soup were hydrolysed fresh.

2.3.3 Extraction and Hydrolysis Conditions

Optimisation of acidic conditions for the hydrolysis of flavonol conjugates has been described by Hertog et al (1992) following an earlier study by Harbourne (1965) on the release of free flavonols by acidic and enzymatic hydrolyses. Preliminary screening was carried out to ascertain the most effective acid hydrolysis conditions for the tissues involved in this study. Samples of tomato fruit and processed products, (20 mg lyophilised tissue), tomato juice and tomato soup, (450 µl) were all hydrolysed at 90 °C for 2 h in a 3 ml glass V-vial containing 2 ml 1.2 M HCl in 50 % aqueous methanol and 20 mM sodiumdiethyldithiocarbamate as an antioxidant. A Teflon coated magnetic stirrer was placed in the vial, which was sealed tightly with a PTFE-faced septum prior to heating in a Reacti-Therm Heating/Stirring Module (Pierce, Rockford, IL, USA). Extract aliquots of 100 µl, taken both before and after acid hydrolysis, were made up to 250 µl with distilled water adjusted to pH 2.5 with trifluoroacetic acid and filtered through a 0.2 µm Anopore filter (Whatman, Maidstone, Kent, UK), prior to the analysis of 100 µl volumes ($1/50^{th}$ aliquot of total sample) by gradient elution reversed phase HPLC. All samples were analysed in triplicate.

2.4 Analysis of the Flavonol Content of Plasma and Urine

2.4.1 Study Design

Three healthy subjects, 1 man and two women (aged 23-27) participated in the study, all gave their written consent. Two flavonol rich sources were investigated, tomato fruits and tomato juice. Tomato fruits selected were Spanish cherry tomato variety Paloma, tomato juice was Del Monte, both were purchased from Safeway, Byres Rd, Glasgow. Three subjects undertook the tomato fruit dosing experiment, one subject participated in the tomato juice study.

In all cases the volunteers followed a low flavonol diet for three days prior to each experiment and fasted overnight. Venous blood samples were collected prior to consumption of the flavonol source and then at 0.5, 1.0, 1.5, 2, 3, 4, 5 and 24 h. Blood samples were collected into heparinised tubes, which were immediately centrifuged at 3000 g at 0 °C for 10 min. Plasma was separated and stored at –80 °C prior to analysis. Urine was collected for 24 h in three aliquots, 0-6 h, 6-12 h and 12-24 h. Urine was stored in plastic bottles and kept at - 20° C prior to analysis. This study protocol was approved by the University of Glasgow Human Ethics Committee for Non-Clinical Research.

2.4.2 Extraction and Hydrolysis Conditions

Samples were hydrolysed as previously described (2.3.3) with the exception of plasma samples, which were hydrolysed for 3 h. Due to the presence of precipitated proteins the plasma samples were centrifuged at 5000 g for 10 min prior to HPLC analysis.

2.5 Flavonol Analysis by High Performance Liquid Chromatography

2.5.1 High Performance Liquid Chromatography and Post-Column Derivatization

Samples were analysed using a Shimadzu (Kyoto, Japan) LC-10A series automated liquid chromatograph comprising a SCL-10A system controller, two LC-10A pumps, a SIL-10A autoinjector with sample cooler, a CTO-10A column oven, and a SPD-10A UV-vis detector linked to a Reeve Analytical (Glasgow, UK.) 2700 data handling system. Reversed phase separations were carried out at 40 °C using a 150 x 3.0 mm i.d., 4 μm Genesis C18 cartridge column fitted with a 10 x 4.0 mm i.d., 4μm C18 Genesis guard column in an integrated holder (Jones Chromatography, Mid-Glamorgan, UK.). The mobile phase was a 20 min, 20-40 % gradient of acetonitrile in distilled water adjusted to pH 2.5 with trifluoroacetic acid, eluted at a flow rate of 0.5 ml/min. Column eluent was first directed to the SPD-10A absorbance monitor operating at 365 nm, after which post-column derivatization was achieved by the addition of 0.1 M methanolic aluminium nitrate containing 7.5 % (v/v) glacial acetic acid (Hollman and Trijp, 1996)

pumped at a flow rate of 0.5 ml/min by a pulse free Model 9802 precision mixer/splitter (Reeve Analytical). The mixture was passed through 1.9 m x 30/1000" i.d. loop of peek tubing in the column oven before being directed to a RF-10A fluorimeter and fluorescent flavonol complexes detected at excitation 425 nm and emission 480 nm. The limit of detection at A_{365} was < 5 ng and linear 5-250 ng calibration curves were obtained for morin, rutin, quercetin, kaempferol and isorhamnetin. The fluorescent intensity of the individual flavonoid derivatives varied, however 0.1-100 ng linear calibration curves were obtained for myricetin, morin, quercetin, kaempferol and isorhamnetin.

2.5.2 Liquid Chromatography-Mass Spectrometry

Samples were analysed using a Shimadzu LC-10A *vp* series automated liquid chromatograph comprising a SCL-10A *vp* system controller, two LC-10A *vp* pumps, a SIL-10AD *vp* autoinjector with sample cooler, a CTO-10AC *vp* column oven and a SPD-10A *vp* UV-vis detector. Reverse phase separations were carried out at 40 °C using a 150 x 3.0 mm i.d. 5 µm Nemesis C_{18} column. The mobile phase was a 20-min gradient of 12-35 % acetonitrile containing 1 % formic acid, maintained for a further five minutes at 35 %. Flow rate: 0.8 ml/min and column eluent was first passed through the SPD-10A *vp* absorbance monitor operating at 371 nm, before being directed to a Shimadzu LCQ 8000 quadrupole mass spectrometer with atmospheric pressure chemical ionisation (APCI) and a nebulising gas flow of 2.5 L/min. Full scan 250-650 m/z negative ion spectra were obtained every 4s. Data obtained were analysed using Shimadzu LCMS QP 8000 software.

2.5.3 Estimates of Free and Conjugated Flavonol Levels

Free flavonols were detected in the unhydrolysed sample while the hydrolysed samples contained both free and conjugated flavonols. Thus conjugated flavonol levels were estimated by subtracting the amount found in the unhydrolysed samples from that detected after acid hydrolysis.

2.5.4 Reference Compounds

Morin, myricetin, quercetin, rutin and kaempferol were purchased from Sigma chemicals (Poole, Dorset, UK.). Isorhamnetin was obtained from Apin chemicals (Abingdon, Oxford, UK.).

2.6 Growth of Plants on Sterile Media

2.6.1 Preparation of Murashige and Skoog (MS) Media

Macronutrients- MS media is prepared containing the following macronutrients; 1.25 mM potassium phosphate (KH_2PO_4), 2.26 mM calcium chloride ($CaCl_2.2H_2O$), 0.73 mM magnesium sulphate ($MgSO_4.\ 7H_2O$). In addition, all media contained 20 mM sodium-iron EDTA, 100 mM sucrose and 8 g/l agar. Media was adjusted to pH 5.7 with 0.1 M potassium hydroxide and immediately autoclaved.

Micronutrients- In order to provide the required micronutrients to support plant growth, the following compounds were added to MS media; 0.1 mM boric acid (H_3BO_3), 0.1 μM cobalt chloride ($CoCl_2$. $6H_2O$), 0.1 μM copper sulphate ($CuSO_4$. $5H_2O$), 0.1 mM manganese sulphate ($MnSO_4$. H_2O), 1.0 μM molybdic acid (Na_2MoO_4. $2H_2O$), 5.0 μM potassium iodide (KI), 0.03 mM zinc sulphate ($ZnSO_4.7H_2O$). A 200x concentrated solution was prepared and 5 ml was added to 1 litre of media.

Altering nitrogen content of media- Standard nitrogen concentration in the media was 60 mM. Media containing five different nitrogen concentrations were prepared, 0, 0.1, 0.6, 6.0 and 60.0 mM. Nitrogen was added to the media in the ratio of 1 mole ammonia: 2 moles nitrate supplied in the form of ammonium nitrate and potassium nitrate. All media contained 1.25 mM potassium phosphate and 100 mM sucrose.

Altering phosphate concentration of media- Media was prepared containing, 0, 0.3, 0.6, 2.5 and 6.3 mM potassium phosphate (KPO_3 monobasic). 1 mM MES buffer was added to the media to enable adjustment of the pH to 5.7. All media contained 60 mM nitrogen and 100 mM sucrose.

2.6.2 Plating Growth Media

Media was heated in a microwave oven to melt and placed in a 55 °C water bath prior to pouring. Plating of media was carried out in a laminar flow hood under sterile conditions. Media to support growth of *Arabidopsis thaliana* was plated onto Petri dishes, media intended for *Lycopersicon esculentum* was poured into sterile magenta vessels.

2.6.3 Surface Sterilisation of *Arabidopsis thaliana* Seed

Sterilisation of seeds was carried out in a laminar flow hood. Seeds contained within filter paper packets were placed within a magenta vessel. They were then immersed for two minutes in 70 % (v/v) ethanol followed by ten minutes in 10 % (v/v) sodium hypochlorite with occasional agitation. Seeds were finally rinsed five times in sterile water and left overnight in the laminar flow hood to dry.

2.6.4 Surface Sterilisation of *Lycopersicon esculentum* Seed

Prior to surface sterilisation, tomato seeds were imbibed for two hours under a flow of constantly running tap water. The container holding the seeds was then covered over with two layers of muslin. Seeds were submerged for two minutes in 70 % (v/v) ethanol, the solution was swirled continuously. Ethanol was poured off and replaced by a 2 % (v/v) hypochlorite solution containing four drops of Tween 20 per 100 ml. Seeds were shaken gently in this solution for ten minutes and then rinsed four times in sterile water. Seeds were then immediately placed onto growth media in a laminar flow hood.

2.6.5 Growth Conditions of Plants on Sterile Media

Arabidopsis seed were sown onto MS media in Petri dishes under sterile conditions. Plates were covered and placed at 4 °C in darkness for three days. Plates were

then transferred to conditions of 24 h white light (100 μEi/m^2/s) provided by warm white fluorescent tubes, at 20 °C for a further ten days.

Lycopersicon esculentum seed were sown onto MS media in magenta vessels allowing the height required for growth of tomato seedlings. Magenta vessels were sealed and transferred to 24 h white light as described above at 20 °C. Plants were grown for either 10 or 21 days before tissue was harvested.

2.6.6 Harvesting of Tissue from Sterile Media

Tissue was harvested on ice and frozen directly in liquid nitrogen. Tissue for flavonol analysis was stored at –20 °C, tissue for RNA analysis was stored at –80 °C.

2.7 Growth of *Lycopersicon esculentum* in Hydroponic Conditions

2.7.1 Study Design

Tomato plants were grown in a commercial environment under glass at Garrion Fruit Farm, Clyde Valley, Lanarkshire. Hydroponic nutrient regimes were designed to control nitrogen and phosphorus availability whilst still allowing plant growth and fruit set. This allowed determination of the effect of nitrogen and phosphorus fertilisers on the flavonol content of tomato fruit and mature vegetative tissue. Tissue sampling was carried out on two occasions one month apart (May-June, 1998). Red, green and breaker

fruits and leaf tissue were collected on each occasion. Tissue was stored at –20 °C prior to determination of flavonol content.

2.7.2 Design of Fertiliser Stock Solutions

Stock solution recipes were based on the use of a 40 gallon (182 l) stock tank, this is the standard stock tank size used by commercial growers. All solutions were diluted 1:100 for application to plants in the trial. Fertilisers for stock solutions were purchased from Clydeside Trading Society, Kirkmuirhill, UK.

2.7.3 Phosphorus Manipulation

A control phosphate regime was designed to produce a nutrient feed with a phosphate content of 30 ppm following dilution. This regime required two stock tanks A and B. Tank A contained calcium nitrate (10.67 kg/182 l), tank B contained potassium nitrate (14.3 kg/182 l), mono-ammonium phosphate (1.97 kg/182 l), ammonium nitrate (1.14 kg/182 l), magnesium sulphate (9.08 kg/182 l) and Solufeed TEC (0.55 kg/182 l). Following 1:100 dilution this regime provides a nutrient content of 100 ppm calcium, 184 ppm nitrogen (nitrate), 24 ppm nitrogen (ammonia), 300 ppm potassium, 50 ppm magnesium, 30 ppm phosphorus and trace elements boron, copper, iron, manganese, molybdenum and zinc supplied by the Solufeed TEC (Clydeside Trading Society).

The low phosphate regime was designed to produce a phosphorus concentration of 5 ppm. This regime matched the control situation as closely as possible differing only in

the concentration of mono-ammonium phosphate (0.33 kg/182 l) and ammonium nitrate (2.27 kg/182 l). This allowed a phosphorus concentration of 5 ppm, 24 ppm nitrogen (ammonia), and 195 ppm nitrogen (nitrate).

The high phosphate regime contained 100 ppm phosphorus. This was achieved by increasing the concentration of mono-ammonium phosphate to 6.56 kg/182 l and omitting ammonium nitrate. Nitrogen levels were thereby reduced to 173 ppm nitrate-N and 45 ppm ammonia-N.

2.7.4 Nitrogen Manipulation

The control nitrogen regime supplied 193 ppm of nitrogen. Stock tank A contained calcium nitrate (10.67 kg/182 l), stock tank B contained potassium nitrate (14.3 kg/182 l), mono-ammonium phosphate (2.95 kg/182 l), magnesium sulphate (9.08 kg/182 l) and Solufeed TEC (0.55 kg/182 l). This produced a nutrient content of 100 ppm calcium, 173 ppm nitrogen (nitrate), 20 ppm nitrogen (ammonia), 300 ppm potassium, 45 ppm phosphorus and 50 ppm magnesium.

The low nitrogen regime contained 79 ppm of nitrogen. To achieve this the concentration of calcium nitrate was decreased to 5.33 kg/182 l, potassium nitrate was decreased to 4.30 kg/182 l and mono-ammonium phosphate was decreased to 1.97 kg/182 l. In addition magnesium sulphate levels were decreased to 5.45 kg/182 l. This regime gave a final nutrient content following 1:100 dilution of, 50 ppm calcium, 66 ppm nitrogen (nitrate), 13 ppm nitrogen (ammonia), 90 ppm potassium, 30 ppm phosphorus and 30 ppm magnesium.

Nitrogen levels in the 'high' regime were increased to 405 ppm. This involved increasing levels of calcium nitrate to 21.34 kg/182 l, potassium nitrate to 27.91 kg/182 l and adding ammonium nitrate at 2.27 kg/183 l. Mono-ammonium phosphate and magnesium sulphate were retained at control levels. Final nutrient concentrations were, 200 ppm calcium, 363 ppm nitrogen (nitrate), 42 ppm nitrogen (ammonia), 585 ppm potassium, 45 ppm phosphorus and 50 ppm magnesium.

2.7.5 Hydroponic Plant Growth Conditions

Scotland's Tomatoes, Lanarkshire, supplied tomato plants of variety Chaser at age 3-4 months. These plants were installed (Feb-Mar 1998) in a commercial glass house receiving nutrients through a controlled drip system. Light levels and temperature would have varied according to outdoor weather conditions. Sampling occurred from May-June 1998, at this time plants would receive 12-14 hours of light per day. Light levels measured on-site at midday during sampling were 800-1000 $\mu Ei/m^2/s$ at the top of the plants and 150 $\mu Ei/m^2/s$ at truss level. Daytime temperatures varied from 20-25 °C.

2.8 Growth of Soil Grown Plants

2.8.1 Growth of Soil Grown *Arabidopsis thaliana*

Soil was first autoclaved and then treated with 0.1 g/l Intercept (Levington, Ipswich, UK.), a water soluble systemic insecticide containing 70 % (w/w) imidacloprid,

giving protection against aphid damage. *Arabidopsis* seeds were sown on treated soil, pots were covered in cling-film and placed in darkness in a cold cabinet at 4 °C for 2 days allowing vernalisation to occur. Plants were then transferred to conditions of 15 $\mu Ei/m^2/s$ white light at 20 °C for three weeks prior to treatment.

2.8.2 Growth of Soil Grown *Lycopersicon esculentum*

Seeds were obtained from the C.M. Rick Centre (California), Simpson's Seeds (Surrey, England) or collected from the fruits of Spanish cherry tomato (variety Paloma) purchased from Safeway, Byres Rd, Glasgow.

Seeds were planted beneath 1 cm of sterile soil and moistened with distilled water. Plant pots were covered with cling film and placed in a growth cabinet with controlled conditions of white light (80-100 $\mu Ei/m^2/s$) at 20 °C. Daylength was fixed at 16 h of light and 8 h of darkness. Plants were grown for three weeks prior to light treatments on seedlings and grown for 4-5 months prior to light treatment of fruits on the vine or analysis of flavonol content of tomato fruits. Following production of the first fruiting truss, plants were fed at alternate waterings with Tomorite liquid tomato fertiliser (Levington, Ipswich, UK).

2.9 Illumination of Plant Material

Illumination of both *Arabidopsis thaliana* and *Lycopersicon esculentum* was carried out under controlled environmental conditions at 20 °C or 10 °C. White light was

provided by warm white fluorescent tubes (Osram, Munich, Germany), UV-A radiation was obtained from Sylvania F35W/B1-2B Black-light Blue fluorescent tubes. UV-B radiation was provided by UVB-313 ultraviolet fluorescent tubes (Q-Panel Co. USA), which were covered with cellulose acetate (Diacel 120 microns FLM 400110, Film Sales) to filter out UV-C radiation. This UV-C filter was replaced every 24 h. Blue light was provided by Sylvania 40W T12 blue fluorescent tubes (GTE, Sylvania, Shipley, UK.). Blue light tubes were covered by a "Moonlight-Blue" filter (No. 183, Lee Filters, England) to screen out UV-A and other wavelengths below 390 nm. Red light was obtained by covering high output white fluorescent tubes with (Phillips, PL-L 55W/83/4P) with 'Deep Golden Amber' filter (No. 135, Lee Filters).

Required fluence rates were achieved by varying the number of fluorescent tubes and the proximity of test plants to the light source.

2.9.1 Fluence Rate Measurement

Fluence rate from white light sources was determined using a hand held Li-Cor quantum sensor, Model Li-185B. Fluence rate from all other radiation sources was measured using a spectroradiometer (Macam SR9910).

2.10 RNA Isolation

2.10.1 Flowgen Purescript RNA Isolation

RNA was extracted using the Flowgen Purescript RNA isolation kit (Flowgen, Staffordshire, UK). Frozen plant tissue, 0.03-0.5 g, was ground to a fine powder in liquid nitrogen in a pestle and mortar. This tissue was quickly transferred to a frozen, sterile Eppendorf tube. Flowgen Cell Lysis solution, 300 µl, was then added (containing citric acid, EDTA and SDS). Samples were briefly vortexed to mix and 100 µl of Flowgen Protein-DNA precipitation solution (containing citric acid and NaCl) was added. The solution was inverted several times and placed on ice for 5 min. Samples were then centrifuged for 3 min in a microfuge at 14,000 rpm. The supernatant was removed into 500 µl chloroform, inverted to mix and centrifuged for 20 min at 14,000 rpm in a microcentrifuge. The upper aqueous layer was then transferred to an Eppendorf containing 300 µl isopropanol, the solution was mixed by inversion and the RNA was pelleted by centrifuging for 3 min at 14,000 rpm. The isopropanol was discarded leaving a small white pellet which was rinsed with 200 µl of 70 % (v/v) ethanol (made with DEPC treated distilled water). The solution was inverted to mix and the RNA was pelleted by 1 min centrifugation. Ethanol was poured off and RNA pellets were allowed to air-dry on the bench for ~ 1 h. RNA was resuspended in 12-25 µl DEPC treated water. Samples were stored on ice for ~ 1 h to allow resuspension and then stored at – 20 or –80 °C.

2.10.2 Determination of RNA Concentration

RNA concentration was determined spectrophotometrically by measuring the optical density of each sample at 260 and 280 nm. It was assumed that an RNA concentration of 40 µg/ml gives an O.D. of 1.

2.10.3 RNA Gel Electrophoresis

Agarose, 1.3 g, was added to 72 ml of distilled water and heated in a microwave to allow the agarose to dissolve. Once the agarose solution had cooled to 60 °C, 10 ml of 10x MOPS (0.2 M MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0) and 18 ml of formaldehyde (37 % v/v, Sigma) was added. The gel was mixed gently to avoid the formation of air bubbles and poured into gel casting apparatus. The gel was allowed a minimum of 30 min to set and was then transferred to a gel tank. Sufficient running buffer (1x MOPS) was then added to submerge the gel.

RNA, 5-10 µg, was aliquoted and made up to 10 µl with DEPC treated water. RNA denaturing buffer, 14.5 µl, was then added (1 µl 10x MOPS, 3.5 µl formaldehyde, 10 µl formamide, 0.1 µl ethidium bromide (10 mg/ml)). Samples were briefly centrifuged at 14,000 rpm in a microfuge and then incubated in a 65 °C water bath for 5 min. Samples were again briefly centrifuged and 4 µl RNA loading buffer (50 % (v/v) glycerol, 1 mM EDTA, 0.25 % Bromophenol Blue, 0.25 % Xylene cyanol FF) was added.

Samples were loaded onto the gel and run at constant 90 volts for ~ 3 h or until the lower dye front reached three quarters of the length of the gel.

2.10.4 Northern Blotting

Blotting apparatus included a reservoir of 20x SSC (3 M NaCl, 0.3 mM tri-sodium citrate) above which a layer of 3 MM filter paper (Whatman) soaked in 20x SSC and in contact with the reservoir was suspended over a glass support. The agarose gel was removed from the gel tank, trimmed to produce smooth edges and carefully laid 'well' side down on the 3MM filter paper avoiding air bubbles. A piece of nylon membrane (Hybond N, Amersham, UK) was laid over the gel. Several pieces of 3 MM filter paper were placed over the nylon membrane. Layers of nescofilm (Bando Chemical Ind.Ltd, Japan) were placed around the agarose gel to prevent 'short-circuiting'. Many layers of dry absorbent tissues were placed on top of the blot and finally a glass plate supporting a 500 g weight was placed on top.

The blot was left overnight to allow transfer of the RNA from the agarose gel onto the nylon membrane. The blot was then disassembled and the filter rinsed in sterile distilled water before fixing the RNA to the nylon membrane using a UV crosslinker (UVP, CL-1000, Ultraviolet crosslinker, 12,000 Joules/m^2/s).

2.11 Amplification and Preparation of Plasmid DNA

2.11.1 Small Scale Preparation of Plasmid DNA

Competent *E.coli* cells were prepared and transformed as described (Sambrook *et al.*, 1989). *E.coli* from glycerol stocks were streaked across LB agar plates and incubated

overnight at 37 °C. One colony was then selected from each plate and transferred to 5 ml L-broth (containing 50 μg/ml ampicillin) in sterile falcon tubes. Falcon tubes were then incubated overnight in a shaker at 37 °C. Plasmid DNA was isolated from bacterial cultures using the Wizard ™ Minipreps DNA purification system (Promega). Bacterial culture, 5-10 ml, was centrifuged at 10,000 g for 10 min to pellet the cells. Supernatant was then removed and cells were resuspended in 400 μl of Cell Resuspension Solution (50 mM Tris (pH 7.5), 10 mM EDTA, 100 μg/ml RNase A). Neutralisation solution, 400 μl, (1.32 M potassium acetate) was then added and the solution was mixed well by inversion. Lysate was centrifuged at 14,000 rpm for 5 min in a microfuge and the supernatant removed into a 2.0 ml Eppendorf.

One Minicolumn/Syringe Barrel assembly was prepared for each *E.coli* culture and attached to a vacuum manifold (Promega). Resuspended DNA Purification Resin, 1 ml, was added to the barrel of the assembly. Cleared lysate was then added into the minicolumn/syringe assembly containing the resin. Vacuum was then applied to pull the resin/ DNA mixture through the minicolumn. Vacuum was then broken and 2 ml of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl, pH 7.5, 40 μM EDTA, 55 % (v/v) ethanol) was added, vacuum was restored to draw the wash solution through the minicolumn. Vacuum was sustained for an additional 30 seconds after the solution had passed through the column to dry the resin, the minicolumn was then transferred to a 1.5 ml Eppendorf. This Eppendorf was centrifuged at 14,000 rpm in a microfuge for 2 min to remove residual Column Wash Solution. The minicolumn was transferred to a fresh Eppendorf and 50 μl of dH2O was added. The Eppendorf was then

centrifuged at 14,000 rpm for 20 seconds to elute the DNA. The minicolumn was discarded and plasmid DNA was stored at –20 °C.

2.11.2 Plasmid Digestion and DNA Isolation

Plasmid DNA, 2 µl, was added to 1 µl of the appropriate buffer (Promega), 0.5 µl of restriction enzyme(s), 4 µl DNA loading buffer (20 % (v/v) glycerol, 0.025 M NaOH, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF) and made up to 10 µl total volume with sterile distilled water. Reactions were incubated for 1 h at 37 °C. Digestions were run out on a TBE gel (0.4 g agarose, 50 ml tris-boric acid EDTA (0.09 M tris-borate, 2 mM EDTA) 1 µl 10 mg/ml ethidium bromide) alongside marker DNA (1 kb ladder, Life Technologies). The gel was then installed in a gel tank with 1 x TBE running buffer and electrophoresis was carried out as described 2.10.3. The gel was visualised using a UV-transilluminator (Spectroline ™ transilluminator, Model TC-312A). Bands of interest were then excised from the gel and stored in an Eppendorf.

2.11.3 Purification of DNA Fragments from Agarose Gels

Agarose gel slices containing DNA were isolated as described (2.11.2). DNA was purified using a QIAquick Gel Extraction Kit (Qiagen). Gel slices were weighed and 300 µl of buffer QG (aqueous solution of guanidine thiocyanate and pH indicator) was added for every 100 mg of gel. Samples were incubated in a water bath at 50 °C for 10 min, and

vortexed at regular intervals during incubation to allow the gel to completely dissolve in the buffer. 100 µl of isopropanol was added for every 100 mg of agarose gel.

The QIAquick spin column was placed within the 2 ml collection tube provided and the agarose gel sample added onto the column. Tubes were centrifuged at 14,000 rpm in a microfuge for 1 min, flow through was discarded and the column was returned to the collection tube. Buffer PE, 750 µl, (aqueous solution of Tris(hydroxymethyl)-aminomethane/hydrochloric acid) was added to the column, tubes were again centrifuged for 1 min. Flow through was discarded and the column was centrifuged for an additional 1 min to remove any residual ethanol. To elute the DNA from the column 50 µl of buffer EB (10 mM Tris (hydroxymethyl)-aminomethane/hydrogen chloride, pH 8.5) was added directly to the membrane of the column, the column was placed in a fresh 1.5-ml Eppendorf and centrifuged for 1 min. The column was discarded and DNA stored at –20 °C.

2.11.4 Determining the Concentration of DNA Inserts Isolated

DNA insert, 4 µl, isolated from plasmid DNA (see 2.11.3), was added to 2 µl DNA loading buffer (20 % (v/v) glycerol, 0.025 M NaOH, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF) and 4 µl sterile water. DNA samples were then run out on a TBE gel (2.11.2). Lambda *Hind* III DNA (Rediprime) of known concentrations, was run alongside DNA samples and a 1 kb ladder (Life Technologies).

The brightness of DNA bands was examined using a UV-transilluminator (Spectroline ™ transilluminator Model TC-312A) to determine the concentration of DNA inserts.

2.11.5 Radiolabelling of dsDNA

Radiolabelled DNA inserts were produced using the Rediprime ™ II DNA labelling kit (Amersham International). DNA was transferred to a screw top Eppendorf and diluted in sterile distilled water to produce a solution of 25 ng DNA in total volume of 45 µl. DNA was denatured by heating to 95-100 °C for 5 min in a boiling water bath. Samples were then briefly spun down in a microcentrifuge. Denatured DNA was added to the Rediprime reaction tube (containing dATP, dGTP, dTTP, exonuclease free Klenow enzyme and random primers) and vortexed to mix. Samples were centrifuged briefly to bring the contents to the bottom of the tube before adding 50 µCi (3,000 Ci mmol^{-1}) of [α-^{32}P] dCTP. Samples were then incubated in a water bath at 37 °C for 10 min. The labelling reaction was stopped by the addition of 5 µl of 0.2 M EDTA. For use in hybridisation reactions, DNA was denatured by heating to 95-100 °C for 5 min and then snap cooled on ice for 5 min. DNA was denatured following the removal of unincorporated radionucleotides.

2.11.6 Removal of Unincorporated Radionucleotides from Radiolabelled DNA

Unincorporated radionucleotides were separated out using a sephadex mini-column. A hole was pierced in the base of a sterile 1.5 ml Eppendorf using a 12 gauge needle. A 0.5 ml sterile Eppendorf with a hole in the base pierced using a 25 gauge needle was suspended within the larger tube. Sterile glass beads, 5-10 µl, (Jencons Ballotini No.11, Jencons (Scientific) Ltd, Leighton, Buzzard, UK) in distilled water were transferred to the 0.5 ml Eppendorf to cover the hole. The Eppendorf was filled with Sephadex TE (sterile Sephadex G50 (Pharmacia, Milton-Keynes, UK) in a 20 fold volume of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The two Eppendorfs were then suspended at the top of a 10 ml test tube and centrifuged at 1,000 g for 4 min. The 0.5 ml Eppendorf was refilled with Sephadex TE avoiding the production of air bubbles and recentrifuged. The now complete Sephadex minicolumn was removed from the 0.5 ml Eppendorf into a screw top Eppendorf before the addition of the solution containing labelled DNA and unincorporated nucleotides. The minicolumn was then spun at 2,000 rpm for 4 min. Unincorporated radionucleotides were retained within the minicolumn, which was discarded, and the labelled DNA was collected in the Eppendorf where it was stored at 4 °C until use.

2.12 Hybridisation of Radiolabelled DNA to Northern Blots.

2.12.1 Hybridisation of Nylon Filters

Nylon filters were moistened with sterile distilled water and placed within Techne hybridisations flasks (Techne, Cambridge, UK). Pre-hybridisation solution, 20 ml, (0.5 M phosphate buffer pH 7.2, 7 % (w/v) SDS, 10 mg/ml BSA) was added using a sterile pipette. Flasks were incubated at 55 °C in Techne hybridisation ovens for at least 2 h. The denatured radiolabelled DNA probe was then added and hybridisation was left to proceed overnight.

2.12.2 Washing Northern Blots

Following hybridisation, filters were washed twice in an appropriate volume of 2 x SSC, 1 % (w/v) SDS at 55 °C in a Techne hybridisation oven for 10 min. Filters were then washed at increasing stringency as required depending on the amount of radioactivity bound to the nylon membrane.

2.12.3 Autoradiography

Filters to be autoradiographed were sealed in heat sealable plastic and exposed to Fuji X-ray film (type RX) in a film cassette with intensifying screens at –80 °C for an appropriate length of time.

2.12.4 Stripping Filters of Bound Radiolabelled Probes

Following autoradiography, a boiling solution of 0.1 % (w/v) SDS was poured over the filter. The solution was allowed to cool to room temperature. Washes were repeated three times or until no further radioactivity could be detected by the Geiger counter. Nylon filters were sealed in plastic and autoradiographed overnight to verify that the radiolabelled probe had been completely removed.

2.12.5 Re-use of Radiolabelled DNA

Following hybridisation, probes were stored in falcon tubes at 4 °C. To re-use, the DNA/hybridisation solution was denatured by heating at 95-100 °C for 10 min and allowed to cool to 55 °C before addition to prehybridised filters. Hybridisation was left to proceed overnight before filters were washed as described in 2.12.2.

2.13 Measurement of *CHS* Activity Using the GUS Reporter Gene

2.13.1 Plant Material

Transgenic *Arabidopsis* line NM4, Non-Mutant 4, (Jackson & Jenkins, 1995) was grown as described in 2.8.1. This transgenic line contains the *uidA* (GUS) coding sequence fused to the *Sinapis alba SA-CH1* gene promoter sequences from positions –907 to +26. NM4 is diploid and homozygous for the transgene at a single heritable locus.

2.13.2 Measurement of GUS Activity

Plant tissue was harvested onto ice and homogenised in 50 µl GUS extraction buffer (50 mM Na_2PO_4 pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1 % (w/v) triton X-100, 0.1 % (w/v) sarcosyl). Fluorometric MUG (4-methyl-umbelliferyl-glucuronide) assay buffer, 450 µl, (2 mM MUG dissolved in GUS extraction buffer) was then added. Samples were incubated in a 37 °C water bath. Sample aliquots of 100 µl were removed after 15 and 60 min. The reaction was stopped in these aliquots by the addition of 900 µl 0.2 M Na_2CO_3. The remaining 300 µl sample was removed from the water bath at 60 min and stored at –20 °C prior to determination of protein concentration.

Samples were mixed well and the concentration of 4-methyl-umbelliferone (4-MU) produced was quantified using a spectrofluorimeter (Perkin Elmer LS50) at excitation 365 nm and emission 455 nm. GUS activity was calculated by calibrating the fluorimeter with solutions of 4-MU (Sigma). A standard curve of fluorescence against 4-MU concentration (nM) was produced. GUS activity was then calculated as pmole 4-MU produced per minute per mg of protein (see 2.13.3).

2.13.3 Measurement of Protein

Protein concentration was measured using the Bradford assay (1976). Concentrated Bradford reagent was prepared by the addition of 100 ml 85 % (w/v) orthophosphoric acid (Sigma) and 50 ml 95 % (v/v) ethanol to 100 mg of coomassie brilliant blue G250. This reagent was stored at 4 °C until required. Before use, concentrated

reagent was made up to 1 litre with distilled water and filtered through Whatman filter paper. Protein samples of 50 µl were added to 950 µl of dilute Bradford reagent. Reactions were mixed by inversion and incubated for 15-20 min at room temperature. The absorbance was then determined at 595 nm in a spectrophotometer.

BSA (Sigma) protein standard (1 mg/ml) was diluted in distilled water to provide protein concentrations, 0, 1, 3, 5, 8, 12, 18, 23, 29 and 35 µg/ml. Standards were incubated with 950 µl of dilute Bradford reagent as previously described and their absorbance at 595 nm was used to construct a standard curve of absorbance against amount of protein (µg). This was used to determine the amount of protein in each sample.

Chapter 3 The Occurrence of Flavonols in Tomato Fruits and Tomato Based Products

3.1 Introduction

In order to investigate both environmental regulation of flavonol production in plant tissues and human absorption of tomato flavonols, it is first necessary to identify those flavonols present in tomato fruits. This chapter describes an HPLC based approach to the identification of tomato flavonols and their distribution within the fruit. The flavonol content of tomato fruits was studied in relation to variety, season and country of origin. In addition flavonols found in commonly consumed processed tomato products were also assessed. The aim of this study was to identify tomato varieties or processed tomato products with a high flavonol content or conditions of cultivation that promote the accumulation of high levels of flavonols within tomato fruits.

3.2 Flavonol Identification by HPLC

Separation of flavonols from crude extracts of tomato tissue taken before and after acid hydrolysis was achieved by reversed phase liquid chromatography. Flavonol detection and identification was by UV and fluorescence detection. UV detection (365 nm) allowed analysis of rutin, quercetin-3-glucoside, quercitrin, myricetin, morin, quercetin, apigenin, kaempferol and isorhamnetin with limits of detection < 5 ng. Chelation of flavonols with methanolic aluminium nitrate followed by fluorescence detection (excitation 425 nm,

emission 480 nm) allowed detection of fewer flavonols (morin, myricetin, quercetin, kaempferol and isorhamnetin) however fluorescence detection provided greater sensitivity and selectivity with limits of detection ~ 0.1 ng. Using reversed-phase HPLC with absorbance and fluorescence detection two flavonols, quercetin and kaempferol, were detected in tomato fruit tissues from Spanish cherry tomatoes. Trace amounts of quercetin and kaempferol were detected in samples prior to acid hydrolysis. However, much larger quantities were detected after acid hydrolysis (Figure 3.1) indicating that the endogenous flavonols are present in tomato tissues primarily as conjugated structures.

3.3 Flavonol Identification by Liquid Chromatography-Mass Spectrometry

To verify the identification of flavonols present within tomato fruits, extracts from the skin of Spanish tomato variety Paloma taken before and after acid hydrolysis, were analysed using an HPLC system linked to a UV/vis monitor (371 nm) and mass spectrometer. This method provided not only UV absorbance chromatographs of flavonols, distinguishable by their retention time but also mass spectra for each peak detected. Analysis of tomato skin extracts by LCMS prior to acid hydrolysis demonstrated that the predominant quercetin conjugate was rutin, quercetin-3-rutinoside, m/z 609 amu (Figure 3.2). No kaempferol conjugates or additional quercetin conjugates were identified. LC-MS analysis (total ion current) of acid hydrolysed samples confirmed the presence of quercetin (m/z 301 amu) and kaempferol (m/z 285 amu) concluded to originate from conjugated forms as the aglycones were not present in extracts analysed prior to hydrolysis (Figure 3.3). Total ion current, LCMS analysis of acid hydrolysed

Figure 3.1 Detection of Flavonols in Tomato Fruits Before and After Acid Hydrolysis by HPLC

Gradient reverse phase HPLC analysis of flavonols. Column: 150 x 3.0 mm i.d. 4-μm Genesis C_{18} column with a 10 x 4.0 mm 4-μm Genesis C_{18} guard cartridge. Mobile phase 20 min gradient of 20-40 % acetonitrile in water containing 0.1 % trifluoroacetic acid. Flow rate: 0.5 ml min^{-1}. Detection by fluorescence (excitation 420 and emission 485 nm) following post-column derivatization with methanolic aluminium nitrate. Samples: (A) 100 ng of (1) myricetin, (2) morin, (3) quercetin, (4) kaempferol, (5) isorhamnetin; (B) 1 mg sample of lyophilised tomato tissue, cv. Paloma. (C) as B but after acid hydrolysis. Numbers indicate peaks that co-chromatograph with standards listed for sample A. Morin was added to samples B and C as an internal standard.

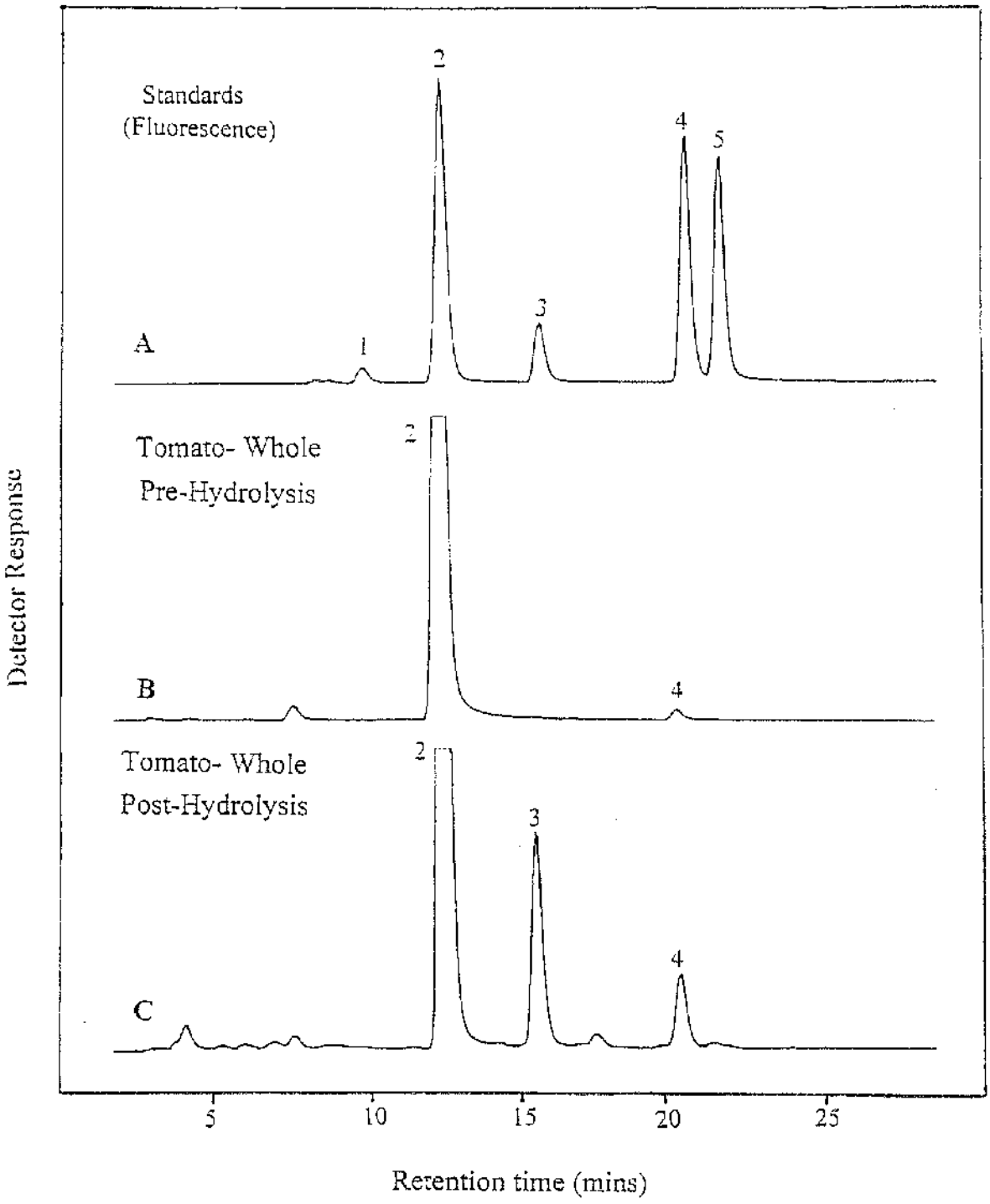
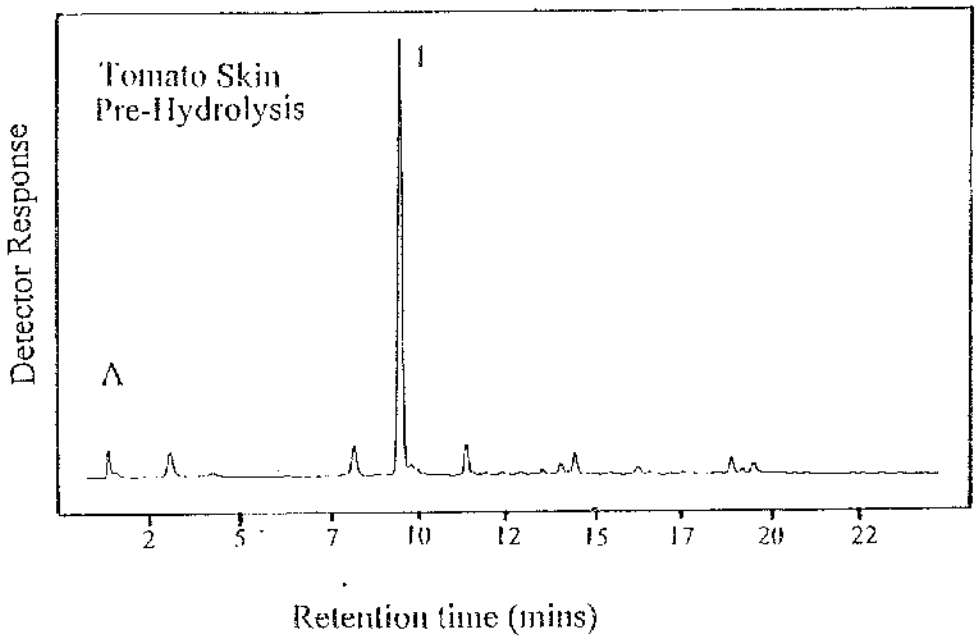

Standards
(Fluorescence)
A
1
2
3
4
5
Tomato- Whole
Pre-Hydrolysis
B
2
4
Tomato- Whole
Post-Hydrolysis
C
2
3
4
Detector Response
5
10
15
20
25
Retention time (mins)

Figure 3.2 Analysis of Flavonols in Tomato Fruits Prior to Acid Hydrolysis Using LCMS

Gradient reversed phase HPLC analysis of flavonols. Column 150 x 3.0 mm i.d. 5-μm Nemesis C_{18} column. Mobile phase: 20 min gradient of 12-35 % acetonitrile containing 1 % formic acid maintained at 35 % for a further 5 mins. Flow rate: 0.8 ml min^{-1}. Detector: absorbance monitor operating at 371 nm, after passing through the flow cell of the absorbance monitor the column eluate was directed to a Shimadzu LCQ 8000 quadrupole mass spectrometer with atmospheric pressure chemical ionisation in negative ion mode operating in full scan mode from 250-650 amu. Spectra obtained were analysed using Shimadzu LCMS QP 8000 software. Samples (A) 1 mg sample of lyophilised tomato fruit skin prior to acid hydrolysis analysed using UV detection at 371 nm. (B) full scan LCMS spectra, m/z 250-650, of rutin standard. (C) full scan spectra of pre-hydrolysed tomato skin sample, peak 1.


Tomato Skin
Pre-Hydrolysis
1
A
Detector Response
2
5
7
10
12
15
17
20
22
Retention time (mins)

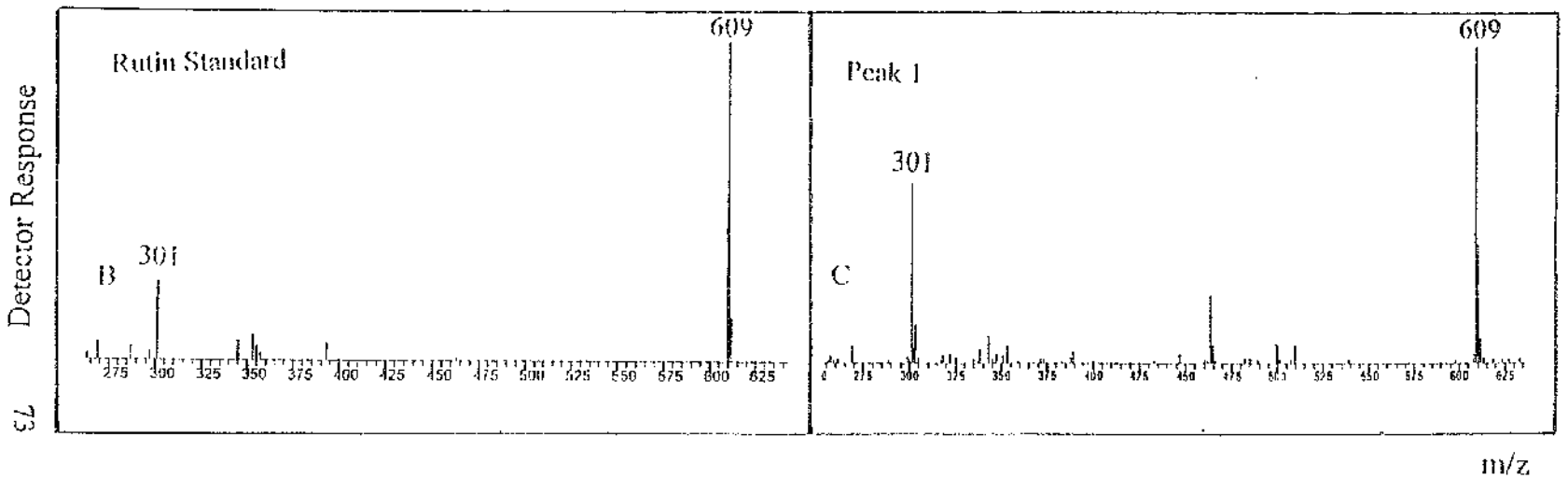
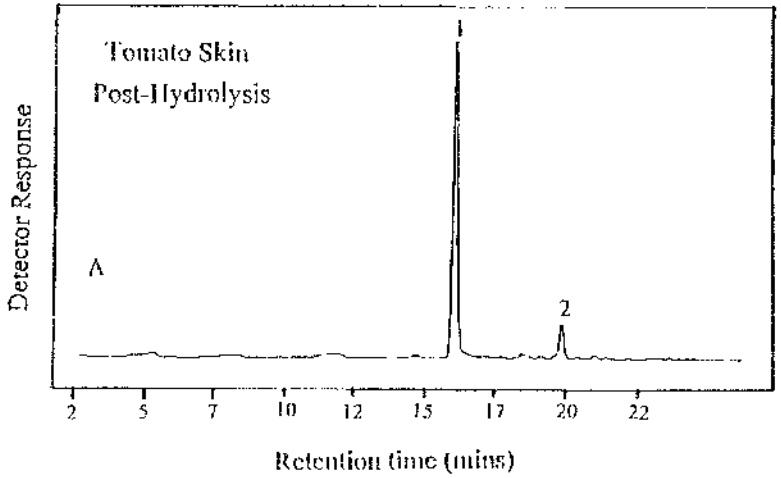
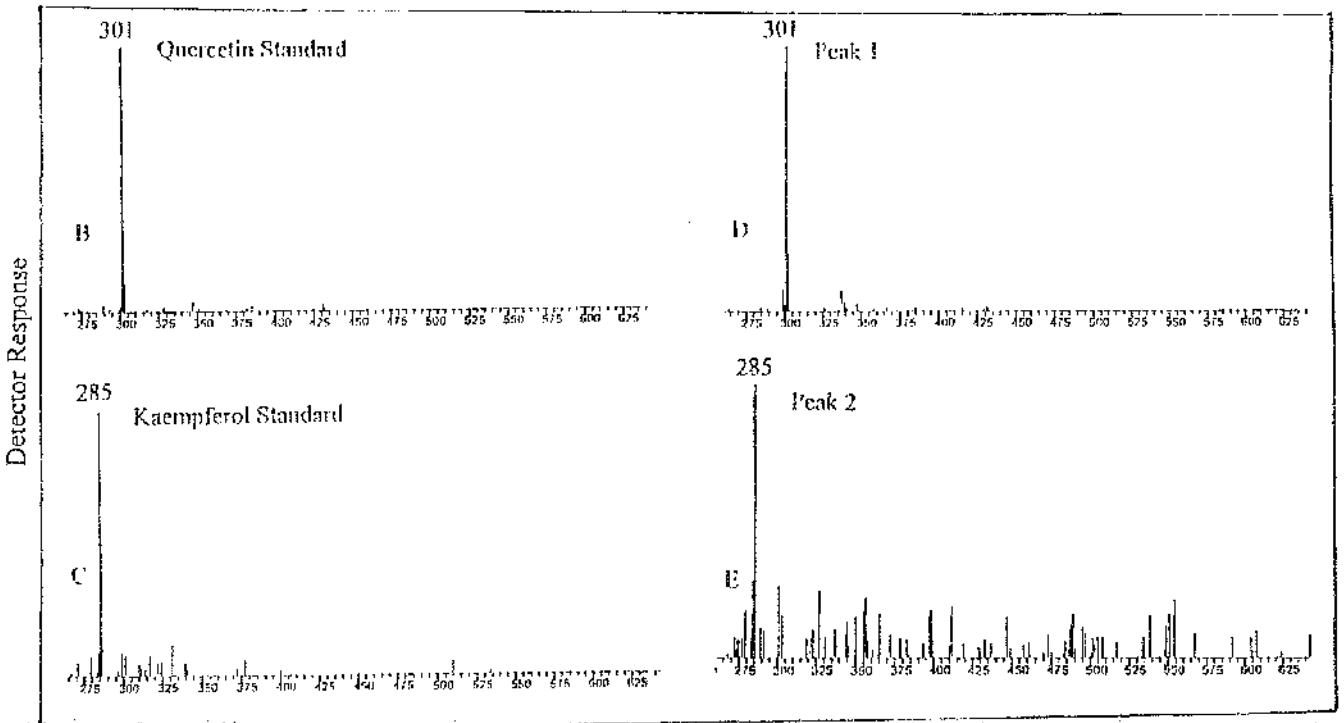

Rutin Standard
609
301
B
Peak 1
609
301
C
Detector Response
m/z

Figure 3.3 Analysis of Flavonols in Acid Hydrolysed Tomato Fruit Extracts Using LCMS

Gradient reversed phase HPLC analysis of flavonols. Column 150 x 3.0 mm i.d. 5-µm Nemesis C_{18} column. Mobile phase: 20 min gradient of 12-35 % acetonitrile containing 1 % formic acid maintained at 35 % for a further 5 mins. Flow rate: 0.8 ml min^{-1}. Detector: absorbance monitor operating at 371 nm, after passing through the flow cell of the absorbance monitor the column eluate was directed to a Shimadzu LCQ 8000 quadrupole mass spectrometer with atmospheric pressure chemical ionisation in negative ion mode operating in full scan mode from 250-650 amu. Spectra obtained were analysed using Shimadzu LCMS QP 8000 software. Samples (A) acid hydrolysed 1 mg sample of lyophilised tomato fruit skin analysed using UV detection at 371 nm. (B) full scan LCMS spectra, m/z 250-650, of quercetin standard. (C) LCMS spectra of kaempferol standard. (D) full scan spectra of hydrolysed tomato skin sample peak 1. (E) as D but with spectra of peak 2.


Tomato Skin
Post-Hydrolysis
A
1
2
Detector Response
2
5
7
10
12
15
17
20
22
Retention time (mins)


Detector Response
301
Quercetin Standard
B
301
Peak 1
D
285
Kaempferol Standard
C
285
Peak 2
E

samples allowed clear identification of the flavonol quercetin in the tomato fruit skin. Kaempferol was found to be present in lower concentrations thereby reducing the definition between the kaempferol peak and background interference in the UV chromatograph and also in the mass spectra (285 amu). LC-MS with selected ion monitoring was used to reduce background interference in acid hydrolysed tomato samples. Monitoring for quercetin at m/z 303 amu and kaempferol at m/z 285 amu allowed a reduction in the scale of the quercetin response whilst allowing a clearer and more sensitive response to kaempferol (Figure 3.4). In each case identification was achieved by comparison of mass spectra with spectra of authentic flavonol standards and by peak retention time. Flavonols in tomato samples were not quantified using this method.

3.4 Distribution of Flavonols within Tomato Fruits

When different parts of Spanish cherry tomatoes were examined, 98 % of the main flavonol, conjugated quercetin, was found in the skin compared to ca. 1 % in the seeds and flesh (Table 3.1). Quercetin contributes ca. 96 % (138.5 $\pm$ 5.6 μg/gfwt) of the skin-derived flavonols with the remainder consisting of kaempferol (4.8 $\pm$ 0.3 μg/gfwt).

Figure 3.4 Analysis of Flavonols in Acid Hydrolysed Tomato Fruit Skin Using LCMS-Selected Ion Monitoring

Gradient reversed phase HPLC analysis of flavonoids. Column: 150 x 3.0 mm i.d. 5 µm Nemesis C18 column. Mobile phase: 20 min gradient of 12-35 % acetonitrile containing 1 % formic acid maintained at 35 % for a further 5 mins. Flow rate: 0.8 ml/min. Detector: absorbance monitor operating at 371 nm, after passing through the flow cell of the absorbance monitor the column eluate was directed to a Shimadzu LCQ 8000 quadrupole mass spectrometer with atmospheric pressure chemical ionisation (APCI) in negative ion mode operating in selected ion monitoring (SIM) mode at m/z 303 and 285. Spectra obtained were analysed using Shimadzu LCMS QP 8000 software. Samples (A) 20 µg of (1) rutin, (2) quercetin-3-glucoside, (3) morin, (4) quercetin analysed using SIM at m/z 301. (B) acid hydrolysed 1 mg sample of lyophilised tomato tissue analysed using SIM at m/z 303. (C) 10 µg of kaempferol analysed using SIM at m/z 285. (D) acid hydrolysed 1 mg sample of lyophilised tomato tissue analysed using SIM at m/z 285. Numbers indicate peaks that co-chromatograph with standards listed for samples A and C.

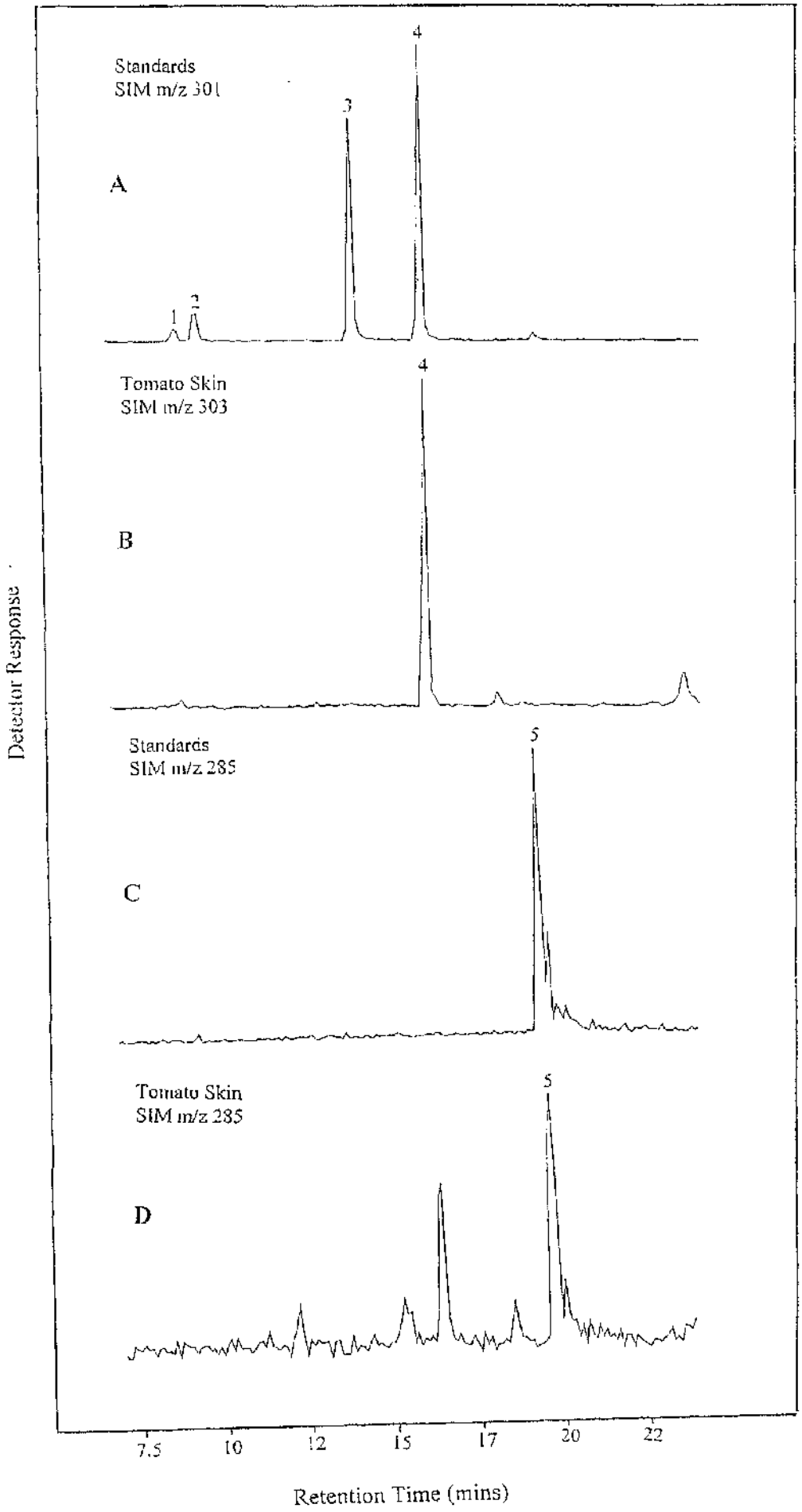

Standards
SIM m/z 301
A
1
2
3
4
Tomato Skin
SIM m/z 303
B
4
Standards
SIM m/z 285
C
5
Tomato Skin
SIM m/z 285
D
5
Detector Response
7.5
10
12
15
17
20
22
Retention Time (mins)

Table 3.1 Distribution of Flavonols in Spanish Cherry Tomatoes (*Lycopersicon esculentum* Mill) c.v. Paloma.

Tomato	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Total Flavonol
Whole	0.2 ± 0.0	23.4 ± 1.2	0.5 ± 0.1	1.2 ± 0.1	25.3 ± 1.3
Skin	0.7 ± 0.0	137.8 ± 5.6	0.4 ± 0.0	4.4 ± 0.3	143.3 ± 5.8
Flesh	n.d	0.9 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	1.2 ± 0.1
Seed	0.1 ± 0.0	1.0 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	1.5 ± 0.1

Tomatoes purchased from Safeway Stores plc. Data expressed as μg/g (fresh weight) ± standard error (n=3).

3.5 Effect of Season on Flavonol Content of Tomato Fruits

The flavonol content of Spanish grown cherry tomato, Paloma, was assessed over a period of 13 months. The levels fluctuated but not markedly with total flavonols ranging from 10.2-36.4 μg/g (Table 3.2). Growing conditions in Spain therefore appear to induce the accumulation of flavonols in tomato fruits throughout the growing season.

3.6 Screening Varieties of Tomato Fruit for Flavonol Content

Screening of tomato fruit varieties for flavonol content was focused mainly on British fruits due to ease of collection. English Village Nurseries (EVN), Southport, supplied the majority of tomato fruits. The large scale of production at this site with varieties grown for sale and field trials allowed a large selection of varieties to be harvested for analysis. Samples were also supplied by Scotland's Tomatoes based in Lanarkshire. Collecting tomato fruits directly from the growers allowed the identification of fruit varieties, plant suppliers and also an insight into methods of cultivation and environmental conditions on-site. Spanish tomato fruits cv Bodar, Bond, Royesta and Havanera were kindly donated by Dr Jesus Chammaro, Institute of Celluology, CSIC, Valencia, Spain. Other tomatoes from Spain as well as Israel and S.Africa, were supplied by Mr. B. Sparkes (English Village Salads plc). Tomatoes from the south of France were obtained in a local market in Toulouse by Sophie Bozonnet. All other fruits were purchased from Safeway, Byres Rd, Glasgow.

Table 3.2 Monthly Variations in the Flavonol Content of Spanish Cherry Tomato cv. Paloma Between February 1997 and February 1998[a]

Date of Harvest	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Total Flavonol
February 1997	n.d	10.9 ± 1.1	0.3 ± 0.0	0.2 ± 0.1	11.4 ± 1.2
March	n.d	19.8 ± 2.2	0.3 ± 0.0	0.4 ± 0.1	20.5 ± 2.2
April	n.d	24.6 ± 2.2	0.3 ± 0.1	0.5 ± 0.0	25.4 ± 2.2
May	0.5 ± 0.1	18.6 ± 1.2	0.3 ± 0.1	0.4 ± 0.1	19.8 ± 1.4
June	0.2 ± 0.0	23.4 ± 1.2	0.5 ± 0.1	1.2 ± 0.1	25.3 ± 1.3
July	0.1 ± 0.0	14.8 ± 0.7	0.2 ± 0.0	0.7 ± 0.1	15.9 ± 0.8
August	0.1 ± 0.0	16.7 ± 1.9	0.2 ± 0.0	0.6 ± 0.1	17.6 ± 2.0
September	0.1 ± 0.0	13.8 ± 1.4	0.2 ± 0.0	1.2 ± 0.2	15.2 ± 1.4
October	0.2 ± 0.0	25.7 ± 1.2	0.3 ± 0.0	1.7 ± 0.2	27.8 ± 1.4
November	0.7 ± 0.3	15.0 ± 0.2	0.2 ± 0.0	1.0 ± 0.0	16.3 ± 0.2
December	0.1 ± 0.0	22.5 ± 0.5	0.3 ± 0.0	1.0 ± 0.0	24.0 ± 0.5
January 1998	0.2 ± 0.0	33.4 ± 1.3	0.4 ± 0.0	2.3 ± 0.3	36.4 ± 1.2
February	0.1 ± 0.0	9.7 ± 0.4	0.2 ± 0.0	0.3 ± 0.0	10.2 ± 0.4

[a] Data expressed as μg/g (fresh weight) ± S.E (n=3).

Screening of tomato varieties from within a single nursery was intended to determine the effect of fruit variety on the flavonol content of fruits grown in very similar conditions. Fruits from different countries were analysed for flavonol content to broadly determine the effect of different environmental conditions and methods of cultivation.

3.7 Analysis of Tomato Fruits from English Village Nurseries

The total flavonol content of tomato fruits grown at EVN in the 1996 season varied from 0.9- 5.2 μg/gfwt. Approximately 90 % of the flavonols identified in English fruits were present in the form of conjugated quercetin with the remaining 10 % composed of both free and conjugated kaempferol (Table 3.3). Kaempferol was present in low levels (0.1-0.6 μg/gfwt) with detection only possible with fluorescence detection following post-column derivatization.

In similar environmental conditions and with similar cultivation methods fruit size appeared to be an important variable influencing flavonol content. Cherry tomato varieties contained higher concentrations of quercetin than other varieties (Table 3.3). Due to the low levels of kaempferol present in tomato fruits, varietal differences were difficult to establish, however the smaller tomato varieties appeared more abundant in kaempferol.

Table 3.3 Free and Conjugated Quercetin and Kaempferol Content of Tomato Varieties Grown at English Village Salads[a]

Fruit Variety	Supplier	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Total Flavonol
Aromata	Bruinsma	n.d	1.2 ± 0.0	0.1 ± 0.0	n.d	1.3 ± 0.1
B-4	Bruinsma	n.d	1.7 ± 0.0	0.1 ± 0.0	n.d	1.8 ± 0.0
Cheetah	S & G Seeds	0.1 ± 0.0	1.6 ± 0.2	0.1 ± 0.0	n.d	1.8 ± 0.2
Cherry Belle[C]	Unknown	n.d	3.9 ± 0.6	0.2 ± 0.0	0.2 ± 0.0	4.3 ± 0.6
E29351	Enza Zaden	n.d	2.0 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	2.2 ± 0.1
Favorita[C]	Unknown	n.d	3.0 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	3.4 ± 0.1
Flavore	Pinetree	n.d	1.6 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	1.8 ± 0.2
GB96T	Rijk Zwaan	n.d	0.9 ± 0.1	0.1 ± 0.0	n.d	1.0 ± 0.1
Melrow	Unknown	n.d	3.3 ± 0.5	0.3 ± 0.0	0.3 ± 0.1	3.9 ± 0.4
Santa[C]	Unknown	n.d	4.9 ± 0.8	0.2 ± 0.0	0.1 ± 0.0	5.2 ± 0.8
Saporo	Pinetree	n.d	1.6 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	1.8 ± 0.1
Solara	Pinetree	n.d	1.2 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	1.4 ± 0.2
Spectra	Rijk Zwaan	n.d	3.0 ± 0.4	0.1 ± 0.0	0.1 ± 0.0	3.2 ± 0.4
Sweetheart	Unknown	n.d	3.4 ± 1.1	0.3 ± 0.0	n.d	3.7 ± 0.9
Vitador	Bruinsma	n.d	1.6 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	1.8 ± 0.1
101-Yellow Pear[Y]	Unknown	0.1 ± 0.0	3.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	3.6 ± 0.2
102-Yellow[Y]	Unknown	n.d	2.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	2.5 ± 0.1
2816	Pinetree	n.d	3.6 ± 0.5	0.1 ± 0.0	0.1 ± 0.0	3.8 ± 0.4
3347	Pinetree	n.d	2.0 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	2.3 ± 0.1
3717	Pinetree	n.d	0.7 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.9 ± 0.1
72/47	Rijk Zwaan	n.d	3.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	3.5 ± 0.0
72/48	Rijk Zwaan	0.1 ± 0.0	0.9 ± 0.1	0.1 ± 0.0	n.d	1.1 ± 0.1
72/49	Rijk Zwaan	n.d	0.8 ± 0.0	0.1 ± 0.0	n.d	0.9 ± 0.0
7488	S & G Seeds	n.d	1.3 ± 0.2	0.1 ± 0.0	n.d	1.4 ± 0.2

[a]Results represent µg/g (fresh weight) ± S.E (n=3). Flavonol content of cherry tomatoes is denoted by [C] and yellow tomatoes[Y] .n.d = none detected. All produce was harvested from English Village Salads in July of 1996.

3.8 Flavonol Analysis of Tomato Fruits from Scotland's Tomatoes

Tomato varieties grown by Scotland's Tomatoes in the 1997 season were analysed for flavonol content (Table 3.4). The total flavonol content of the fruits varied from 1.1 –12.0 μg/gfwt. Quercetin was mainly present in a conjugated form with low levels of free quercetin detected in samples 72/47, E27681 and Vanessa Beefsteak. On average quercetin contributed 83.1 % of the total flavonol content of Scottish tomato fruits with the remainder present in the form of kaempferol. Cherry tomato varieties, E27681 and Favorita were found to contain the highest levels of flavonols, 12.0 and 6.6 μg/gfwt respectively. Beefsteak tomato varieties Vanessa 2000 and Vanessa Beefsteak contained similar flavonol levels to normal sized Scottish fruits (1.8 and 2.3 μg/gfwt respectively).

3.9 Flavonol Analysis of Tomato Fruits Grown in Different Countries

Fruits from different countries were analysed for flavonol content to broadly determine the effect of different environmental conditions and methods of cultivation, (Table 3.5). As observed for British fruits quercetin was the major flavonol detected, contributing ~ 92 % of the total flavonol content. Quercetin was present mainly in a conjugated form with low levels of free quercetin (1-2 % of total) detected in some samples.

Analysis of the red cherry tomato Favorita obtained not only from England but also Spain, S.Africa, and Scotland showed that fruits from Spain and S.Africa contained 4-5 fold more quercetin than British fruits. The smaller cherry varieties contained higher

Table 3.4 Free and Conjugated Quercetin and Kaempferol Content of Tomato Fruits Produced by Scotland's Tomatoes[a]

Fruit Variety	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Total Flavonol
Capita	n.d	1.1 ± 0.0	n.d	n.d	1.1 ± 0.0
E27681[C]	0.1 ± 0.0	10.9 ± 0.0	0.2 ± 0.0	0.7 ± 0.0	12.0 ± 0.1
Favorita[C]	n.d	5.4 ± 0.1	0.2 ± 0.0	1.0 ± 0.1	6.6 ± 0.1
Liberto	n.d	1.3 ± 0.2	0.2 ± 0.0	0.3 ± 0.0	1.8 ± 0.2
Lovatt	n.d	2.1 ± 0.5	0.2 ± 0.0	1.2 ± 0.3	3.5 ± 0.6
Spectra	n.d	1.8 ± 0.2	0.1 ± 0.0	0.3 ± 0.0	2.2 ± 0.2
Vanessa Beefsteak[B]	0.2 ± 0.1	1.8 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	2.3 ± 0.1
Vanessa 2000[B]	n.d	1.6 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	1.8 ± 0.2
72/47	0.2 ± 0.1	3.0 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	3.7 ± 0.1

[a]Results represent µg/g (fresh weight) ± S.E where n=3. Flavonol content of cherry tomato varieties is denoted by [C] and beefsteak varieties by [B]. n.d= none detected. Tomatoes were harvested from Scotland's Tomatoes in June of 1997.

Table 3.5 Flavonol Content of Tomato Fruits Produced in Various Countries[a]

Country of Origin	Fruit Variety	Date of Harvest	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Total Flavonol
Canary Islands	Unknown	Jan 1999	n.d	3.6 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	4.1 ± 0.1
Holland	Unknown	Jan 1999	n.d	3.9 ± 1.0	0.3 ± 0.0	0.2 ± 0.0	4.4 ± 1.0
Israel	Unknown[C]	Mar 1997	n.d	21.5 ± 0.8	0.2 ± 0.1	0.5 ± 0.0	22.2 ± 0.8
S.Africa	Favorita[C]	Feb 1997	n.d	15.0 ± 0.7	0.2 ± 0.1	0.8 ± 0.0	16.0 ± 0.7
S.France	Unknown (Market)	Sept 1997	n.d	10.3 ± 0.4	0.2 ± 0.0	1.3 ± 0.0	11.8 ± 0.5
	Unknown (Toulouse)	Sept 1997	0.2 ± 0.0	13.1 ± 1.1	0.2 ± 0.0	0.4 ± 0.0	13.9 ± 1.1
Spain	Baez	July 1997	0.2 ± 0.1	6.3 ± 0.4	0.2 ± 0.0	0.3 ± 0.0	7.0 ± 0.4
	Bodar	July 1997	0.1 ± 0.0	7.7 ± 0.9	0.2 ± 0.0	0.4 ± 0.1	8.4 ± 1.0
	Bond	July 1997	0.1 ± 0.0	10.2 ± 0.5	0.3 ± 0.0	0.4 ± 0.0	10.9 ± 0.5
	Claudia	July 1997	0.3 ± 0.1	8.7 ± 1.1	0.2 ± 0.0	0.4 ± 0.0	9.6 ± 1.1
	Favorita[C]	Jan 1997	n.d	20.7 ± 0.6	0.2 ± 0.0	0.6 ± 0.1	21.5 ± 1.8
	Havanera	July 1997	n.d	5.6 ± 0.9	0.2 ± 0.0	0.8 ± 0.1	6.6 ± 1.0
	Paloma[C]	May 1997	0.5 ± 0.1	18.6 ± 1.2	0.3 ± 0.1	0.4 ± 0.1	19.8 ± 1.3
	Royesta	July 1997	0.1 ± 0.0	12.7 ± 0.4	0.3 ± 0.0	0.7 ± 0.1	13.8 ± 0.4
	Unknown[B]	Jan 1999	n.d	4.4 ± 0.3	0.3 ± 0.0	0.3 ± 0.0	5.0 ± 0.3

[a]Results represent µg/g (fresh weight) ± S.E (n=3). Flavonol content of cherry tomatoes is denoted by [C] and beefsteak tomatoes by [B]. n.d= none detected.

levels of flavonols as compared to fruits of regular size (Table 3.5). The Spanish tomato fruit with the lowest quercetin concentration was the large beefsteak fruit. Within the Spanish group the effect of variety on flavonol levels can be observed. Bond and Havanera are both normal sized, field grown Spanish tomatoes obtained from plants cultivated alongside each other on the same plot near Valencia (July 1997), none-the-less the total flavonol content of Bond fruit was 10.9 ± 0.5 μg/g compared to 6.6 ± 1.0 μg/g in Havanera.

3.10 Flavonol Analysis of Tomato Fruits with Different Skin Colours

The concentration of flavonols in tomato fruits with deep red and purple skins was investigated. It was hypothesised that the skins of such varieties may contain substantial amounts of anthocyanins, and as flavonols originate from the same branch of the phenylpropanoid pathway as anthocyanins (Holton & Cornish, 1995; Duthie &Crozier, 2000) the skins might also contain elevated levels of flavonols. Plants were grown in a growth room in conditions of constant high white light (80-100 μmol/m^2/s) at 20°C. Fruits were harvested at ripeness and the flavonol content of their skins analysed. The darkly pigmented skin of Noire Charbonneuse had a total flavonol content of 440 μg/g (Table 3.6 & Figure 3.5). The flavonol content of Anthocyanin Gainer, a deep red fruit with yellow 'freckles' was also found to be high (Figure 3.6). However not all of the darkly pigmented fruits were high in flavonols; skin from Aubergine, a variety characterised by purple striations contained only 108 μg flavonol/g (Figure 3.7). In contrast, skin from Anthocyanin Free and Dark Green, which were not heavily pigmented

Table 3.6 Flavonol Content of Skins from Tomato Varieties with Different Coloured Skins[a]

Tomato Variety	Skin Colour	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Total Flavonol
Noire Charbonneuse	red/purple	3.9 ± 0.2	402 ± 14	0.2 ± 0.0	14.2 ± 2.0	440 ± 29
Anthocyanin Gainer	deep red	3.0 ± 0.4	252 ± 30	0.4 ± 0.0	20.9 ± 1.0	276 ± 32
Aubergine	red/dark patches	0.3 ± 0.0	103 ± 7	n.d	4.5 ± 0.2	108 ± 7
Anthocyanin Free	red	0.6 ± 0.0	206 ± 20	0.1 ± 0.0	17.3 ± 0.9	224 ± 19
Dark Green	red/yellow	0.8 ± 0.1	183 ± 11	n.d	4.9 ± 0.4	189 ± 12

[a] Data expressed as µg/g (fresh weight) ± S.E (n=3).

Figure 3.5

Tomato fruit of variety Noire Charbonneuse. Fruit depicted during ripening, the lower part of the fruit ripened to become a dark red/purple colour, the upper part of the fruit retained its dark striations. This pattern was also observed in the flesh of the fruit.

Figure 3.5 Tomato Fruit of Variety Noire Charbonneuse

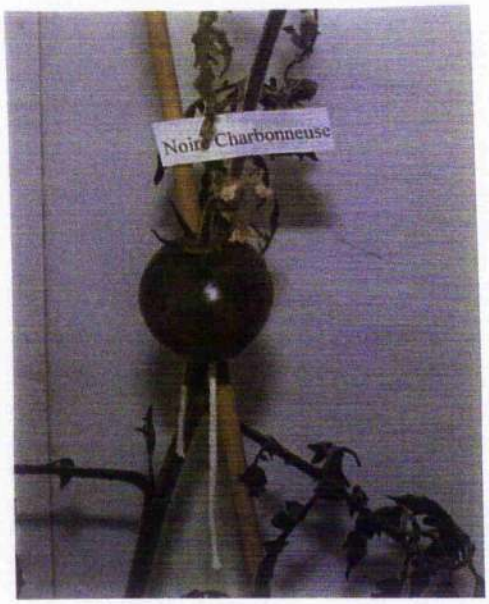


Figure 3.6

Tomato fruit of Anthocyanin Gainer. (A) immature tomato fruits during ripening. (B) a ripe fruit of variety Anthocyanin Gainer, deep red in colour with yellow 'freckles'.

Figure 3.6 Tomato Fruit of Anthocyanin Gainer

A

B

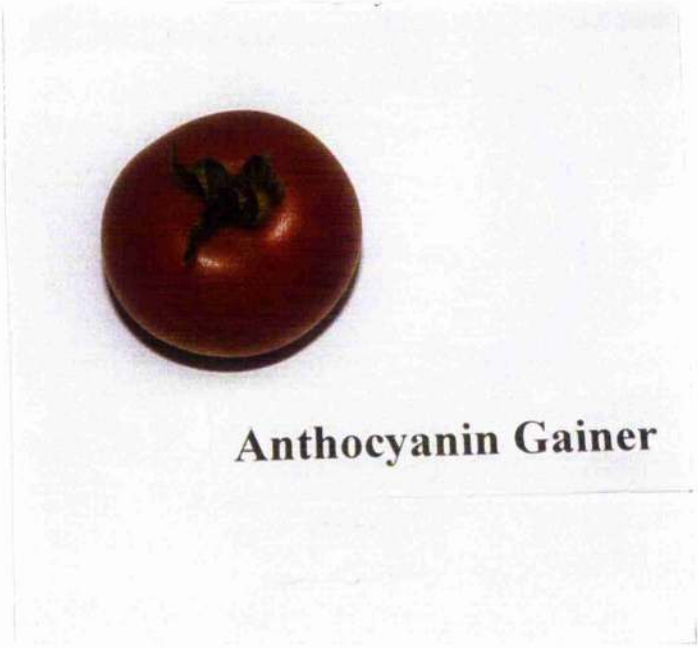


Figure 3.7

Tomato fruit of variety Aubergine. (A) fruit at the mature green stage of development, purple striations are observed on the upper part of the fruit. (B) ripe fruit of tomato variety Aubergine. Fruits ripen to become red/purple in colour with purple striations remaining on the upper part of the fruit.

Figure 3.7 Tomato Fruit of Variety Aubergine

A

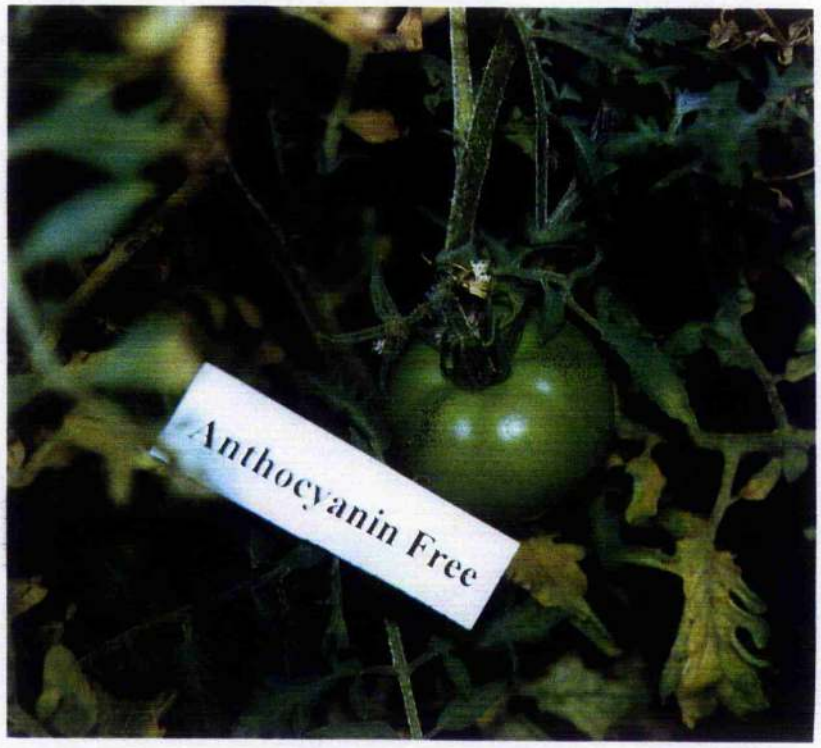
B

Figure 3.8

Tomato fruit of variety Anthocyanin Free. (A) fruit at the mature green stage of development, black 'freckles' are observed on the upper part of the fruit. (B) ripe fruit of tomato variety Anthocyanin free, black 'freckles' are no longer observed with fruits ripening to become red.

Figure 3.8 Tomato Fruit of Variety Anthocyanin Free

A



B

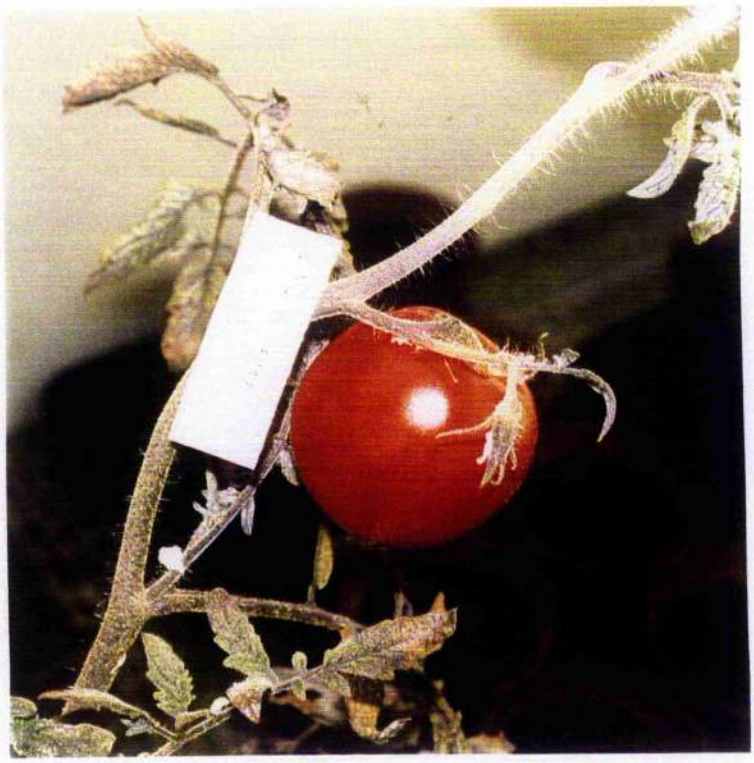
Figure 3.9

Tomato fruit of variety Dark Green. (A) tomato fruit at the mature green stage of development, fruits were very dark green with yellow striations. (B) ripe fruit of tomato variety Dark Green, contrary to its name this variety ripens to become red/yellow in colour.

Figure 3.9 Tomato Fruit of Variety Dark Green

A

B



contained 224 and 189 μg/g, respectively (Figures 3.8 & 3.9). Although leaves from these plants were not analysed in this study many also showed increased pigmentation (Figure 3.10).

3.11 Effect of Different Light Regimes on the Flavonol Content of Detached Tomatoes

Data obtained in this study (section 3.9) indicate that those tomato fruits grown in warm sunny climates contain high levels of flavonols. The accumulation of flavonols in these fruits may be enhanced by their exposure to high levels of sunlight. High levels of white light and in particular UVB, UVA and blue light are known to induce the biosynthesis of flavonoids (Fuglevand *et al.*, 1996).

The effect of light quality on the flavonol content of tomato fruits was investigated by exposing detached fruits to high or low white light, red, blue, UVA or UVB light. Tomato fruits of variety Spectra, obtained from Scotland's Tomatoes, were picked at the fully grown green stage of maturation, transferred to a growth room and allowed to ripen over 12 days at 20 °C under one of the chosen light qualities. Fruits were removed after 3, 6, 9 and 12 days for flavonol analysis (Table 3.7). All fruits had ripened normally after 12 days exposure.

The flavonol content of all the tomatoes declined slightly under all six light regimes indicating that the detached fruit are relatively insensitive to alterations in light quality.

Figure 3.10

Leaf pigmentation of tomato plants grown to produce tomato fruits with different skin colours. A typical leaf was removed from each plant after 4 weeks of growth in conditions of white light (80-100 $\mu mol/m^2/s$). Increased pigmentation was observed on the leaves of Anthocyanin Gainer, Intense Pigment and Aubergine. Some pigmentation was observed on the veins of Dark Green and the tips of the leaflets of Anthocyanin Free. Plant variety Intense Pigment was very small compared to the other varieties employed in this study and was unable to set fruit.

Figure 3.10 Leaf Pigmentation of Tomato Plants Grown to Produce Tomato Fruits with Different Skin Colours

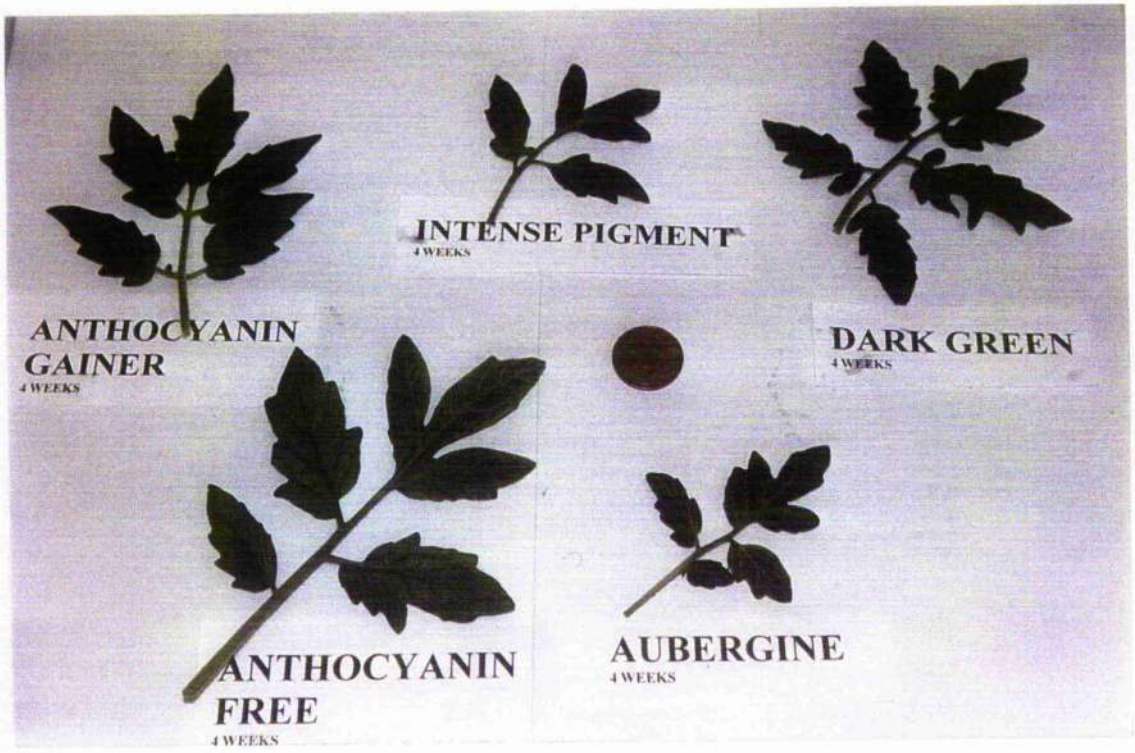


Table 3.7 Effect of Different Light Regimes on the Flavonol Content of Detached Tomato Fruits[a]

Light Treatment	Incubation Time (days)			
	3	6	9	12
High White	3.8 ± 0.7	2.6 ± 0.3	3.6 ± 0.7	3.6 ± 0.4
Low White	2.7 ± 0.3	3.9 ± 0.3	2.0 ± 0.2	2.9 ± 0.8
Red	3.2 + 0.5	2.3 ± 0.6	2.2 ± 0.1	3.6 ± 0.8
Blue	3.0 ± 0.1	1.9 ± 0.2	2.7 + 0.1	3.2 ± 0.2
UVA	3.5 ± 0.3	2.3 ± 0.2	3.0 ± 0.6	3.3 ± 0.2
UVB	3.1 ± 0.3	1.9 ± 0.3	4.6 ± 0.8	3.7 ± 0.1

[a]Total flavonoid content of detached tomato fruits, variety Spectra, obtained from Scotland's Tomatoes November, 1996, at mature green stage of development and incubated for up to 12 days at 20 °C under high white light (80 µmol/m^2/s), low white light (20 µmol/m^2/s), red light (85 µmol/m^2/s), blue light (50 µmol/m^2/s), UVA light (80 µmol/m^2/s) or UVB light (4 µmol/m^2/s). Prior to Treatment tomato fruits contained 5.0 ± 0.9 µg flavonol/g/fwt Results represent µg/g/fwt ± standard error where n=3.

3.12 Flavonol Content of Various Tomato Based Foods

A range of processed tomato products were analysed and the data obtained are summarised in Table 3.8. Tomato juice was found to be a rich source of flavonols with a total flavonol content 14-16 μg/ml, comparable with that of red wine which can vary from 4.6-41.6 μg/ml (McDonald *et al.*, 1998). The flavonols detected in tomato juice comprised 95 % quercetin, 4 % kaempferol and 1 % isorhamnetin, trace levels of morin were also detected. In contrast to tomato fruit, which contains almost exclusively conjugated quercetin, up to 30 % of the quercetin in processed produce was in the free form. Hydrolysis of flavonol conjugates during cooking of tomatoes was not observed in an earlier study (Crozier *et al.*, 1997) so the accumulation of quercetin in juices, puree and paste may be a consequence of enzymatic hydrolysis of rutin and other quercetin conjugates during pasteurisation and processing procedures. The concentration of flavonols in tomato juice is likely to depend on the extraction of flavonols from the skin into the juice during initial processing, which often involves heating, and also on the amount of skin remaining in the tomato juice following filtration. Safeway brand tomato puree was also identified as being particularly rich in flavonols, containing 70 μg/g of which 98 % was quercetin and ~ 2 % kaempferol. Analysis of tinned tomatoes revealed that tinned cherry tomatoes contained five times more quercetin than tinned peeled plum tomatoes (Napolina), 1.7 and 0.3 μg/gfwt respectively. This may indicate the contribution of the tomato skin to the flavonol content of the food product and may also indicate that the cherry tomato varieties were higher in flavonol content than the plum tomatoes. However both tinned tomato products contained flavonols in very low levels indeed

Table 3.8 Free and Conjugated Quercetin, Kaempferol and Isorhamnetin Content of Tomato-Based Food Products[a]

Tomato Product	Brand	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Free Isorhamnetin	Conjugated Isorhamnetin	Free Flavonol %	Total Flavonol
Tomato Soup	Safeway	0.3 ± 0.0	1.2 ± 0.1	n.d	n.d	n.d	n.d	19.6	1.5 ± 0.1
Tomato Juice	Del Monte	2.9 ± 0.1	11.5 ± 1.8	0.4 ± 0.0	0.4 ± 0.1	n.d	0.1 ± 0.0	21.6	15.3 ± 1.9
	Libby's	3.5 ± 0.2	12.7 ± 1.0	0.4 ± 0.0	0.3 ± 0.0	n.d	0.1 ± 0.0	22.9	17.0 ± 1.0
Tin Cherry Tomatoes	Napolina	n.d	1.7 ± 0.1	n.d	0.1 ± 0.0	n.d	n.d	0	1.8 ± 0.1
Tin Plum Tomatoes	Napolina	n.d	0.3 ± 0.0	n.d	n.d	n.d	n.d	0	0.4 ± 0.0
Pasta Sauce	Dolmio	1.2 ± 0.2	7.9 ± 0.6	n.d	0.1 ± 0.0	n.d	0.3 ± 0.0	12.6	9.5 ± 0.5
Ketchup	Heinz	0.4 ± 0.0	8.2 ± 0.5	n.d	0.1 ± 0.0	n.d	n.d	4.5	8.8 ± 0.5
Puree	Casino	3.8 ± 0.2	32.5 ± 4.0	0.6 ± 0.3	0.2 ± 0.0	n.d	n.d	11.9	37.1 ± 4.3
	Masque D'or	5.4 ± 0.5	10.9 ± 2.1	n.d	0.3 ± 0.0	n.d	n.d	32.5	16.6 ± 1.7
	Safeway	9.5 ± 1.6	61.4 ± 5.5	n.d	1.3 ± 0.2	n.d	n.d	13.2	72.2 ± 5.8

[a]Data for tomato juice and tomato soup expressed as μg/ml ± S.E (n=3), all other data expressed as μg/g (fresh weight) ± S.E (n=3). n.d = not detected

compared to most varieties of fresh fruit. This could be due to boiling of the fruit prior to canning as cooking in this manner results in up to an 80 % loss of flavonols (Crozier *et al.*, 1997), presumably by leaching from the skins.

3.13 Discussion

Screening of the flavonol content of fruits and vegetables by Hertog *et al.* (1992), included the quantification of flavonols in Dutch tomatoes at four time points over a twelve month period. The amounts detected were between 4.6 and 8.2 μg quercetin/g (fresh weight) and <2 μg kaempferol/g. The samples were analysed only after acid hydrolysis and therefore provided no information on the relative proportion of free and conjugated flavonols. More recently work by Crozier *et al.* (1997) on the flavonol content of Spanish, Scottish and Dutch tomatoes showed that quercetin is present almost exclusively as conjugates, an observation confirmed by the present study. Mass spectrometric analysis of the flavonol content of tomato extracts, enabled the identification of quercetin and rutin (Justesen *et al.*, 1998; Mauri *et al.*, 1999).

With the addition of fluorescence detection following post-column derivatization with aluminium nitrate, free quercetin and kaempferol although present at low levels could be detected in most tomato samples prior to hydrolysis. After acid hydrolysis, quercetin was identified as the major flavonol with lower levels of kaempferol also detected. Analysis of tomato extracts prior to hydrolysis allowed identification of the quercetin conjugate rutin (UV detection, 365 nm).

The production of flavonols in plant tissues is influenced by environmental factors. In many plant species the accumulation of flavonols is enhanced in response to elevated light levels, in particular to increased UV-B radiation (Lois & Buchanan, 1994; Brandt *et al.*, 1995). There is evidence that quercetin is present in much higher concentrations in the skin of Pinot Noir grapes grown in the open rather than shaded clusters (Price *et al.*, 1995). Exposure to high levels of sunlight may therefore result in the accumulation of increased amounts of protective compounds, including flavonols in the skin of tomato fruit. Commercial tomato growing in Scotland and England usually involves the use of greenhouses with plants tightly packed together and fruits shaded by the foliage of surrounding plants (Figure 3.11). Fruit grown in greenhouses is effectively grown in conditions of relatively low light (~35 $\mu Ei/m^2/s$) with UV-B from sunlight filtered first through glass and then through surrounding leaf tissue. This may limit the induction and reduce the accumulation of flavonols in the skin of the tomatoes. In contrast, fruit from warmer sunnier countries such as Spain are usually field grown and if necessary they are shielded from the elements using plastic rather than glass. The developing fruits would receive more sunlight and would be exposed to UV-B radiation. Cherry tomato E27 681, obtained from Scotland's Tomatoes was found to contain higher levels of flavonols (11.9 μg quercetin/g fresh weight) than the other Scottish grown cherry tomatoes. However, E27 681 was grown in a small experimental greenhouse with plants well spaced out with little shading of fruit.

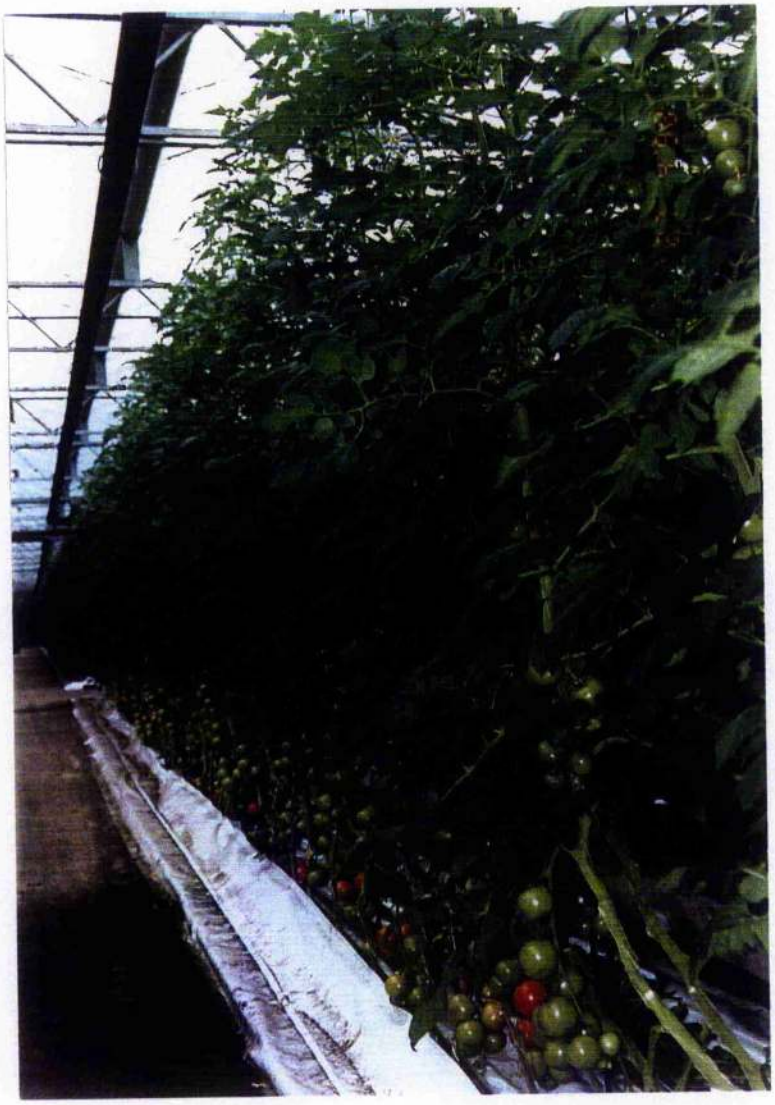
No effect of high light intensity was observed on the flavonol content of fruits detached from the parent plant. It is possible that when detached from the parent plant, tomato fruits do not have the reserves required to synthesise increased quantities of

Figure 3.11

Typical commercial conditions for the production of tomato fruits in Scotland

Due to the expense of maintaining glasshouses with tightly controlled systems for maintaining optimum conditions of temperature and nutrition, tomatoes are produced in row upon row of tightly packed plants to maximise yields. Tomato fruits are concentrated towards the lower part of the plant and are therefore shaded by the foliage of surrounding plants. This photograph was taken at Garrion Fruit Farm, Lanarkshire and is typical of commercial tomato fruit growing in Scotland.

Figure 3.11 Typical Commercial Conditions for the Production of Tomato Fruits in Scotland



flavonols. Alternatively, young fruits may respond to potentially damaging levels of light by accumulating protective pigments such as flavonols in order to protect the development of seeds within the fruit. As ripening is a process of dying during which the seeds reach maturity, the induction of protective compounds may no longer be necessary.

It is difficult to draw conclusions on the effects of environmental influences on the flavonol content of tomato fruits. No environmental data could be obtained for sites abroad and even within the UK, conditions would vary greatly within a single growing season. However fruits grown in warm sunny countries appeared to contain higher levels of flavonols.

Factors influencing the flavonol content of tomato fruits include variety and fruit size, such factors may be of greater importance in warmer sunnier climates such as Spain. Flavonol levels in British fruits may be restricted to a greater extent by a lack of light induction rather than the capacity of the type of tomato fruit being grown to synthesise flavonols. It is clear from the analysis of Spanish fruits that the highest flavonol concentrations are found in cherry tomatoes, with lower levels in normal sized and beefsteak tomato fruit varieties. The total flavonol content of Spanish Favorita, a red cherry tomato, was 21.5 μg/g, compared to the normal sized fruit variety, Bond, which contained 10.9 μg/g (Table 3.5). Compared with an earlier study that included commercial English and Spanish cherry tomatoes (Crozier *et al.*, 1997), the flavonol contents of the English grown cherry varieties, Favorita and Cherry Belle were very low indeed, with the levels no different from those detected in normal sized fruits from the same source (Table 3.3). One factor which may explain the elevated levels of flavonols in cherry tomatoes compared to normal varieties reported by Crozier *et al.*, (1997) is the

higher skin: volume ratio of small tomatoes compared to larger varieties. There is evidence that red wines derived from small thick skinned grapes, such as Cabernet Sauvignon, contain much higher levels of flavonols than wines made from large thin skinned berries such as Grenache and Cinsault (McDonald *et al.*, 1998).

Flavonols within tomato fruits were able to survive industrial processing methods and could be detected in a range of tomato based food products such as pasta sauce, ketchup and soup. Tomato puree was a particularly rich source of flavonols, which may be due to the concentrated nature of this product. Previous studies (Hertog *et al.*, 1993) on the flavonol content of beverages, identified quercetin (1.3 mg/l) and low levels of myricetin (< 0.5 mg/l) in tomato juice. Results presented (Table 3.8) identified much higher concentrations of quercetin (14-16 mg/l) with kaempferol, isorhamnetin and trace levels of morin also detected. Myricetin was not identified in tomato juice despite the increased sensitivity of fluorescence detection. The concentration of flavonols in tomato juice is likely to depend on the extraction of flavonols from the skin into the juice during initial processing, which often involves heating, and on the proportion of skin remaining in the juice following filtration. The concentration of flavonols found in tomato products may depend upon tomato variety used, proportion of skin remaining in final product or extraction of flavonols from fruit skin during processing.

3.14 Conclusions

Tomatoes and tomato-based products are a rich source of conjugated quercetin and kaempferol. Flavonol contents were found to vary according to fruit variety, size and country of origin, with cherry tomatoes originating from warm, sunny climates such as Spain containing the highest concentrations. Tomato flavonols were able to withstand industrial processing methods allowing their detection in a variety of tomato based products. Tomato juice (16.6 μg/ml), and tomato puree (72.2 μg/g fresh weight), were found to be particularly rich in flavonols. Because of the addition of tomato sauce to many foods, and the widespread use of concentrated tomato pastes in dishes such as pizza and lasagne, tomatoes may directly and indirectly make a more sizeable contribution to daily flavonol intake than was previously realised.

Chapter 4 Absorption and Excretion of Flavonols from Tomato Fruits and Tomato Juice

4.1 Introduction

Although studies of the flavonol content of fruits are clearly important with respect to potential nutritional value, a key factor is whether flavonols are absorbed during digestion. Early studies on the ability of flavonoids to be absorbed by the human body produced widely conflicting results, (Gugler *et al.*, 1975, Kuhnau, 1976). The situation has been clarified by recent work focusing on the absorption of flavonoids from food. Hollman (1995) employed ileostomy patients thereby eliminating any colonic degradation that could lead to an overestimation of the amount of flavonoids absorbed. Results showed that onion conjugates were better absorbed than the corresponding aglycone. A similar study with healthy subjects demonstrated the variable absorption and excretion of onion-derived quercetin and isorhamnetin glucosides (Aziz *et al.*, 1998).

The work presented in this chapter aimed to establish the bioavailability of flavonols from flavonol rich cherry tomato fruits and tomato juice. This was achieved by determining the levels of flavonol accumulation in plasma and excretion in urine using sensitive HPLC procedures.

4.2 Flavonol Intake from Tomato Fruits and Tomato Juice Consumed in this Study

All foods and beverages employed in this study were well accepted by volunteers and caused no adverse reaction. They were easily available and commonly consumed brands found to contain consistently high levels of flavonols. The amount of each product ingested was not designed to provide each subject with a similar dose, instead these simply represent the maximum that could be consumed within the time available (Table 4.1).

4.3 Flavonol Content of Tomato Fruits and Tomato Juice

Tomatoes were found to contain quercetin and kaempferol, with total flavonol content 11.7 and 13.7 μg/g fresh weight in samples one and two respectively. Tomato juice was found to contain low levels of the additional flavonol isorhamnetin and had a total flavonol content of 16.3 μg/ml. Isorhamnetin is present mainly in the green tissue of the tomato, although low levels of isorhamnetin are detected in tomato fruit skin. The presence of isorhamnetin in tomato juice may represent contamination of the juice with vegetative tissue during processing. In general tomato based products were found to contain low levels of free flavonols with most of the quercetin locked up as conjugates, principally as quercetin-3-rutinoside, (rutin), (Table 4.2).

Table 4.1 Flavonol Intake from Tomato Fruits and Tomato Juice Selected for Feeding Studies[a]

Tomato Product	Brand	Amount Consumed	Flavonol Content	Flavonol Intake (mg)
Tomato Juice	Del Monte	860 ml	16.40 ± 0.60	13.95
Tomato Subject A	Safeway Spanish cherry	367 g	11.45 ± 0.37	4.20
Tomato Subject B	Safeway Spanish Cherry	257 g	13.47 ± 1.26	3.31
Tomato Subject C	Safeway Spanish Cherry	250 g	13.47 ± 1.26	3.22

[a]Flavonol content expressed as μg/ml for beverages and μg/g(fresh weight) for solids. Errors represent standard error where n=3 for all cases. Flavonol Intake was calculated on the basis of the flavonol content of the product consumed.

Table 4.2 Free and Conjugated Flavonol Content of Tomatoes and Tomato Juice Employed in Feeding Studies[a]

Food Product	Quercetin		Kaempferol		Isorhamnetin	
	Free	Conjugated	Free	Conjugated	Free	Conjugated
Tomato Juice	0.1 ± 0.0	15.3 ± 0.6	n.d	0.5 ± 0.0	n.d	0.4 ± 0.0
Tomato Sample 1	0.2 ± 0.0	10.6 ± 0.6	0.2 ± 0.0	0.7 ± 0.1	n.d	n.d
Tomato Sample 2	0.1 ± 0.0	12.8 ± 1.2	0.2 ± 0.0	0.6 ± 0.0	n.d	n.d

[a]Flavonol content expressed as μg/ml for beverages and μg/g(fresh weight) for solids. Errors represent standard errors where n=3. Tomato sample 1 was consumed by subject A, sample 2 was consumed by subjects B and C. nd- not detectable.

4.4 Accumulation of Flavonols in Plasma

Absorption profiles clearly demonstrate the accumulation of flavonols in plasma following consumption of tomato fruits (Figure 4.1) and tomato juice (Figure 4.2). In each case two major peaks in accumulation of conjugated quercetin were observed with subsequent decline. These observations are in keeping with previous work on the absorption of flavonols from onions into human plasma (Aziz *et al.*, 1998). Subjects consuming Spanish cherry tomatoes displayed peak accumulation of quercetin conjugates between 1.0-1.5 h and then again at 3.0-4.0 h. A similar absorption profile was observed following the consumption of tomato juice, peak flavonol accumulation was observed at 0.5 and 3.0 h. The main quercetin conjugate present is believed to be rutin, however HPLC techniques were not sufficiently sensitive to detect rutin in plasma samples.

Free quercetin was detected in plasma between 1.0 and 2.0 h after consumption of tomatoes (Figure 4.1). This may reflect either the absorption of free quercetin or the absorption and subsequent metabolism of conjugated quercetin. Subjects A and C absorbed only quercetin whereas plasma from subject B was found to contain low levels of conjugated kaempferol.

Figure 4.1

Detection of free and conjugated quercetin in human plasma from three volunteers following consumption of Safeway Spanish cherry tomatoes. Data expressed as percentage of the intake based on the flavonol content of Spanish cherry tomatoes ± S.E (n=3) and calculated on the basis of 3000 ml of plasma per person.

Figure 4.1 Detection of Free and Conjugated Quercetin in Human Plasma Following Consumption of Spanish Cherry Tomatoes

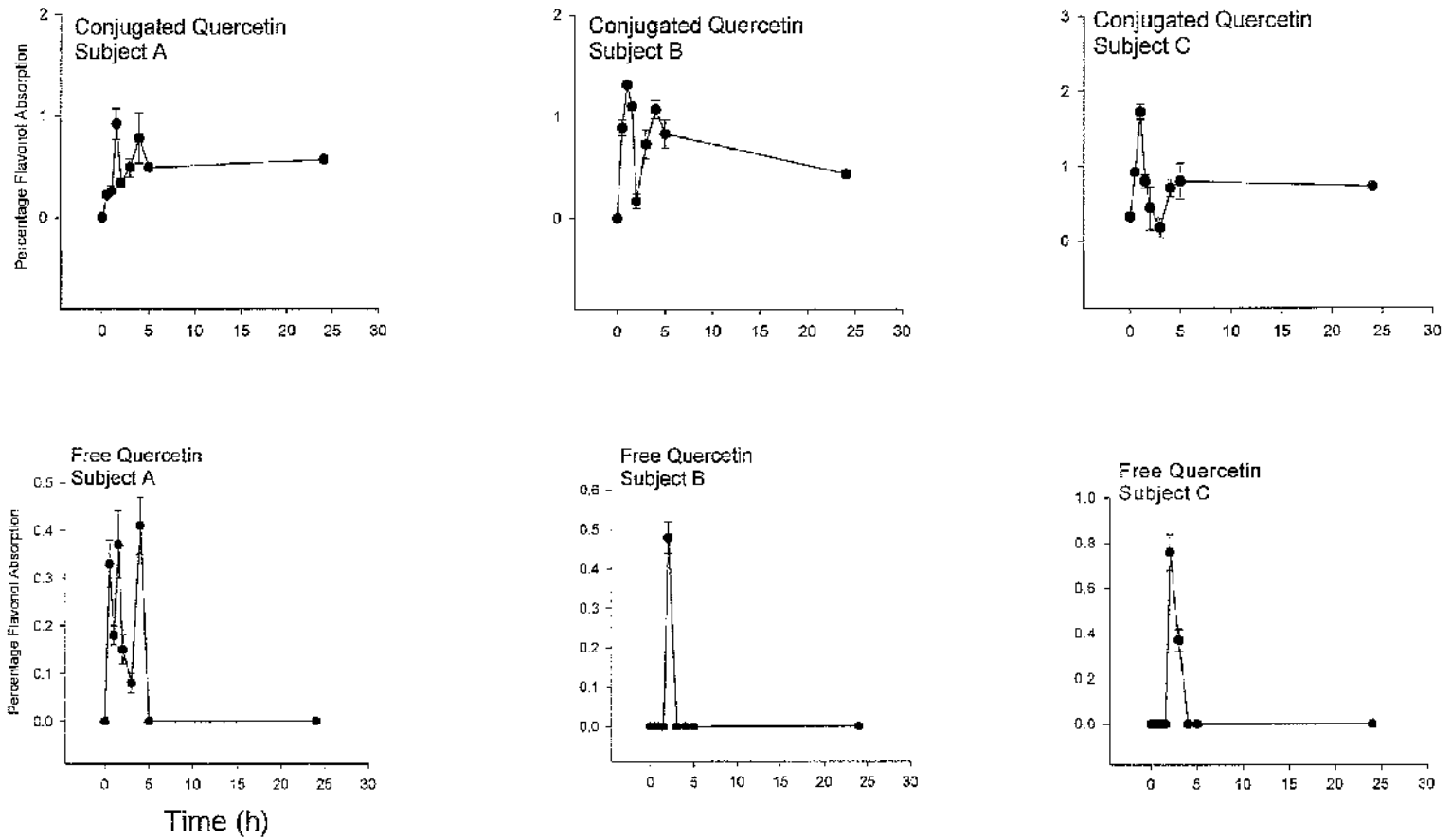
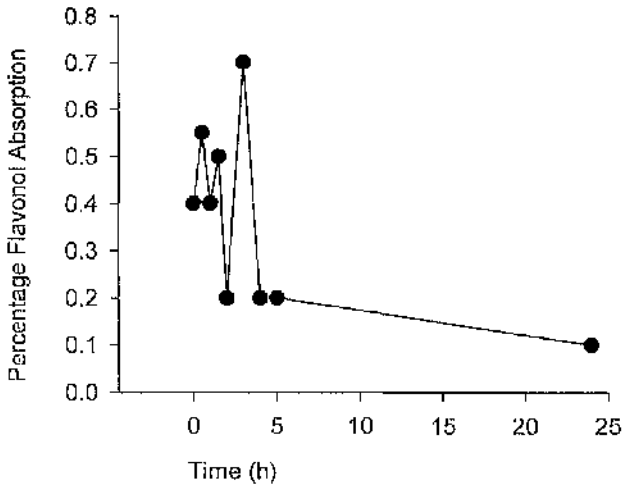


Figure 4.2

Flavonol absorption into human plasma following consumption of tomato juice (Del Monte). Data expressed as percentage of intake based on the flavonol content of product consumed and calculated on the basis of 3000 ml of plasma per person.

Figure 4.2 Flavonol Absorption into Human Plasma Following Consumption of Tomato Juice



4.5 Excretion of Flavonols in Urine

Flavonols are detected unchanged in urine following feeds with tomatoes and tomato juice verifying that they are indeed absorbed. Flavonol levels were found to be high throughout the course of urine collection (Table 4.3).

4.6 Discussion

Plasma flavonols detected in this study consisted mostly of conjugated quercetin believed to be derived from rutin, with kaempferol appearing not to be absorbed or absorbed at very low levels (Figure 4.3). Following the consumption of both tomato fruits and tomato juice two absorption maxima in plasma were observed. Plasma profiles obtained from healthy volunteers following consumption of capsules containing 220 mg of rutin showed peak quercetin accumulation at 9 hrs with slow subsequent decline (Hollman, 1997). The slow absorption kinetics of rutin led to the conclusion that bacteria in the colon are required to break β-glycosidic bonds before the quercetin liberated could be absorbed by the body. Rutin is believed to be the primary quercetin conjugate present in tomatoes, quercetin accumulation in plasma following consumption of tomato fruits may also be expected to show late peak maxima. Two peak maxima were determined 1.0-1.5 h and 3.0-4.0 h. The first peak appears to indicate absorption in the small intestine, the second peak is possibly due to absorption further down the digestive tract however hydrolysis by colonic bacteria is unlikely to play a major role as quercetin detected at this later stage was still present in a conjugated form.

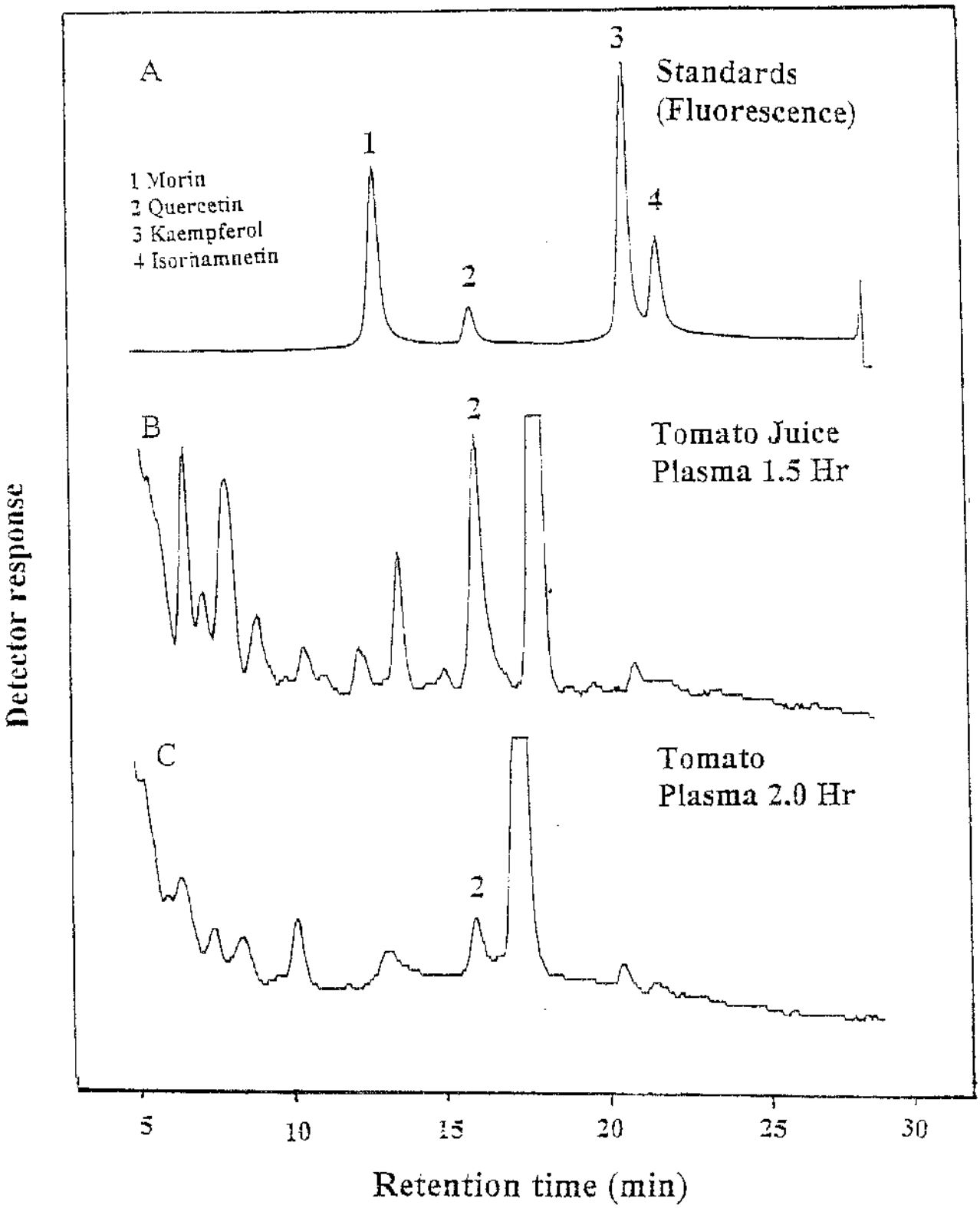
Table 4.3 Percentage Flavonol Excretion[a]

Time Period hrs	Tomato Juice	Tomato Subject A	Tomato Subject B	Tomato Subject C
0-6	0.13 ± 0.04	0.69 ± 0.02	0.06 ± 0.01	0.10 ± 0.01
6-12	0.05 ± 0.01	0.23 ± 0.00	0.03 ± 0.00	0.00
12-24	0.24 ± 0.01	0.53 ± 0.03	0.11 ± 0.01	0.00

[a]Results expressed as flavonols excreted in urine as a percentage of flavonols consumed. Errors represent standard errors where n=3.

Figure 4.3 Detection of Quercetin in Human Plasma Following Consumption of Tomato Fruits or Tomato Juice Using HPLC

Gradient reverse phase HPLC analysis of flavonols. Column: 150 x 3.0 mm i.d. 4-μm Genesis C18 column with a 10 x 4.0 mm 4-μm Genesis C18 guard cartridge. Mobile phase 20 min gradient of 20-40 % acetonitrile in water containing 0.1 % trifluoroacetic acid. Flow rate: 0.5 ml/min. Detector: absorbance monitor operating at 365 nm and, following post-column derivatization with methanolic aluminium nitrate, a fluorimeter operating at excitation 420 and emission 485 nm. Samples (A) 100 ng of (1) morin, (2) quercetin, (3) kaempferol and (4) isorhamnetin with fluorescence detection following post-column derivatization. (B) acid hydrolysed 12 μl aliquot of plasma collected 1.5 h following consumption of 860 ml of tomato juice. (C) as B but with plasma collected 2.0 h following consumption of 367 g of Spanish cherry tomatoes. Numbers indicate peaks that co-chromatograph with standards listed for sample A.


A
Standards
(Fluorescence)
1 Morin
2 Quercetin
3 Kaempferol
4 Isorhamnetin
1
2
3
4
B
Tomato Juice
Plasma 1.5 Hr
2
C
Tomato
Plasma 2.0 Hr
2
Detector response
5
10
15
20
25
30
Retention time (min)

The presence of low levels of free quercetin in plasma may reflect either the absorption of free quercetin or the absorption and subsequent metabolism of conjugated quercetin.

Variation between the results of the three subjects who consumed tomatoes may be due to subject-subject variability or incomplete collection. Alternatively this may represent differences in fluid intake between subjects during the course of this study. Excretion patterns should not necessarily be expected to resemble the time course for accumulation in plasma as a significant portion of the flavonols absorbed may be sequestered by the body or radically metabolised to facilitate excretion.

The results indicate that flavonol conjugates contained within tomato fruits and tomato juice are absorbable and bioavailable.

4.7 Conclusions

Following consumption of tomato fruits and tomato juice, conjugated quercetin was detected in plasma. Flavonols were also detected unchanged in urine. Results indicate that the flavonols present in tomato fruits are absorbable and bioavailable.

Chapter 5 The Effect of Nutrient Deficiency on Flavonol Accumulation in Plant Tissues

5.1 Introduction

Previous studies have identified a link between nutrient deficiency and flavonoid accumulation in plant tissues, primarily the deposition of highly coloured anthocyanins on leaves and stems. Information on the exact nature of these flavonoids is lacking, most studies have identified only flavonoid groups such as flavonols, flavones and flavonones (Carpena *et al.*, 1982, Zornoza & Esteban, 1984). In addition, although tomato has commonly been used as a model system in which to study the effects of nutrient deprivation, there is little information on the effect of nutrient stress on tomato fruits.

The aim of this study was to determine whether nutritional deficiency could be employed to increase flavonol accumulation in plant tissues. The effects of nitrogen and phosphorus deficiency were determined initially using seedlings of *Arabidopsis thaliana* as a model system due to its rapid life cycle and relatively high flavonol content. Studies were then transferred to tomato seedlings and leaves and fruits of mature tomato plants grown in a commercial situation.

5.2 Effect of Nitrogen and Phosphorus Deficiency on Seedlings of *Arabidopsis thaliana*

Initial experiments to determine any effect of reduced nitrogen or phosphate availability on the flavonol content of plant tissues were carried out using seedlings of *Arabidopsis thaliana* (Columbia). Sterile seeds were plated onto MS media containing either 0-60 mM nitrogen or 0-6.3 mM phosphate. All media contained 100 mM sucrose. Seedlings were then harvested after 11 days in conditions of white light (100 $\mu Ei/m^2/s$) at 20 °C for flavonol analysis. Those seedlings grown on phosphorus deficient media appeared normal in size but were darker green in colour than those grown in standard conditions of 2.5 mM phosphorus. At the highest phosphorus concentrations, seedlings were pale yellow in colour. Seedlings grown on nitrogen deficient media appeared to be greatly reduced in size and purple in colour, indicating increased anthocyanin content. As the nitrogen concentration of the media was increased, seedlings became light green and then of normal colour and size from 6.0 mM nitrogen onwards.

Acid hydrolysed *Arabidopsis* tissue samples were analysed by HPLC with UV detection (365 nm) and fluorescence detection (excitation 425 nm, emission 480 nm) following post column derivatisation with methanolic aluminium nitrate. Identification of flavonols in *Arabidopsis* was by HPLC retention time and co-chromatography with authentic flavonol standards. The flavonol aglycones quercetin, kaempferol and isorhamnetin were identified in whole seedling tissue from *Arabidopsis*. Although no attempt was made in this study to analyse the effects of nutrient deficiency on individual flavonol conjugates, analysis of *Arabidopsis* samples taken before and after acid

hydrolysis gave an indication of the proportion of flavonols present in free and conjugated forms. Quercetin and kaempferol were detected almost exclusively as conjugates (~ 99 %), while isorhamnetin was found in a conjugated form.

Effect of Nitrogen Deficiency- The standard nitrogen concentration found in MS media is 60 mM, *Arabidopsis* seedlings grown in these conditions were found to contain a total flavonol content of 109 μg/g fresh weight (Table 5.1). Seedlings grown in conditions of zero nitrogen had a total flavonol content of 394 μg/g fresh weight. Clearly, limiting nitrogen availability induced higher concentrations of quercetin, kaempferol and isorhamnetin in *Arabidopsis* seedlings. The lowest flavonol content (44.6 ± 12.0) was observed in seedlings grown on 6.0 mM nitrogen, perhaps indicating that the 60 mM regime imposed a stress on the plants due to elevated nitrogen availability.

Effect of Phosphorus Deficiency- Those *Arabidopsis* seedlings grown on media with zero phosphate showed a clear increase in total flavonol content compared to plants grown on 6.3 mM phosphate (105.1 and 27.5 μg/g fresh weight respectively). Limiting phosphate availability produced a clear increase in quercetin, kaempferol and isorhamnetin (Table 5.2). The flavonol content of *Arabidopsis* seedlings in the nitrogen trial was generally higher than those from the phosphate trial. The altered phosphate media contained 60 mM nitrogen, whilst the altered nitrogen media contained 1.25 mM phosphate. Those plants growing in levels of high phosphate with 60 mM nitrogen would receive the least nutrient stress and were found to contain the lowest concentration of flavonols (27.5 μg/g fresh weight). Conversely, plants grown in conditions of zero N and 1.25 mM phosphate may be considered to be under the greatest stress and indeed these plants show the highest flavonol content (394 μg/g fresh weight). Nitrogen limitation

Table 5.1 Free and Conjugated Quercetin, Kaempferol and Isorhamnetin Content of *Arabidopsis thaliana* Seedlings in Conditions of Reduced Nitrogen Availability[a]

Nitrogen Conc (mM)	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Free Isorhamnetin	Conjugated Isorhamnetin	Total Flavonol
0	0.2 ± 0.1	273.0 ± 4.0	0.1 ± 0.0	103.7 ± 3.1	n.d	16.6 ± 0.3	393.6 ± 0.8
0.1	0.1 ± 0.0	160.3 ± 25.6	n.d	78.1 ± 18.2	n.d	10.8 ± 2.6	249.3 ± 46.4
0.6	0.1 ± 0.0	70.0 ± 9.6	n.d	72.0 ± 15.6	n.d	5.7 ± 0.9	147.8 ± 21.3
6.0	0.1 ± 0.0	14.7 ± 4.3	n.d	29.8 ± 10.3	n.d	n.d	44.6 ± 12.0
60	0.1 ± 0.0	37.4 ± 7.2	0.1 ± 0.0	67.3 ± 14.4	n.d	4.0 ± 0.8	108.9 ± 18.2

[a]Results represent μg/g (fresh weight) ± S.E (n=3). n.d= none detected.

Table 5.2 Free and Conjugated Quercetin, Kaempferol and Isorhamnetin Content of *Arabidopsis thaliana* Seedlings Grown in Conditions of Reduced Phosphate Availability[a]

Phosphorus Conc (mM)	Free Quercetin	Conjugated Quercetin	Free Kaemperol	Conjugated Kaempferol	Free Isorhamnetin	Conjugated Isorhamnetin	Total Flavonol
0	0.1 ± 0.0	36.1 ± 4.5	n.d	64.9 ± 5.5	n.d	4.0 ± 0.6	105.1 ± 11.3
0.3	0.1 ± 0.0	32.0 ± 4.0	n.d	49.8 ± 1.0	n.d	3.9 ± 0.2	85.8 ± 8.8
0.6	0.1 ± 0.0	30.8 ± 1.2	n.d	46.6 ± 2.5	n.d	3.5 ± 0.2	81.0 ± 3.8
2.5	0.1 ± 0.0	29.0 ± 1.1	n.d	37.2 ± 0.3	n.d	3.6 ± 0.4	69.9 ± 2.8
6.3	0.1 ± 0.0	11.8 ± 1.4	0.2 ± 0.1	13.9 ± 3.0	n.d	1.6 ± 0.2	27.5 ± 3.3

[a]Results represent µg/g (fresh weight) ± S.E where n=3. nd= none detected.

appears to provide greater induction of flavonols than a reduced supply of phosphate. However, in conditions of high nitrogen a reduction of phosphate facilitates flavonol induction.

This study provided clear evidence that the flavonol content of plant tissues is influenced by their nutritional status.

5.3 Effect of Nitrogen and Phosphorus Deficiency on Seedlings of *Lycopersicon esculentum*

Flavonols present in tomato seedlings were identified by HPLC with UV and fluorescence detection. Identification was by retention time and co-chromatography with flavonol standards. The flavonols quercetin, kaempferol and isorhamnetin were detected, however these compounds were present in tomato seedlings in much lower concentrations than in *Arabidopsis*. These flavonols were present almost exclusively in the form of conjugates with only very low levels of free quercetin detected. As found in tomato fruits, the only flavonol conjugate detected was a quercetin conjugate, quercetin-3-rutinoside (rutin). Tomato seedlings were grown on nitrogen and phosphate deficient media for 21 days prior to harvest of whole seedlings for flavonol analysis (Figure 5.1).

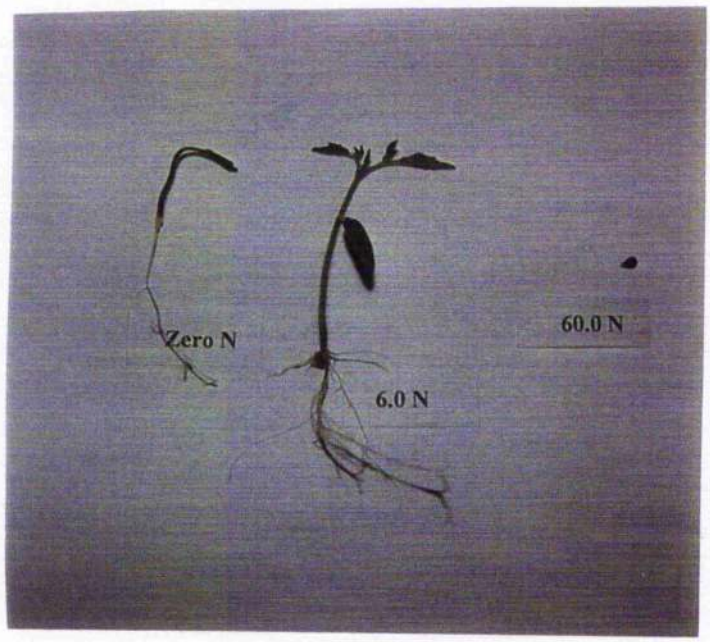
Effect of Nitrogen Deficiency- Due to the increased duration of the experiments and larger containers required for growth of tomato seedlings over three weeks it was not possible to perform experiments with the five concentrations of nitrogen previously used. Instead three concentrations were selected, zero, 6.0 and 60.0 mM nitrogen MS media. Due to poor germination of tomato seedlings on 60.0 mM MS media it was only possible

Figure 5.1

Tomato seedlings grown on MS media with varying nitrogen or phosphorus availability. (A) Tomato seedlings grown for three weeks on MS media containing 0, 6.0 or 60.0 mM nitrogen. (B) Tomato seedlings grown for three weeks on MS media containing 0, 2.5 or 6.3 mM phosphorus. Seedlings were grown at 20 □C under white light (80-100 μmol/m^2/s).

Figure 5.1 Tomato Seedlings Grown on MS Media with Varying Nitrogen or Phosphorus Availability

A



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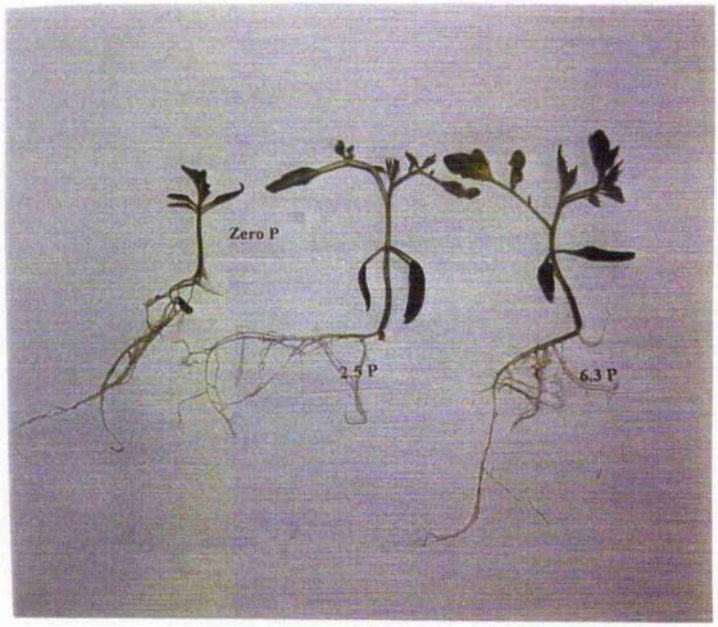


to harvest tissue from zero and 6.0 mM nitrogen treatment regimes. Decreasing the nitrogen concentration of the MS media from 6.0 to zero caused a four-fold increase in the flavonol content of tomato seedlings (Table 5.3).

Effect of Phosphorus Deficiency- Three concentrations of phosphate MS media, zero, 2.5 and 6.3 mM were selected to determine any effect of phosphate deficiency on three week old tomato seedlings. Flavonol contents overall were lower in seedlings grown on reduced phosphate media than on reduced nitrogen media. However flavonol levels showed ~ 2 fold increase in conditions of zero phosphate as compared to 2.5 and 6.3 mM phosphate [6.2, 3.7 and 3.0 μg flavonol/g fresh weight respectively], (Table 5.4). The results indicate that the flavonol response of tomato seedlings in conditions of nutrient stress is very similar to that of *Arabidopsis*. Both species contain the flavonols quercetin, kaempferol and isorhamnetin, the concentration of which is increased in response to either nitrogen or phosphate stress.

5.4 The Effect of Nitrogen and Phosphate Deficiency on the Flavonol Content of Mature Vegetative and Fruit Tissue of *Lycopersicon esculentum*

A trial was established at Garrion Fruit Farm, Lanarkshire, to determine any effects of decreased nitrogen and phosphate availability on leaf and fruit tissues of tomato plants grown in a commercial situation (Figure 5.2). Plants were grown alongside commercial tomato plants in a glass-house. Nutrients were supplied by a hydroponic system (Figure 5.3). Nutrient regimes were designed to represent high, control and low nitrogen and

Table 5.3 Free and Conjugated Quercetin, Kaempferol and Isorhamnetin Content of Tomato Seedlings cv. Paloma Grown in Conditions of Reduced Nitrogen Availability[a]

Nitrogen Concentration (mM)	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Free Isorhamnetin	Conjugated Isorhamnetin	Total Flavonol
0	0.2 ± 0.0	10.6 ± 0.5	0.1 ± 0.0	2.5 ± 0.1	n.d	2.5 ± 0.1	15.9 ± 0.7
6.0	n.d	2.6 ± 0.1	n.d	0.7 ± 0.0	n.d	0.3 ± 0.0	3.6 ± 0.2

[a]Results represent μg/g (fresh weight) ± S.E where n=3. n.d = none detected.

Table 5.4 Free and Conjugated Quercetin, Kaempferol and Isorhamnetin Content of Tomato Seedlings cv. Paloma Grown in Conditions of Reduced Phosphate Availability[a]

Phosphate Conc (mM)	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Free Isorhamnetin	Conjugated Isorhamnetin	Total Flavonol
0	0.1 ± 0.0	3.7 ± 0.4	n.d	1.8 ± 0.3	n.d	0.6 ± 0.1	6.2 ± 0.4
2.5	0.1 ± 0.0	2.3 ± 0.2	n.d	0.9 ± 0.1	n.d	0.4 ± 0.0	3.7 ± 0.0
6.3	0.1 ± 0.0	2.0 ± 0.4	n.d	0.6 ± 0.0	n.d	0.3 ± 0.0	3.0 ± 0.6

[a]Results represent μg/g (fresh weight) ± S.E where n=3. n.d = none detected.

Figure 5.2

Tomato variety Chaser growing under commercial conditions, Garrion Fruit Farm. Photograph shows tomato plants of variety Chaser used to determine the effect of nitrogen and phosphorus deficiency on the flavonol content of tomato leaves and fruits. Plants were grown using the same methods of cultivation as commercial tomato plants.

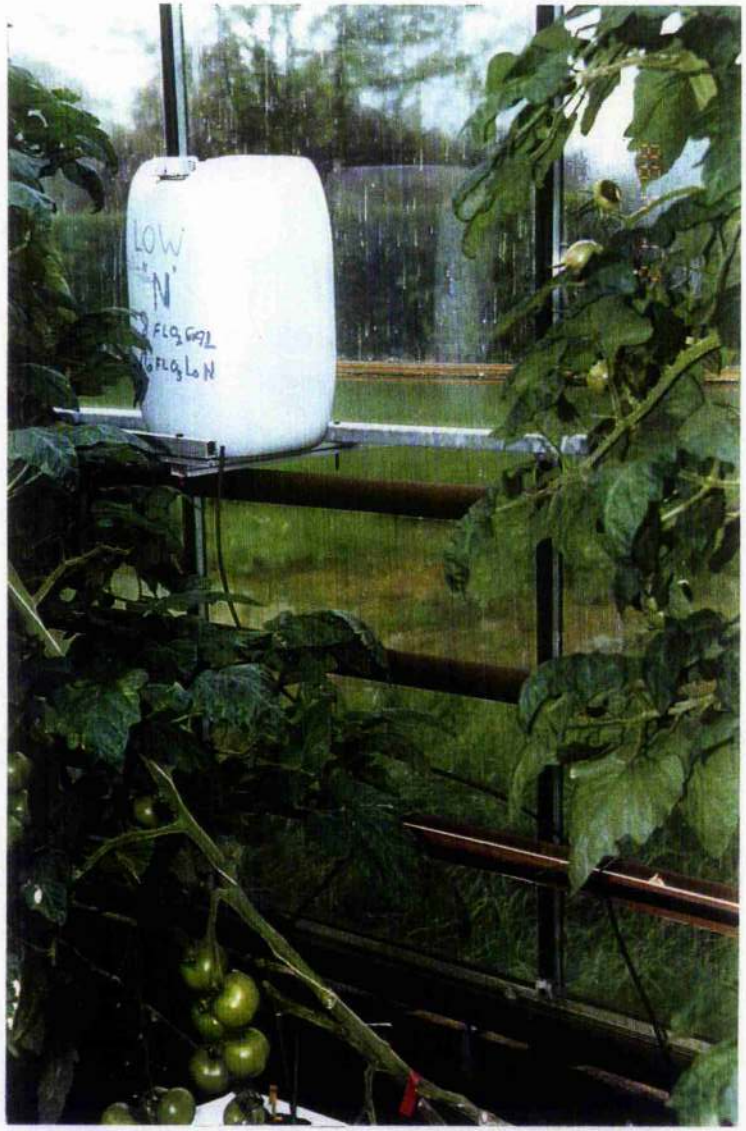
Figure 5.2 Tomato Variety Chaser Growing under Commercial Conditions, Garrion Fruit Farm

Figure 5.3

Drip-feeding system supplying nutrients to tomato plants of variety Chaser, Garrion Fruit Farm. (A) drip-feeding system with lines running from the nutrient tank to the root bulk of the tomato plants. (B) large stock tank in which individual fertilisers were mixed to produce the required nutrient feed.

Figure 5.3 Drip Feeding System Supplying Nutrients to Tomato Plants of Variety Chaser, Garrion Fruit Farm

A



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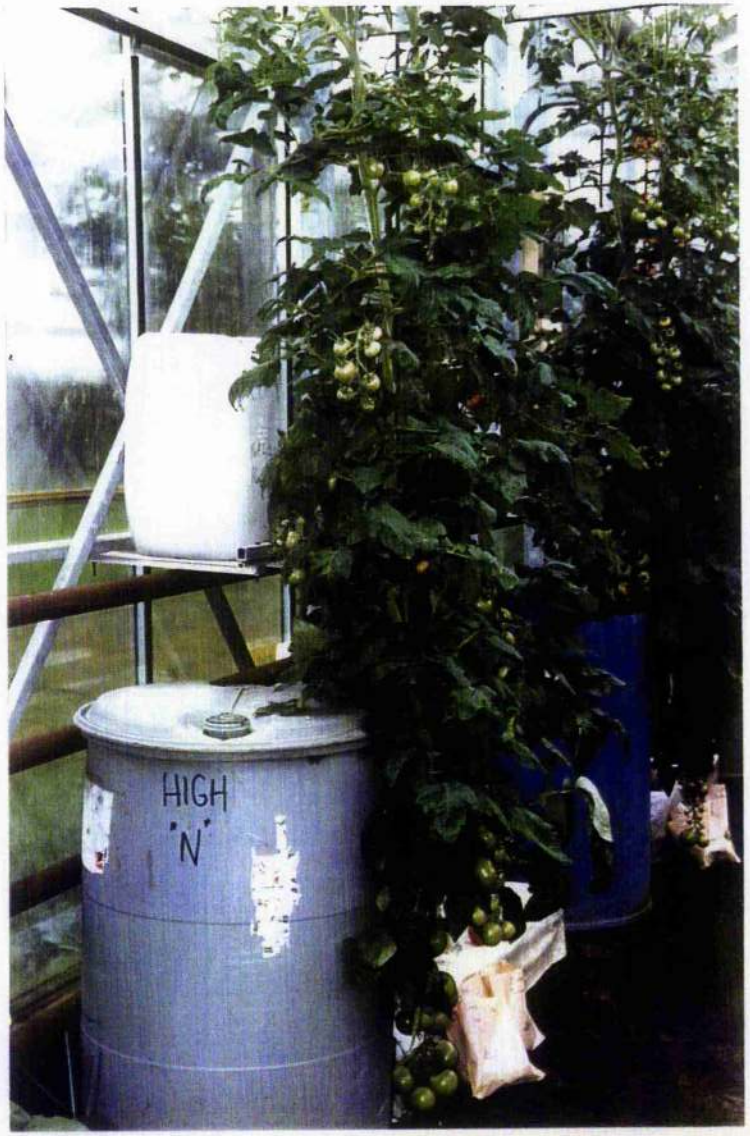


phosphate concentrations. Phosphate concentrations selected were: high 100 ppm, control 30 ppm and low 5 ppm. Nitrogen concentrations selected were: high 405 ppm, control 193 ppm and low 79 ppm. These concentrations were selected to provide the highest and lowest nitrogen and phosphate levels with which it is possible to support plant growth and fruit set. Such conditions would not be expected to support long term cultivation. Full nutrient, conductivity and pH analyses were carried out on all hydroponic solutions to ensure that the desired nitrogen and phosphate levels were achieved (Table 5.5). Nitrogen levels were found to adhere closely to values expected from feed recipes, phosphorus levels were generally lower than anticipated but were deemed to be satisfactory for this study. The actual phosphate levels were found to be, high 64.8 ppm, control 6.13 ppm and low 1.04 ppm. The reason for the lower phosphate concentrations may be due to inaccurate weighing out of the various fertilisers required on-site. Phosphate levels employed for commercial use in the Clyde Valley are generally ~ 50 ppm, with nitrogen levels ~ 300 ppm. Both would approximate most closely with the high treatment regimes. Three tomato plants cv. Chaser were grown for each treatment level. Tissue sampling was carried out on two separate occasions one month apart (May & June 1998). On each occasion red, green and breaker tomato fruits were removed randomly from the plants in each nutrient regime. Leaf samples were also removed from the top of the plants. Red, breaker and green fruits were also harvested from tomato variety Chaser grown in standard commercial conditions for flavonol analysis (June 1998).

Table 5.5 Analysis of Hydroponic Fertiliser Solutions Used in the Nitrogen and Phosphorus Trial, Garrion Fruit farm May-June 1998

Factor	Target[a]	Low N	Control N	High N	Low P	Control P	High P
Acidity	6.0	6.14	5.84	5.43	5.75	6.24	5.75
Nitrogen (ppm)	150.0	112	215	369	112	130	125
Phosphorous (ppm)	30.0	15.6	24.3	34.9	1.04	6.13	64.8
Potassium (ppm)	250.0	90.8	293.0	748.0	254	271	368
Calcium (ppm)	100.0	40.1	75.4	144.0	79.2	78.4	84.7
Magnesium (ppm)	25.0	27.7	39.3	45.8	41.8	28.3	44.2
Sodium (ppm)	<100.0	7.7	8.5	8.7	8.3	8.7	9.6
Iron (ppm)	3.0	3.5	3.8	3.6	4.4	3.3	4.5
Manganese (ppm)	1.0	0.5	0.6	0.8	0.8	0.6	0.9
Boron (ppm)	0.5	0.3	0.4	0.4	0.5	0.4	0.5
Copper (ppm)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Zinc (ppm)	0.5	0.4	0.5	0.5	0.6	0.5	0.6

[a] Target nitrogen concentration represents the desired nitrogen content of hydroponic solutions with altered phosphate concentrations. Target phosphorous concentration represents the desired phosphate concentration of hydroponic solutions with altered nitrogen content.

5.4.1 Effect of Nitrogen and Phosphate Availability on the Flavonol Content of Tomato Leaf Tissue

The flavonol content of mature tomato leaves harvested from the tops of tomato plants involved in the nitrogen and phosphate nutrient trial was assessed using HPLC analysis. As found in tomato seedlings, leaf tissue contains conjugated quercetin, kaempferol and isorhamnetin. The predominant flavonol was quercetin (80-90 %) followed by kaempferol (8-9 %) with relatively low levels of isorhamnetin present.

Effect of Nitrogen deficiency- It is clear that the total flavonol content of tomato leaves was increased in response to reduced nitrogen availability. In trial 1 the total flavonol content varied from 51.3 in the high N regime to 117.1 μg/g fresh weight in the low N regime, in trial 2 the flavonol content increased from 113.8 to 175.7 μg/g fresh weight (Table 5.6). Leaves harvested in trial 2 were found to contain higher flavonol concentrations than those collected in trial 1.

Effect of Phosphate Deficiency-The flavonol content of tomato leaves collected from plants grown in conditions of reduced phosphate did not show a clear trend from high to low phosphate regimes. Samples collected in May of 1998 showed a decreasing flavonol content as phosphate availability was reduced (105.2 to 54.5 μg/g fresh weight). Those samples collected in June of 1998 showed an increase in flavonol content from high to low phosphate regimes (133.0 to 188.0 μg/g fresh weight). In the conditions of our study, high and control phosphate levels (64.8 and 6.13 ppm respectively) appear to be the most inductive phosphate concentrations for vegetative tomato tissue (Table 5.7). Again

Table 5.6 Quercetin, Kaempferol and Isorhamnetin Content of Tomato Leaf Tissue cv. Chaser Grown in a Hydroponic System Supplied with High, Low or Control Levels of Nitrogen[a]

Nutrient Regime	Free Quercetin		Conjugated Quercetin		Free Kaempferol		Conjugated Kaempferol		Conjugated Isorhamnetin		Total Flavonol	
Trial	1	2	1	2	1	2	1	2	1	2	1	2
High	0.1 ± 0.0	0.3 ± 0.0	44.6 ± 8.2	103.3 ± 20.3	0.1 ± 0.0	n.d	4.6 ± 0.9	8.0 ± 1.9	1.1 ± 0.2	2.2 ± 0.5	50.5 ± 9.4	113.8 ± 21.6
Control	0.5 ± 0.0	0.3 ± 0.0	39.4 ± 3.8	144.5 ± 18.0	n.d	n.d	3.5 ± 0.4	11.4 ± 1.5	0.8 ± 0.1	3.6 ± 0.4	44.2 ± 4.2	159.8 ± 19.1
Low	0.7 ± 0.1	0.5 ± 0.0	104.2 ± 20.3	158.4 ± 6.9	0.1 ± 0.0	n.d	9.6 ± 1.6	11.6 ± 0.8	2.6 ± 0.4	5.2 ± 0.5	117.2 ± 22.2	175.7 ± 9.3

[a]Results represent µg/g fresh weight ± S.E (n=3). n.d = none detected. Leaves for trial 1 were harvested in May 1998, leaves in trial 2 were harvested in June of 1998.

Table 5.7 Quercetin, Kaempferol and Isorhamnetin Content of Tomato Leaf Tissue cv. Chaser Grown in a Hydroponic System Supplied with High, Low and Control Levels of Phosphate[a]

Nutrient Regime	Free Quercetin		Conjugated Quercetin		Free Kaempferol		Conjugated Kaempferol		Conjugated Isorhamnetin		Total Flavonol	
Trial	1	2	1	2	1	2	1	2	1	2	1	2
High	0.8 ± 0.0	0.4 ± 0.0	93.3 ± 19.8	119.7 ± 19.9	0.1 ± 0.0	n.d	8.8 ± 1.8	9.2 ± 1.4	2.2 ± 0.3	3.8 ± 0.6	105.2 ± 17.9	133.1 ± 18.1
Control	0.7 ± 0.0	0.6 ± 0.0	71.3 ± 6.5	204.8 ± 11.9	0.1 ± 0.0	n.d	6.4 ± 0.2	19.6 ± 1.1	1.2 ± 0.1	4.9 ± 0.2	79.7 ± 6.6	229.9 ± 10.2
Low	0.7 ± 0.0	0.6 ± 0.0	48.7 ± 3.7	169.1 ± 14.2	0.1 ± 0.0	n.d	4.2 ± 1.3	13.4 ± 0.9	0.8 ± 0.3	4.8 ± 0.7	54.5 ± 4.6	187.9 ± 14.7

[a] Results represent μg/g fresh weight ± S.E (n^{-3}). n.d = none detected. Leaves for trial 1 were harvested in May 1998, leaves in trial 2 were harvested in June of 1998.

samples collected in June generally contained a higher flavonol content than samples collected in May.

5.4.2 Effect of Nitrogen and Phosphate Deficiency on the Flavonol Content of Tomato Fruit Skin

Tomato fruits at three stages of ripening, green, breaker and red, were harvested randomly from plants in each nutrient regime in May and June 1998 (trial 1 and 2 respectively). Despite the altered nutritional status of the plants in the trial, fruiting was abundant and the tomato fruits appeared normal (Figure 5.4). Fruit skins were removed for flavonol analysis. Skins were found to contain primarily conjugated quercetin with lower levels of conjugated kaempferol. Very low levels of free quercetin and kaempferol were also detected.

Nitrogen Deficiency- Skins from mature green fruits showed a small increase in total flavonol content in the low nitrogen regime as compared to the control and high regimes (Table 5.8). Altering nitrogen availability had no obvious effect on the flavonol content of tomato fruits at the breaker and red stage of ripening. The flavonol content of green tomato fruit skins was found to be higher in fruits from the second trial (June) than those sampled in May. This may indicate increasing nitrogen stress on the plants over the duration of this experiment and increasing light levels. This effect was no longer observed as plants reached the breaker and red stage of ripening.

Phosphate Deficiency- reduced phosphate availability caused an increase in flavonol accumulation in fruit skins early in fruit development (mature green stage). The flavonol

Figure 5.4

Tomato variety Chaser grown under commercial conditions, Garrion Fruit Farm. (A) truss of developing green fruits from plants grown in the high phosphate nutrient regime. (B) ripening fruits of plants grown in the control phosphate regime.

Figure 5.4 Tomato Variety Chaser Grown under Commercial Conditions, Garrion Fruit Farm

A

B

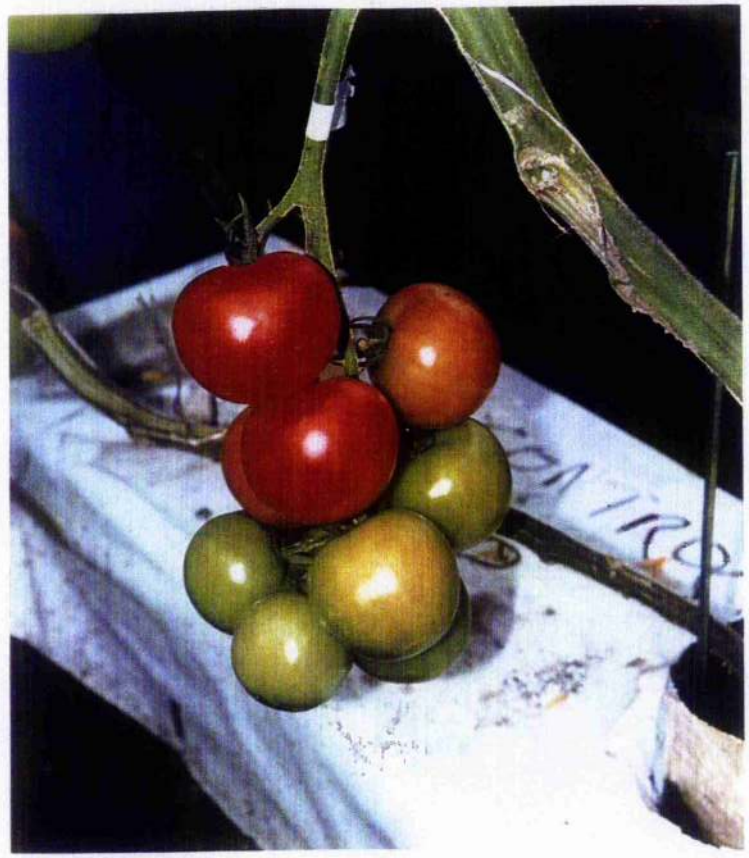


Table 5.8 Free and Conjugated Quercetin and Kaempferol Content of Tomato Fruit Skins c.v Chaser Grown in High, Low or Control Nitrogen Conditions in the Clyde Valley May-June 1998[a]

Developmental Stage	Nutrient Regime	Free Quercetin		Conjugated Quercetin		Free Kaempferol		Conjugated Kaempferol		Total Flavonol	
Trial		1	2	1	2	1	2	1	2	1	2
Green	High	0.2 ± 0.0	0.1 ± 0.0	16.4 ± 1.6	26.2 ± 2.2	0.5 ± 0.0	0.4 ± 0.0	3.5 ± 0.7	2.8 ± 0.4	20.6 ± 2.2	29.5 ± 1.9
	Control	0.1 ± 0.0	0.1 ± 0.0	17.5 ± 2.1	20.8 ± 1.2	0.4 ± 0.0	0.4 ± 0.0	4.0 ± 0.2	5.2 ± 0.8	22.0 ± 1.9	26.5 ± 0.7
	Low	0.2 ± 0.0	0.1 ± 0.0	22.2 ± 0.9	29.6 ± 4.6	0.4 ± 0.0	0.3 ± 0.0	5.3 ± 0.2	2.7 ± 0.3	28.1 ± 1.1	32.7 ± 4.9
Breaker	High	0.2 ± 0.0	0.1 ± 0.0	23.6 ± 3.9	23.3 ± 0.8	0.1 ± 0.0	0.4 ± 0.0	5.9 ± 2.2	2.8 ± 0.4	29.8 ± 6.1	26.6 ± 1.0
	Control	0.1 ± 0.0	n.d	19.3 ± 2.6	23.4 ± 0.6	0.1 ± 0.0	0.4 ± 0.0	3.5 ± 0.5	3.5 ± 0.4	23.0 ± 3.3	27.3 ± 1.0
	Low	0.2 ± 0.0	n.d	18.8 ± 2.8	24.5 ± 1.5	0.1 ± 0.0	0.3 ± 0.0	3.0 ± 0.6	3.7 ± 0.0	22.1 ± 3.4	28.5 ± 1.5
Red	High	0.2 ± 0.0	n.d	28.0 ± 0.9	26.9 ± 2.9	0.4 ± 0.0	0.3 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	32.7 ± 1.1	31.3 ± 3.0
	Control	0.1 ± 0.0	n.d	30.8 ± 1.8	22.5 ± 0.9	0.5 ± 0.0	0.3 ± 0.0	4.2 ± 0.5	4.1 ± 0.3	35.6 ± 2.6	26.9 ± 1.1
	Low	0.1 ± 0.0	n.d	24.5 ± 3.4	24.9 ± 1.0	0.5 ± 0.0	0.3 ± 0.0	4.3 ± 0.6	4.0 ± 0.2	29.4 ± 4.0	29.2 ± 1.1

[a]Results represent μg/g fresh weight ± S.E (n=3). n.d= none detected. Tomatoes in trial 1 collected May, trial 2 in June 1998.

content in the high regime compared to the low nitrogen regime was found to increase from 19.5 to 24.2 μg/g fwt (trial 1) and 25.2 to 42.5 μg/g fwt (trial 2). Reduced phosphate availability caused flavonol induction at the breaker stage of fruit development but to a lesser extent than that observed at the green stage, 17.2 to 20.3 μg in trial 1 and 23.0 to 31.6 μg/g in trial 2. There was no evidence for flavonol induction due to phosphate nutrition at the red stage of ripening (Table 5.9). The flavonol content of fruit skins from tomato variety Chaser grown in standard commercial nutrient conditions under glass in the Clyde Valley was assessed in samples harvested in June of 1998. Total flavonol content was found to vary from 27.3 to 30.1μg/g fwt according to developmental stage (Table 5.10). The flavonol content of these commercial fruits is comparable with flavonol contents determined for fruits harvested from plants employed in nutrient studies. Nitrogen deprivation appeared to produce little effect on the flavonol content of fruit skin. Phosphate deprivation affected the flavonol content of tomato fruit skin at the mature green and breaker stage of development. Comparing the commercial samples with the fruits collected from the phosphate trial, the flavonol contents equated most closely with the high or control situations.

Table 5.9 Free and Conjugated Quercetin and Kaempferol Content of Tomato Fruit Skins c.v Chaser Grown in Conditions of High, Low and Control Phosphate in the Clyde Valley May-June 1998[a]

Developmental Stage	Nutrient Regime	Free Quercetin		Conjugated Quercetin		Free Kaempferol		Conjugated Kaempferol		Total Flavonol	
Trial		1	2	1	2	1	2	1	2	1	2
Green	High	0.1 ± 0.0	0.1 ± 0.0	14.7 ± 2.2	22.4 ± 2.1	0.4 ± 0.0	0.4 ± 0.0	4.4 ± 0.3	2.3 ± 0.1	19.6 ± 2.4	25.2 ± 2.1
	Control	0.1 ± 0.0	0.2 ± 0.0	13.6 ± 3.0	34.1 ± 0.4	0.4 ± 0.0	0.4 ± 0.0	3.6 ± 1.0	3.5 ± 0.4	17.7 ± 4.0	38.2 ± 0.6
	Low	0.2 ± 0.0	0.2 ± 0.0	19.3 ± 1.5	36.8 ± 3.3	0.5 ± 0.0	0.4 ± 0.0	4.3 ± 0.4	5.2 ± 0.7	24.3 ± 1.9	42.6 ± 2.5
Breaker	High	0.1 ± 0.0	0.1 ± 0.0	14.9 ± 1.0	20.0 ± 0.9	0.3 ± 0.1	0.4 ± 0.0	2.0 ± 0.2	2.4 ± 0.4	17.3 ± 1.0	22.9 ± 1.2
	Control	0.1 ± 0.0	0.1 ± 0.0	16.4 ± 0.2	15.6 ± 1.2	0.1 ± 0.0	0.4 ± 0.0	3.2 ± 0.3	2.8 ± 0.3	19.8 ± 0.1	18.9 ± 1.5
	Low	0.1 ± 0.0	0.1 ± 0.0	17.4 ± 1.7	28.7 ± 4.1	0.1 ± 0.0	0.4 ± 0.0	2.7 ± 0.3	2.4 ± 0.1	20.3 ± 2.0	31.6 ± 3.9
Red	High	0.2 ± 0.0	n.d	26.4 ± 1.8	23.6 ± 2.2	0.5 ± 0.0	0.3 ± 0.0	3.3 ± 0.5	3.4 ± 0.5	30.4 ± 2.3	27.3 ± 2.5
	Control	0.3 ± 0.0	n.d	23.2 ± 1.7	16.8 ± 0.9	0.5 ± 0.0	0.4 ± 0.0	2.7 ± 0.5	3.5 ± 0.6	26.7 ± 2.2	20.7 ± 1.0
	Low	0.3 ± 0.0	0.1 ± 0.0	20.7 ± 3.2	23.9 ± 3.4	0.6 ± 0.0	0.4 ± 0.0	4.0 ± 0.3	3.6 ± 0.1	25.6 ± 3.5	28.0 ± 3.4

[a]Results represent µg/g fresh weight ± S.E (n=3). n.d = none detected. Trial 1 = tomatoes harvested May, trial 2 June 1998.

Table 5.10 Free and Conjugated Quercetin and Kaempferol Content of Tomato Fruit Skins c.v Chaser Grown in a Commercial Situation in the Clyde Valley, June 1998[a]

Developmental Stage	Free Quercetin	Conjugated Quercetin	Free Kaempferol	Conjugated Kaempferol	Total Flavonol
Green	0.1 ± 0.0	22.8 ± 1.0	0.4 ± 0.0	4.5 ± 0.4	27.8 ± 1.3
Breaker	0.1 ± 0.0	23.3 ± 1.2	0.4 ± 0.0	3.5 ± 0.5	27.3 ± 0.7
Red	0.1 ± 0.0	23.8 ± 2.4	0.4 ± 0.0	5.9 ± 0.4	30.2 ± 2.8

[a]Results represent μg/g fresh weight ± S.E (n=3).

5.5 Discussion

Initial experiments using seedlings of *Arabidopsis thaliana* and *Lycopersicon esculentum* allowed a rapid screening of the effect of nitrogen and phosphorus deficiency on the flavonol content of plant tissues to be carried out. Seedling tissue from *Arabidopsis* and tomato was found to contain the flavonols quercetin, kaempferol and isorhamnetin. These compounds were found almost exclusively in the form of conjugates. The flavonol content of *Arabidopsis* seedlings was far higher than that of tomato, ~ 25 fold higher in conditions of zero nitrogen and ~ 16 fold higher in conditions of zero phosphate. *Arabidopsis* was therefore an excellent system in which to quickly determine the flavonol response of plant tissues to nutrient stress. Results clearly demonstrated an inverse relationship between nitrogen and phosphorus nutrition and flavonol content. The flavonol content of *Arabidopsis* seedlings grown for eleven days on nitrogen deficient media was found to be ~ 9 fold higher than that of seedlings grown on media containing 6.0 mM nitrogen. In addition the flavonol content of *Arabidopsis* seedlings grown on phosphorus deficient media was found to be ~ 4 fold higher than that of seedlings grown on media containing 6.3 mM phosphorus. In each case nutrient stress caused induction of quercetin, kaempferol and isorhamnetin. Although this study was concerned with the flavonol content of plant tissues, the purple colouration of seedlings grown on media deficient in nitrogen indicates the induction of anthocyanins in response to nutrient stress.

When tomato seedlings were exposed to nitrogen and phosphorus deficient media over eleven days, no flavonol induction was observed. It was only when tomato seedlings were exposed to the nutrient deficient media for 21 days that flavonol levels were found to vary according to the imposed nutrient regime. Total flavonol

content was found to be ~ 4 fold higher in the zero nitrogen treatment as compared to the 6.0 mM nitrogen regime. Flavonol content was ~ 2 fold higher in seedlings grown on zero phosphate media as compared to 6.3 mM phosphate media. It is possible that the extended growth period required for tomato seedlings to induce flavonols in response to nutrient stress as compared to *Arabidopsis* may indicate an effect of the longer life cycle of tomato plants. Three weeks may be the length of time required for seed reserves to be exhausted and for the treatment to cause a noticeable effect.

In order to determine any effect of nutrient stress on the flavonol content of mature leaf tissue and tomato fruit tissue at various stages of ripening, a trial was established under commercial conditions. The aim of this trial was to determine the effect of nitrogen and phosphorus deficiency on a commercially grown tomato cultivar adhering as closely as possible to commercial growing conditions. Mature tomato leaf tissue was found to contain the flavonols quercetin, kaempferol and isorhamnetin. These flavonols were mainly present as conjugates with only very low levels of free flavonols detected. Nitrogen deficiency was found to cause 1.5-2 fold induction in flavonol content as compared to the high nitrogen regime. In addition leaf samples, harvested in trial 2 (June 1998) contained a higher flavonol content than those harvested in trial 1 (May 1998). This increase may be due to increasing nitrogen stress over the additional month that plants had been growing in conditions of reduced nitrogen. In addition light levels may have been increasing throughout May-June, those samples collected in June may have shown greater light induction of flavonols. Phosphorus deficiency did not produce a clear induction of flavonols. Instead the high and control phosphate regimes appeared to induce the highest flavonol concentrations in tomato leaves. A study by Carpena *et al*

(1982) included analysis of the effect of phosphate deficiency on mature leaves of tomato plants. Levels of total flavonol were determined by measurement of the UV absorption of methanolic leaf extracts at 290, 330 and 360 nm. No attempt was made to identify individual flavonols. Leaves were harvested after 11, 13, 16 and 19 weeks of plant exposure to nutrient deficient media. Samples collected at 19 weeks contained a higher putative flavonol content than those previously collected, however the flavonol content at all stages was lower than that of leaves from plants grown in control conditions of phosphate. There was therefore no evidence in their study for the induction of flavonols in tomato leaves due to phosphate deficiency. A more recent study by Bongue-Bartelsman & Phillips (1995) determined the effect of nitrogen deficiency on the anthocyanin and flavonol content of tomato leaves using an HPLC based approach. They reported that anthocyanin content increased 3.4 fold in response to nitrogen stress. The only flavonol identified was a flavonol glucoside, quercetin-3-glucoside (Q3G) which was found to double in response to nitrogen stress.

It was not within the scope of this study to identify the flavonol conjugates present in tomato leaves. Instead samples were analysed before and after acid hydrolysis to allow determination of the proportion of flavonols present in free and conjugated forms. Q3G was therefore not identified in tomato leaves in this study however the resulting flavonol aglycone, quercetin, was identified along with the flavonols kaempferol and isorhamnetin. All three flavonols were found to increase in response to nitrogen deficiency.

The effect of nitrogen and phosphorus deprivation on the flavonol content of tomato fruits was also assessed. To the best of the author's knowledge, this is the first

study to assess the effect of nutrient stress on the individual flavonols of tomato fruits in a commercial setting. The skins of red, breaker and mature green fruits were analysed for flavonol content. Exposure to the low nitrogen regime and low phosphate regime was found to cause an increase in the flavonol content of tomato fruit skins early in the ripening process (mature green stage). Any effect of nutrient stress on the flavonol content of fruits appeared to be lost as ripening progressed. It is possible that green fruits may have to compete with other plant sinks for available nutrients and may therefore suffer from a lack of available nutrients. During ripening the sink strength of the fruit is likely to increase such that the nutrient deficiency no longer has any effect on flavonol induction. Alternatively, induction of flavonols in the skins of green fruits may be important to protect the fruit tissues and developing seeds from penetration by potentially damaging radiation. As ripening is a process of senescence during which the tomato seeds within the fruit reach maturity, the induction of such protective compounds may carry no further advantage.

A study by Zornoza & Esteban (1984) analysed the effect of phosphate deficiency on the flavonol content of tomato fruits. In conditions of zero phosphate the flavonol content of tomato fruits was found to increase from 9.4 mg/g to 11.0 mg/g dry weight. In their study, total flavonol content was calculated on the basis of UV absorption at 290, 330 and 360nm. No attempt was made to identify individual flavonol compounds. The results obtained by this method produced surprisingly high flavonol concentrations e.g 11 mg/g dwt, which may be expected to represent a concentration of 1.1 mg/g fwt. The highest total flavonol content of a whole tomato fruit sample determined in the screening described in chapter 3 was 22.2 µg/g fresh weight. The methodology employed

by Zornoza & Esteban (1984) is likely to have overestimated the flavonol content of tomato fruits. UV absorbance determined at 280 and 330 nm would detect not only flavonols but also a wide range of phenolics. Absorbances determined at 360 nm would pick up the second absorbance maxima of flavonols but would also detect flavones and related compounds.

Two theories to explain the increase in flavonoid synthesis in response to nitrogen deficiency have been proposed. It has been suggested that enhanced PAL activity would release ammonia for amino-acid metabolism with the resulting carbon products shunted into the flavonoid biosynthetic pathway (Margna, 1977). Alternatively, it has been suggested that nitrogen stress would reduce rates of photosynthesis for example by decreasing available chlorophyll and disrupting photosynthetic membranes due to starch accumulation. This may lead to increased sensitivity to high light levels. Production of pigments such as anthocyanins and flavonols may afford protection against light induced oxidative damage (Guidi *et al.*, 1998). No theories have been proposed in the literature to explain the increase in flavonoid accumulation in response to phosphorus deficiency. It is possible that as phosphate plays an integral part in membrane stability, reduced phosphate levels may lead to a weakening of cell membranes, cell leakage and possibly an increased chance of pathogen attack. Increasing flavonoid production may allow the accumulation of compounds important for defence against pathogen attack.

5.6 Conclusions

The present study provides clear evidence that the flavonol content of plant tissues is influenced by their nutritional status. Nitrogen and phosphorus deprivation caused a clear increase in flavonols in seedlings of *Arabidopsis thaliana* and *Lycopersicon esculentum*. Nitrogen deprivation was also able to increase flavonol accumulation in mature vegetative tissue of tomato plants. Phosphorus deficiency could not elicit this response. Nutrient deficiency appeared to produce an increase in flavonols in tomato fruit tissues only in the early stages of fruit development, as ripening progressed no increase was observed.

Nutrient stress can be employed to manipulate the flavonol content of vegetative plant tissues. Further studies may be required to determine the longevity of the flavonol increase in response to a period of nutrient stress and investigate factors such as reduction in yield.

Increased dietary intake of flavonols is believed to be linked to potential health benefits. An understanding of the factors influencing the production of such compounds in plant tissues may allow the production of vegetables rich in flavonols.

Chapter 6 An Investigation into the Effect of Light Quality and Low Temperature on Chalcone Synthase Promoter Activity in *Arabidopsis thaliana.*

6.1 Introduction

The action of the enzyme chalcone synthase is the first committed step in the biosynthesis of flavonoids (Heller & Forkman, 1994). Monitoring the activity of the chalcone synthase gene promoter provides a rapid method of analysing plant responses to changing environmental conditions. Previous studies (Fuglevand *et al.*, 1996) determined that blue, UVA and UVB light given separately could induce *CHS* promoter activity. When these light treatments were combined, interactions between phototransduction pathways produced additive or synergistic increases in *CHS* promoter activity. In addition, increased expression of *PAL* and *CHS* genes following exposure to low temperature has been reported, although this increase required the presence of light (Leyva *et al.*,1995). The work presented in this chapter is an investigation into the effects on *CHS* promoter activity of combining various light qualities with low temperature. *Arabidopsis thaliana* was used as a model system as it contains a single *CHS* gene. In addition, the *Arabidopsis hy4* mutant was employed to determine any effect of low temperature on the CRY1-mediated UVA/blue phototransduction pathway. Effects on *CHS* promoter activity were determined using the transgenic *Arabidopsis* line NM4 (Non-mutant 4) containing the

Sinapis alba chalcone synthase promoter linked to the GUS reporter gene (Jackson *et al.*, 1995). This gene fusion allows rapid biochemical determination of the activity of the *CHS* promoter. In addition, molecular biological methods were employed to determine whether patterns of *CHS-GUS* transgene expression were representative of *CHS* transcript accumulation.

6.2 *CHS-GUS* Promoter Activity in Response to Blue, UVA or Blue/UVA Light

Previous studies by Fuglevand *et al* (1996) investigated the effect of blue, UVA or UVB light on *CHS-GUS* promoter activity and *CHS* transcript accumulation. When given separately each of these light treatments was found to stimulate *CHS* transcription. When blue and UVA light treatments were combined the subsequent increase in *CHS-GUS* promoter activity was found to be additive. However, when UVB treatment was combined with either UVA or blue light the resulting increase in *CHS-GUS* promoter activity was found to be synergistic. When these light treatments were applied to the *Arabidopsis hy4* mutant it was found that although this mutant is impaired in its inductive response to blue and UVA radiation, it retained the synergistic response to blue or UVA radiation combined with UVB. These results indicate that there are at least four distinct phototransduction pathways mediating perception of blue, UVA and UVB radiation. The first involves blue/UVA signal transduction via the CRY1 photoreceptor. The second pathway involves UVB perception and interacts synergistically with two further

pathways mediating blue or UVA signal transduction pathways not involving CRY1 (Figure 1.6).

An initial study was carried out to verify that blue, UVA and UVB treatment does indeed switch on *CHS* transcription in *Arabidopsis thaliana* and that combination of blue and UVA light treatments provides an additive increase in *CHS-GUS* promoter activity.

Arabidopsis NM4 seedlings, grown for three weeks in non-inductive conditions of white light (20 μmol/m^2/s), were exposed to blue, UVA or blue/UVA light at 20 °C. Samples were removed at time zero and after 24 and 48 hours of exposure, GUS activities were then determined (Table 6.1).

Blue- Exposure to blue light (50 μmol/m^2/s) caused a 2.3 fold increase in *CHS-GUS* promoter activity after 24 hours at 20 °C (63.9 ± 12.0 pmol 4-MU/mg/min). Similar levels of GUS activity were detected after 48 hours of exposure.

UVA- Following exposure to UVA light (20 μmol/m^2/s) at 20 °C *CHS-GUS* promoter activity was observed to increase 3.5 fold after 24 hours and 4.9 fold after 48 hours reaching 97.5 ± 23.5 and 134.0 ± 44.0 pmol 4-MU/mg/min respectively.

Blue/UVA- Exposing seedlings to blue and UVA light together (50/20 μmol/m^2/s) caused an increase in GUS activity of 4.6 fold after 24 hours reaching 10.6 fold after 48 hours (128.0 ± 16.6 and 292.0 ± 29.1 pmol/4-MU/mg/min respectively). As expected from previous studies (Fuglevand *et al.*, 1996) exposing plants to blue and UVA radiation simultaneously produced stimulation of *CHS*-promoter activity in an approximately additive manner.

Table 6.1 Induction of *CHS-GUS* Promoter Activity Following Exposure to Blue, UVA or Blue/UVA Light at 20 °C[a]

Exposure Time (Hrs)	CHS-GUS Activity		
	Blue	UVA	Blue/UVA
0	27.6 ± 5.0	27.6 ± 5.0	27.6 ± 5.0
24	63.9 ± 12.0	97.5 ± 23.5	128.0 ± 16.6
48	58.8 ± 15.0	134.0 ± 44.0	292.0 ± 29.1

[a]Results represent pmol 4-MU/mg/min following exposure to blue radiation (50 μmol/m^2/s), UVA radiation (20 μmol/m^2/s) or blue/UVA radiation (50/20 μmol/m^2/s) at 20 °C. Errors represent standard errors where n=10.

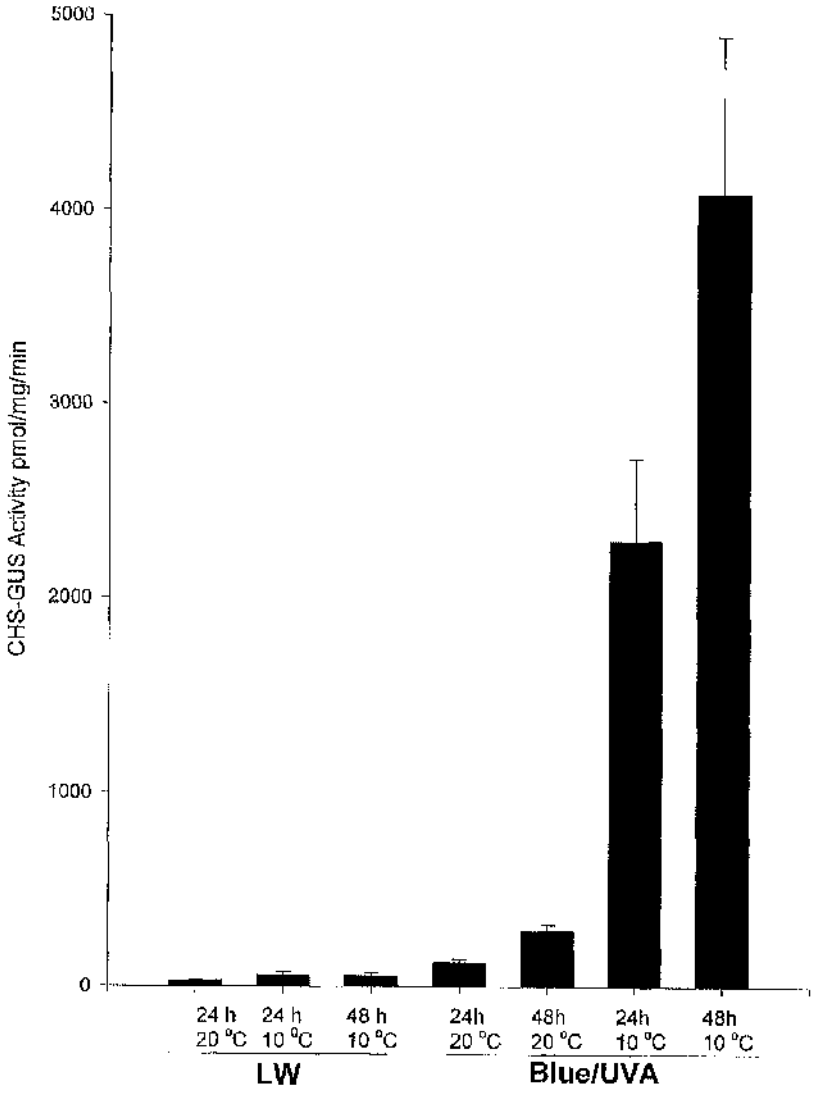
6.3 Synergistic Interaction between Light and Low Temperature on *CHS-GUS* Induction

Arabidopsis NM4 seedlings were exposed to a combination of blue and UVA light (50/20 μmol/m^2/s) at 20 °C. Samples were removed at time zero, 24 h and 48 h and GUS activities were determined (Figure 6.1). As described in Section 6.2, exposure of plants to blue/UVA radiation at 20 °C led to a 10.5 fold increase in *CHS-GUS* promoter activity after 48 hours. Exposing plants to blue/UVA radiation at low temperature (10 °C) stimulated an 83 fold increase in *CHS-GUS* promoter activity after 24 hours rising to 148 fold induction after 48 hours of exposure (2295 ± 430 and 4088 ± 810 pmol 4-MU/mg/min respectively). When plants were exposed to blue/UVA radiation at low temperature the magnitude of the *CHS-GUS* response was far greater than that observed when plants were exposed to blue/UVA or cold separately. Furthermore, levels of *CHS-GUS* expression were greater than the effects of blue/UVA and cold added together. Thus, exposure of plants to blue/UVA radiation at low temperature produced a strong synergistic response indicating that blue/UVA light and low temperature regulate *CHS* expression through separate but interacting signal transduction mechanisms.

Figure 6.1

Synergistic interaction between B/UVA light and low temperature on *CHS-GUS* induction. *Arabidopsis* NM4 seedlings grown in white light (20 μmol/m^2/s) at 20 °C for three weeks were transferred for 24 or 48 hours to white light (20 μmol/m^2/s) or blue/UVA light (50/20 μmol/m^2/s) at 20 or 10 °C. GUS activity was determined and is expressed as pmol 4-MU/mg/min. Bars indicate standard errors where n=10.

Figure 6.1 Synergistic Interaction Between B/UVA Light and Low Temperature on *CHS-GUS* Induction



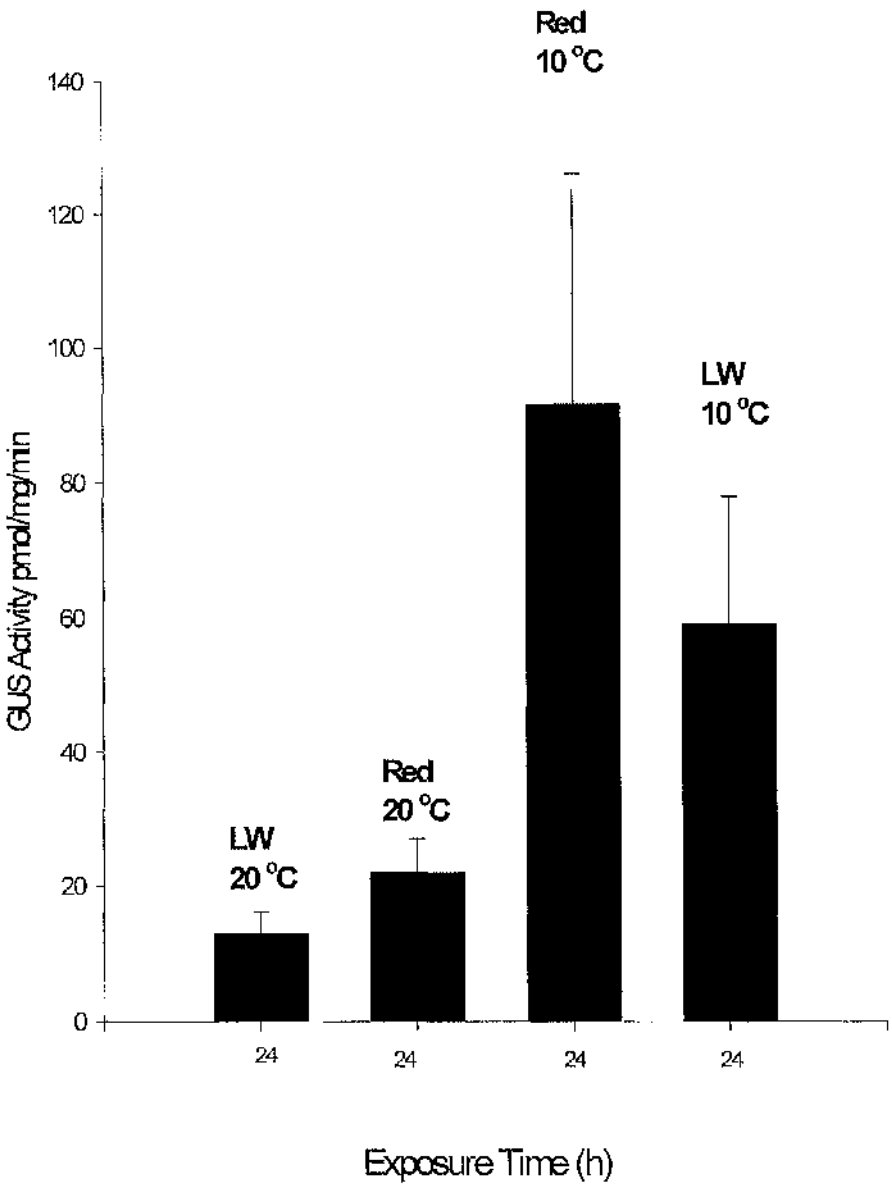
6.4 Effect of Red light and Low Temperature on *CHS-GUS* Promoter Activity

It was important to determine whether the synergistic interaction between light and cold was restricted to B/UVA radiation. *Arabidopsis* seedlings were exposed to red light (85 $\mu mol/m^2/s$) over 24 hours at 20 °C or 10 °C. Samples were removed after 24 hours and GUS activities determined (Figure 6.2). After 24 hours exposure at 20 °C GUS activity was found to be 22.0 ± 5.0 pmol 4-MU/mg/min, following treatment at 10 °C activity was found to be 91.6 ± 34.6 pmol 4-MU/mg/min. *CHS* gene transcription shows very little induction in response to red light (Jackson *et al.*, 1995). Therefore the ~4 fold increase observed may have been induced by the cold treatment alone. Seedlings exposed to non-inductive white light (20 $\mu mol/m^2/s$) for 24 hours at 10 °C were observed to increase *CHS* promoter activity from 19.8± 3.8 to 59.1 ± 18.9 pmol/mg/min, so cold can stimulate *CHS* expression to a small extent even in non-inductive light treatments. The GUS activities observed under these conditions are much lower than those observed following blue/UVA/cold treatments.

Figure 6.2

Effect of red light and low temperature on *CHS-GUS* promoter activity. *Arabidopsis* NM4 plants were grown in conditions of non-inductive white light (20 μmol/m^2/s) for 3 weeks at 20 °C before being transferred for 24 hours to conditions of red light (85 μmol/m^2/s) at 10 or 20 °C or white light (20 μmol/m^2/s) at 10 or 20 °C. GUS activity was determined and is expressed as pmol 4-MU/mg/min. Bars indicate standard errors where n=10.

Figure 6.2 Effect of Red Light and Low Temperature on *CHS-GUS* Promoter Activity



6.5 Effect of UVB Illumination at Low Temperature on *CHS-GUS* Promoter Activity

UVB illumination was combined with low temperature (10 °C) to determine any effect on *CHS-GUS* promoter activity. *Arabidopsis* NM4 seedlings were exposed to UVB radiation (3.5 μmol/m^2/s) at 20 °C or 10 °C. Samples were removed after 24 and 48 hours and GUS activities were determined (Table 6.2).

Plants treated with UVB radiation at 20 °C increased *CHS-GUS* promoter activity within the first 24 hours, however by 48 hours these plants were very obviously wilted and dying and *CHS* promoter activity was found to decline. Plants exposed to UVB radiation at 10 °C continued to increase *CHS-GUS* promoter activity up to 48 hours and although obviously in distress these plants were not yet dying. Beyond 48 hours of exposure to UVB radiation at low temperature these seedlings had deteriorated to such an extent that it was impossible to remove an intact seedling for analysis. The values of GUS activity observed in plants given UV-B and low temperature were again much lower than in plants exposed to blue/UVA and low temperature. Thus, no comparable interaction was observed in this study between UVB phototransduction and cold perception.

Table 6.2 Effect of UVB Illumination at Low Temperature on *CHS-GUS* Promoter Activity[a]

Temperature	Duration of Treatment (hr)	GUS Activity
20 °C	0	33.9 + 7.2
	24	118.4 ± 67.0
	48	45.9 ± 25.3
10 °C	0	33.9 ± 7.2
	24	164.6 ± 54.9
	48	178.7 ± 47.9

[a]Results represent GUS activity (pmol 4-MU/mg/min) following exposure of *Arabidopsis* NM4 seedlings to UVB radiation (3.5 μmol/m^2/s). Errors represent standard errors where n= 10.

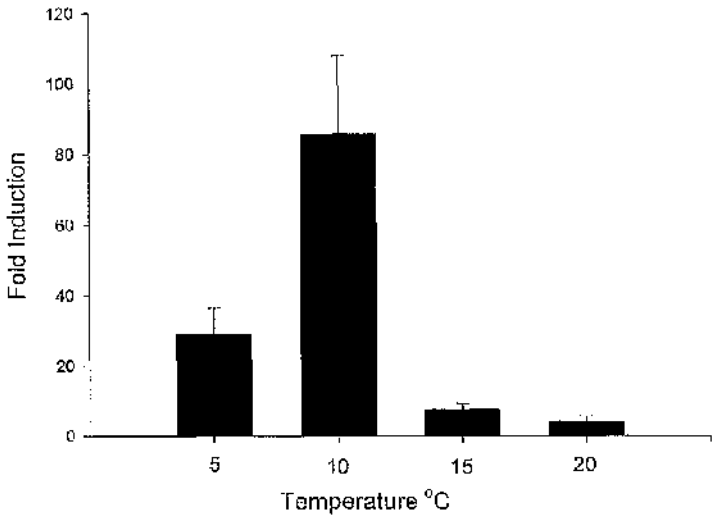
6.6 Determination of the Optimum Temperature for Blue/UVA Induction of *CHS-GUS* Promoter Activity

Having observed a synergistic increase in *CHS-GUS* promoter activity in the presence of blue/UVA radiation when given in combination with low temperature, further studies were carried out to determine the optimum temperature for this effect. Seedlings were exposed to blue/UVA radiation (50/20 μmol/m^2/s) for 24 hours at 5, 10, 15 or 20 °C before seedlings were harvested for determination of GUS activity (Figure 6.3). The fold induction of *CHS-GUS* promoter activity was found to increase between 5 and 10 °C with a subsequent decline as the temperature was increased further. Peak activity was observed following blue/UVA illumination at 10 °C (2311.0 ± 604.0 pmol/mg/min).

6.7 Determination of the Optimum Fluence Rate of Blue Radiation for Blue/UVA Induction of *CHS-GUS* Promoter Activity at 10 °C

The optimum fluence rate of blue radiation was determined by exposing seedlings to B/UVA radiation over 24 hours at 10 °C. UVA fluence rate was kept constant throughout this experiment (20 μmol/m^2/s). The fluence rate of blue radiation was tested at 30, 50, 60, 70, 90, 100 and 150 μmol/m^2/s (Figure 6.4). *CHS-GUS* promoter activity was observed to increase between 30 and 50 μmol/m^2/s (98.2 ± 32.2 and 365.9 ± 113.0 pmol/mg/min respectively). Between 50 and 90 μmol/m^2/s induction of *CHS-GUS*

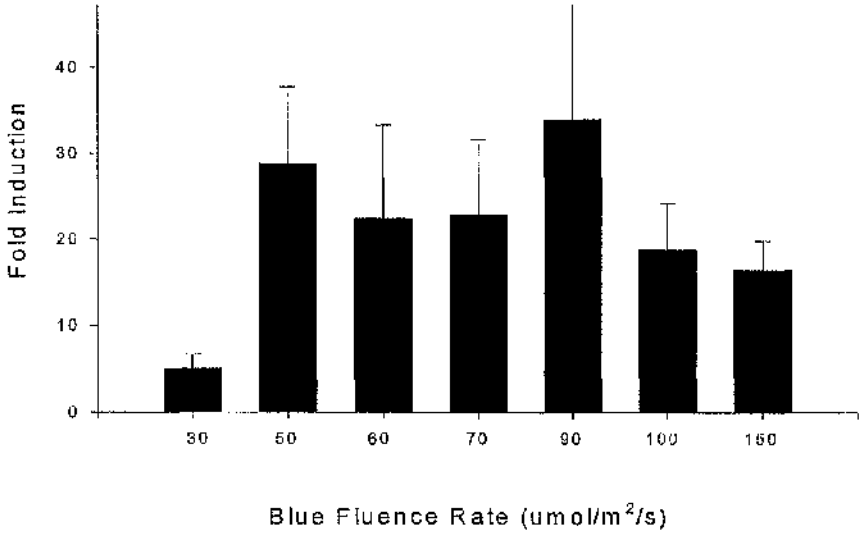
Figure 6.3 Optimum Temperature for Blue/UVA induction of *CHS-GUS* Promoter Activity in NM4 *Arabidopsis* Seedlings[a]



Exposure Time (Hrs)	Temperature	GUS Activity
0	5	54.1 ± 14.0
24		1571.0 ± 402.0
0	10	27.0 = 3.9
24		2311.0 ± 604.0
0	15	106.0 ± 36.0
24		809.6 ± 187.0
0	20	104.0 ± 37.8
24		429.0 ± 166.0

[a]Results represent pmol 4-methyl-umbelliferone (4-MU) produced per mg/min following exposure to blue radiation (50 µmol/m^2/min) and UVA radiation (6.4 µmol/m^2/min) at 5,10,15 or 20 °C. Errors represent standard errors where n=10.

Figure 6.4 Optimum Blue Fluence Rate for Blue/UVA Induction of *CHS-GUS* Promoter Activity in NM4 *Arabidopsis* Seedlings at 10°C[a]



Exposure time (Hrs)	Blue Fluence Rate	GUS Activity
0	30	19.5 ± 6.2
24		98.2 ± 32.2
0	50	12.7 ± 2.5
24		365.9 ± 113.0
0	60	16.4 ± 4.0
24		368.1 ± 178.0
0	70	22.2 ± 5.9
24		505.7 ± 194.6
0	90	8.9 ± 2.9
24		293.5 ± 117.7
0	100	15.2 ± 2.8
24		283.7 ± 85.1
0	150	27.8 ± 4.8
24		455.4 ± 94.2

[a]Results represent pmol 4-MU/mg/min produced following exposure to UVA radiation (20 µmol/m^2/min) and blue radiation (30, 50, 60, 70, 90, 100 or 150 µmol/m^2/min) for 24 hours at 10°C. Errors represent standard errors where n=10.

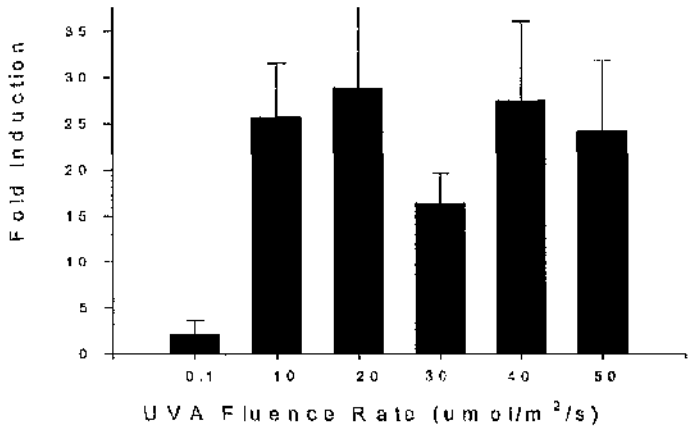
promoter activity remained relatively stable fluctuating between ~22 fold and ~32 fold induction. Induction of *CHS-GUS* promoter activity began to decline as the fluence rate of blue radiation was increased further. As there was no great increase in promoter activity beyond 50 μmol/m^2/s, this fluence rate was chosen for all further blue/UVA experiments as it required fewer light sources.

6.8 Determination of the Optimum UVA Fluence Rate for Blue/UVA Induction of *CHS-GUS* Promoter Activity at 10 °C

In order to determine the optimum fluence rate of UVA required to obtain maximum induction of GUS activity using blue/UVA irradiation at low temperature, seedlings were exposed to blue/UVA treatment at 10 °C over 24 hours. The fluence rate of blue radiation was kept constant (50 μmol/m^2/s) whilst the fluence rate of UVA radiation was tested at 0.1, 10, 20, 30, 40 and 50 μmol/m^2/s (Figure 6.5). At fluence rates of 10 μmol/m^2/s and above GUS activities remained fairly stable with induction ranging from ~16 fold to 29 fold. In this study the greatest *CHS-GUS* induction was observed at 20 μmol/m^2/s (29 fold induction). This fluence rate was therefore selected for future blue/UVA studies.

By increasing blue or UVA fluence rates a dose response curve is observed which becomes saturated presumably as the CRY1 photoreceptor response becomes saturated.

Figure 6.5 Optimum UVA Fluence Rate for Blue/UVA Induction of *CHS-GUS* Promoter Activity in NM4 *Arabidopsis* Seedlings at 10°C[a]



Exposure Time (Hrs)	UVA Fluence Rate	GUS Activity
0	0.1	33.8 ± 6.0
24		69.4 ± 50.7
0	10	16.4 ± 8.7
24		421.0 ± 95.0
0	20	12.7 ± 2.5
24		365.9 ± 113.0
0	30	32.2 ± 5.7
24		528.5 ± 103.0
0	40	16.3 ± 2.9
24		448.1 ± 140.0
0	50	11.5 ± 2.3
24		278.0 ± 87.0

[a]Results represent pmol 4-MU/mg/min following exposure to blue radiation (50 μmol/m^2/min) and UVA radiation (0.1,10,20,30,40 or 50 μmol/m^2/min) for 24 hours at 10°C. Errors represent standard errors where n=3.

A fluence rate of blue (50 μmol/m^2/s) and UVA (20 μmol/m^2/s) giving a total fluence rate of 70 μmol/m^2/s appears sufficient to produce the maximum *CHS-GUS* response to blue/UVA light at low temperature.

6.9 Effect on *CHS-GUS* Promoter Activity of a 20 °C Pre-treatment with B/UVA Radiation Prior to the Addition of a 10 °C Cold Treatment

Previous studies on the synergistic interaction between blue or UVA phototransduction and UVB phototransduction identified an effect of the order of illumination on *CHS-GUS* expression (Fuglevand *et al.*, 1996). The level of GUS activity was found to be greater when the blue light treatment preceded the UVB treatment rather than vice-versa. It was concluded that the blue light treatment produces a signal capable of enhancing the subsequent response to UVB treatment. UVA treatments had to be given simultaneously with the UVB treatment in order to obtain a synergistic increase in *CHS-GUS* expression.

It was conceivable that exposure of NM4 seedlings to blue/UVA radiation prior to the addition of cold may prime the signal transduction mechanism so as to enhance the subsequent response to cold stress. To test this possibility, seedlings were exposed to blue/UVA radiation at 20 °C for 8 hours before being transferred to blue/UVA radiation at 10 °C for a further 16 or 24 hours. Samples were removed after 8, 24 and 32 hours of treatment and GUS activities were determined (Table 6.3). As a control, seedlings were also exposed to 24 hours of blue/UVA radiation at 10 °C.

Table 6.3 Effect on *CHS-GUS* Promoter Activity of 20 °C Pretreatment with Blue/UVA Light Prior to the Addition of 10 °C Cold Treatment[a]

Duration of 20 °C Pretreatment (h)	Duration of Cold Treatment (10°C) (h)	GUS Activity
8	0	29.3 ± 34.8
8	16	554.9 ± 143.7
8	24	698.9 ± 319.3
0	24	2295.0 ± 430.3

[a]Results represent pmol 4-MU/mg/min following exposure to blue/UVA light (70 µmol/m^2/s). Samples received 8 hr 20 °C blue/UVA pre-treatment prior to 16 or 24 hours of blue/UVA/cold (10 °C) with the exception of the final blue/UVA sample, which received no pre-treatment and 24 hr blue/UVA/cold. Errors represent standard errors where n=10.

Samples at time zero, grown under 20 μmol/m^2/s of white light, were found to have an average GUS activity of 19.8 ± 3.8 pmol 4-MU/mg/min.

Applying the 20 °C blue/UVA pre-treatment to seedlings produced *CHS-GUS* promoter activities of 554.9 ± 143.7 and 698.9 ± 319.3 pmol 4-MU/mg/min after 16 and 24 hours of blue/UVA/cold treatment respectively. This can be compared to the *CHS-GUS* promoter activity determined in seedlings exposed to 24 hours (2295.0 ± 430.3 pmol/mg/min) of blue/UVA radiation at 10 °C. It appears that prior exposure to blue/UVA light at 20 °C limits the extent of the subsequent response to low temperature.

6.10 Effect on *CHS-GUS* Promoter Activity of Blue or UVA 20 °C Pre-treatment Prior to Exposure to B/UVA Radiation at 10 °C

As described in Section 6.9, previous studies by Fuglevand *et al* (1996) identified an effect of the order of illumination on *CHS-GUS* expression. It was conceivable that exposure of NM4 seedlings to blue or UVA light may produce a signal that enhances the subsequent response to B/UVA and cold stress. Exposure to blue/UVA light at low temperature produces a synergistic increase in *CHS-GUS* promoter activity. Therefore plants were pre-treated with blue or UVA radiation prior to exposure to blue/UVA/cold treatment to determine any effect on GUS activity.

NM4 seedlings were transferred from conditions of non-inductive white light (20 μmol/m^2/s) to blue (50 μmol/m^2/s) or UVA (20 μmol/m^2/s) light for 8 hours at 20 °C. Seedlings were then exposed to a further 16 or 24 hours of blue/UVA light at 10 °C. Samples were removed after the 8 hour pre-treatment and after 16 or 24 hours of blue/UVA/cold treatment, GUS activities were then determined (Table 6.4).

Blue pre-treatment- Exposure to blue radiation at 20 °C produced little induction of *CHS-GUS* expression. Subsequent exposure to blue/UVA radiation at low temperature induced an increase in GUS activity reaching peak levels after 24 hours of blue/UVA exposure (551.5 ± 93.3 pmol 4-MU/mg/min).

UVA-pre-treatment- *CHS-GUS* expression following UVA treatment at 20 °C and blue/UVA treatment at 10 °C reached peak levels of GUS activity after 16 hours of blue/UVA radiation at 10 °C (463.0 ± 156.0 pmol 4-MU/mg/min).

No pre-treatment- GUS activities in control plants exposed to blue/UVA radiation at 10 °C with no pre-treatment were found to greatly exceed those of plants receiving the 20 °C pre-treatment. 24 hours of exposure to blue/UVA radiation at 10 °C induced a GUS activity of 2295.0 ± 430.3 pmol 4-MU/mg/min, this is ~ 4-5 times higher than the peak activity detected in plants that received blue/UVA radiation at 10 °C after the blue or UVA 20 °C pre-treatment.

Table 6.4 Effect on *CHS-GUS* Promoter Activity of Blue or UVA Light Treatment at 20 °C Prior to Exposure to Blue/UVA Light at 10 °C[a]

Pre-treatment	Duration of Pre-treatment (h)	Duration of Blue/UVA/Cold Treatment (h)	GUS Activity
LW	8	0	37.8 ± 13.0
Blue	8	0	42.1 ± 8.5
	8	16	415.6 ± 92.9
	8	24	551.5 ± 93.3
UVA	8	0	47.7 ± 17.4
	8	16	463.0 ± 156.0
	8	24	351.6 ± 130.6
No Pretreatment	0	24	2295.0 ± 430.3

[a]Results represent GUS activity pmol 4-MU/mg/min following exposure to B/UVA (50/20 μmol/m^2/s) at 10 °C with an 8 hr pre-treatment with blue (50 μmol/m^2/s), UVA (20 μmol/m^2/s) at 20 °C or with no pre-treatment. Errors represent standard errors where n=10.

Thus, blue or UVA pre-treatment at 20 °C did not produce any further induction of *CHS-GUS* promoter activity than blue/UVA radiation with cold treatment alone. Indeed pre-treatment appeared to impair the subsequent response to blue/UVA light and low temperature.

6.11 Synergistic Interaction between Light and Low Temperature on *CHS* Transcript Levels

It was previously demonstrated that blue/UVA/cold treatment produced a strong synergistic increase in *CHS-GUS* promoter activity (Section 6.3). It was therefore of interest to determine the effect of this treatment at the transcript level.

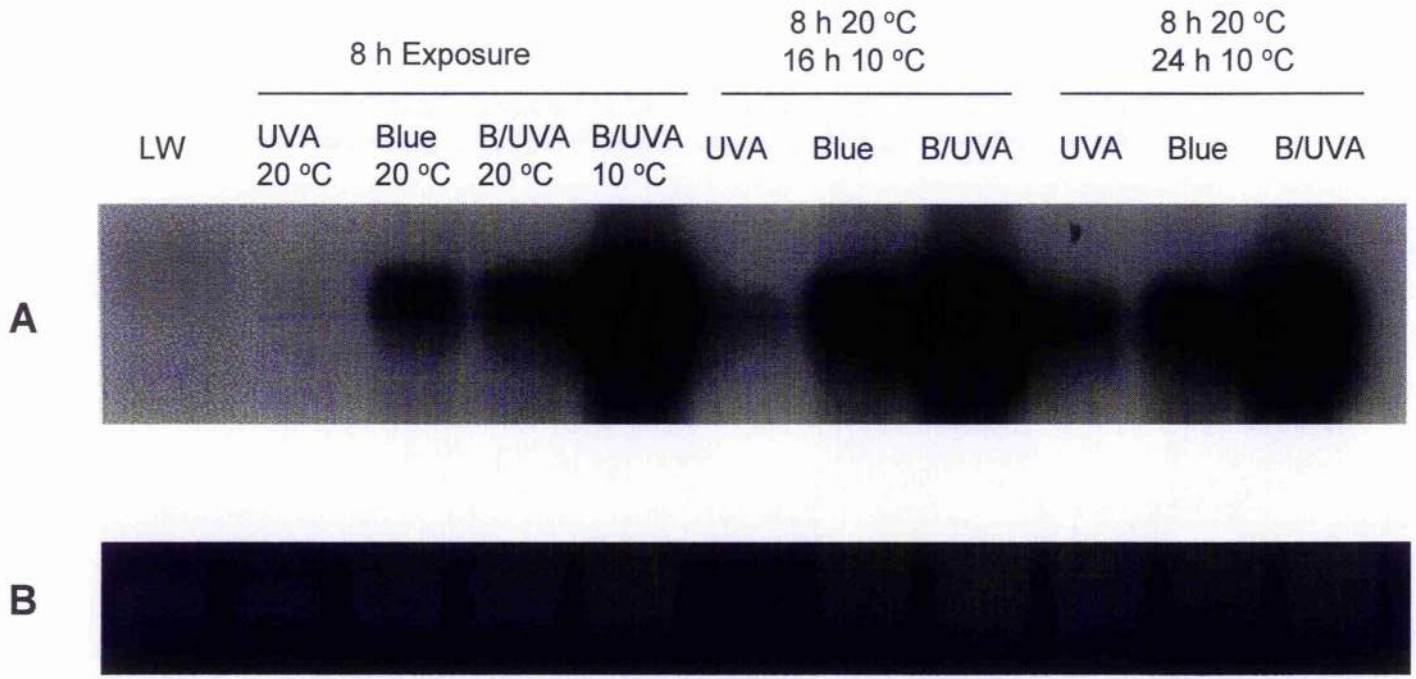
Exposure of *Arabidopsis* seedlings, grown in non-inductive white light (20 $\mu mol/m^2/s$), to 8 hours of blue light at 20 °C produced an increase in *CHS* transcript accumulation as compared to low white light. The effect of UVA light was barely detectable at this exposure of the autoradiograph.

Exposure of plants to blue/UVA light at 10 °C and 20 °C clearly demonstrated the strong inductive effect of blue/UVA/cold treatment on *CHS* transcript accumulation (Figure 6.6).

Figure 6.6

CHS transcript levels following blue, UVA or B/UVA pre-treatment at 20 °C prior to the addition of low temperature. Wild type *Arabidopsis* seedlings were grown in conditions of non-inductive white light (20 μmol/m^2/s) at 20 °C for three weeks before being transferred to LW 20 °C (lane 1), 8 hours of UVA (20 μmol/m^2/s) 20 °C (lane 2), blue (50 μmol/m^2/s) 20 °C (lane 3), Blue/UVA (50/20 μmol/m^2/s) at 20 °C (lane 4) or Blue/UVA (50/20 μmol/m^2/s) at 10 °C (lane 5), 8 hr light treatment at 20 °C followed by 16 hours of light treatment at 10 °C with UVA light (lane 6), Blue light (lane 7), Blue/UVA (lane 8), 8 hours of light treatment at 20 °C followed by 24 hours of light treatment at 10 °C with UVA (lane 9), blue (lane 10) or Blue/UVA (lane 11). (A) *CHS* transcript levels in total leaf RNA (5 μg per lane) were measured by hybridisation of a *CHS* probe to RNA gel blots. *Arabidopsis thaliana derived CHS* probe was isolated from pUC 19 plasmid described by Trezzini *et al.*, 1993. (B) total rRNA detected by UV fluorescence following reaction with ethidium bromide.

Figure 6.6 *CHS* Transcript Levels Following Blue, UVA or B/UVA Pre-Treatment at 20 °C Prior to the Addition of Low Temperature



6.12 Effect on *CHS* Transcript Levels of a 20 °C Pre-treatment with Blue, UVA or Blue/UVA Radiation Prior to the Addition of a 10 °C Cold Treatment

The effect of exposing plants to blue, UVA or blue/UVA light prior to the addition of cold on *CHS* transcript accumulation was determined. It was conceivable that exposure to light prior to cold may affect the subsequent response to low temperature.

Wild type *Arabidopsis* seedlings, grown in non-inductive white light (20 μmol/m^2/s), were exposed to blue (50 μmol/m^2/s), UVA (20 μmol/m^2/s) or blue/UVA (70 μmol/m^2/s) radiation for 8 hours at 20 °C. Light conditions were then kept constant whilst temperatures were reduced to 10 °C. Samples were removed after the 8 hour pre-treatment and following 16 and 24 hours of the light/cold treatment and *CHS* transcript levels were determined. As a control, plants were exposed to 8 hours of blue/UVA light at 10 °C.

Blue/UVA light treatment at 10 °C for 8 hours appeared sufficient for induction of *CHS* transcript accumulation. Prior exposure to blue, UVA or blue/UVA light at 20 °C did not stimulate transcript accumulation further (Figure 6.6).

6.13 *CHS* Transcript Levels Following Blue or UVA 20 °C Pre-treatment Prior to Blue/UVA Radiation at Low Temperature

The effect, on *CHS* transcript accumulation, of exposing seedlings to blue or UVA light prior to blue/UVA/cold treatment was assessed. As described by Fuglevand *et al* (1996) providing blue light prior to UVB light enhanced the subsequent *CHS* response to UVB. It was possible that exposure of plants to UVA or blue light prior to the highly inductive blue/UVA/cold treatment may prime the signal transduction mechanism so as to enhance the response to light and cold stress. Although no effect was seen on *CHS-GUS* promoter activity, it was important to establish whether any effects could be observed at the transcript level.

Wild type *Arabidopsis* seedlings were taken from non-inductive conditions of white light (20 μmol/m^2/s) at 20 °C and exposed to either blue (50 μmol/m^2/s) or UVA (20 μmol/m^2/s) light at 20 °C for 8 hours. Plants were then transferred to conditions of blue/UVA light (70 μmol/m^2/s) at 10 °C for 16 or 24 hours. Samples were removed after the 8 hour pre-treatment and following 16 or 24 hours of blue/UVA/cold treatment. *CHS* transcript levels were determined. As a control *Arabidopsis* seedlings were exposed to 8 hours of blue/UVA light at 20 °C followed by 24 hours of blue/UVA light at 10 °C.

Exposure of seedlings to 8 hours of blue or UVA light at 20 °C produced little

induction of *CHS* transcript accumulation during the exposure time of the autoradiograph (Figures 6.7 & 6.8).

Exposure of seedlings to blue or UVA light at 20 °C prior to blue/UVA/cold treatment produced a strong induction of *CHS* transcript accumulation. Induction appeared to be greater than that determined in seedlings exposed to 8 hours of blue/UVA light at 20 °C followed by 24 hours of blue/UVA light at 10 °C.

Thus, order of illumination appears to affect *CHS* transcript accumulation with a blue or UVA light treatment at 20 °C enhancing the subsequent response to blue/UVA/cold treatment.

6.14 *CHS* Transcript Accumulation in Response to Red Light

Exposure of NM4 seedlings to red light and low temperature had little effect on GUS activity (Section 6.4). This experiment was repeated using WT *Arabidopsis* seedlings to determine any effect on *CHS* transcript levels.

Wild-type *Arabidopsis* seedlings were taken from conditions of non-inductive white light (20 μmol/m^2/s) at 20 °C and exposed to red light (85 μmol/m^2/s) for 24 hours at 20 or 10 °C. At low temperature in the presence of red light, *CHS* transcript accumulation

Figure 6.7

CHS transcript levels following blue 20 °C pre-treatment prior to B/UVA radiation at low temperature. Wild type *Arabidopsis* seedlings were grown in conditions of non-inductive white light (20 μmol/m^2/s) at 20 °C for 3 weeks before being transferred to white light (20 μmol/m^2/s) at 20 °C (lane 1), 8 hours blue light (50 μmol/m^2/s) at 20 °C (lane 2), 8 hours of blue light 20 °C followed by 16 hours of Blue/UVA light (50/20 μmol/m^2/s) at 10 °C (lane 3), 8 hours of blue light at 20 °C followed by 24 hours of Blue/UVA light at 10 °C (lane 4). As a control, plants were exposed to 8 hours of Blue/UVA light (50/20 μmol/m^2/s) at 20 °C followed by 24 hours of Blue/UVA treatment at 10 °C (lane 5). (A) *CHS* transcript levels in total leaf RNA (5 μg per lane) were measured by hybridisation of a *CHS* probe to RNA gel blots. (B) total rRNA detected by UV fluorescence following reaction with ethidium bromide.

Figure 6.7 *CHS* Transcript Levels Following Blue 20 °C Pre-Treatment Prior to B/UVA Radiation at Low Temperature

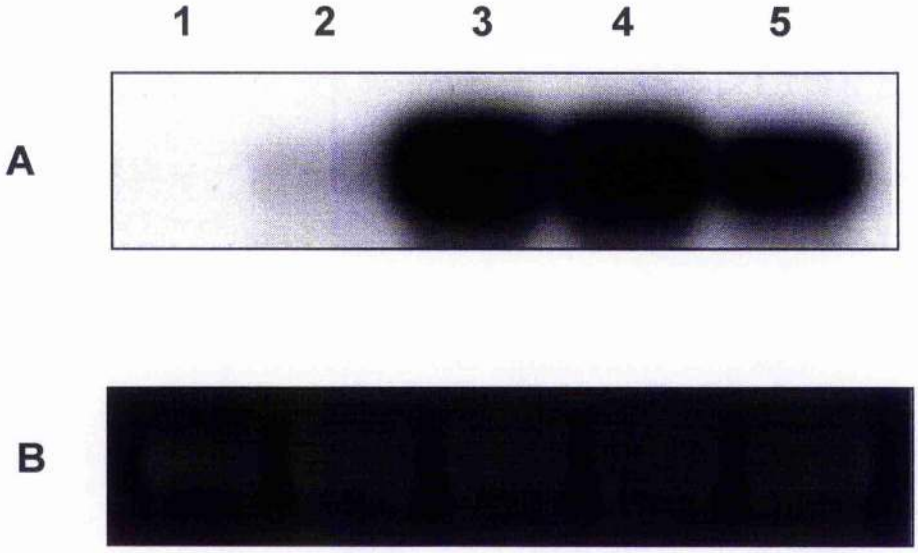
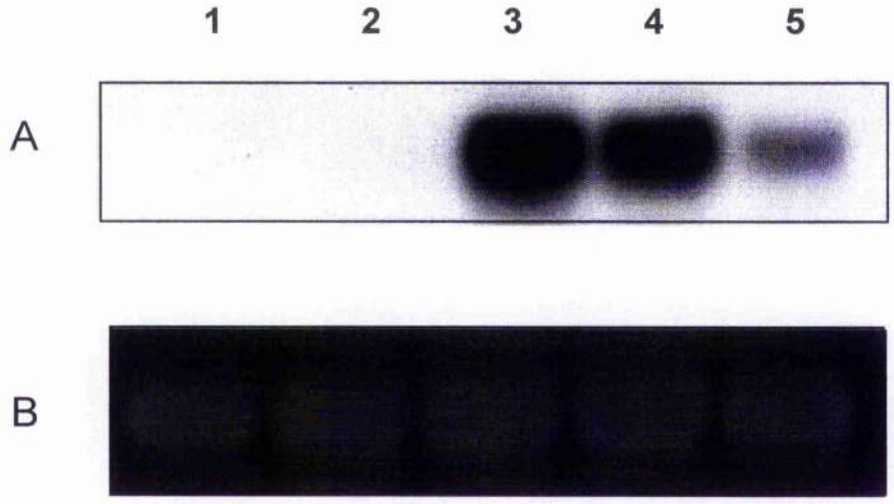


Figure 6.8

CHS transcript levels following UVA 20 °C pre-treatment prior to exposure to B/UVA radiation at 10 °C in *Arabidopsis thaliana*. Wild type *Arabidopsis* seedlings were grown in non-inductive conditions of white light (20 μmol/m^2/s) at 20 °C for 3 weeks before being transferred to white light (20 μmol/m^2/s) at 20 °C (lane 1), 8 hours of UVA light (20 μmol/m^2/s) at 20 °C (lane 2), 8 hours of UVA light treatment at 20 °C followed by 16 hours of Blue/UVA light (50/20 μmol/m^2/s) at 10 °C (lane 3), 8 hours of UVA light at 20 °C followed by 24 hours of Blue/UVA light at 10 °C (lane 4). As a control, plants were exposed to 8 hours of Blue/UVA light (50/20 μmol/m^2/s) at 20 °C followed by 24 hours of Blue/UVA light at 10 °C (lane 5). (A) *CHS* transcript levels in total leaf RNA (5 μg per lane) were measured by hybridisation of a *CHS* probe to RNA gel blots. (B) total rRNA detected by UV fluorescence following reaction with ethidium bromide.

Figure 6.8 *CHS* Transcript Levels Following UVA 20 °C Pre-Treatment Prior to Exposure to B/UVA Radiation at 10 °C in *Arabidopsis thaliana*



appeared greater than that induced by red light at 20 °C (Figure 6.9A). This induction of *CHS* transcripts is likely to result from exposure to low temperature alone. Exposure of seedlings to low temperature in white light (20 μmol/m^2/s) induced a similar level of *CHS* transcripts (Figure 6.9 C). The level of *CHS* transcripts stimulated by exposure to low temperature alone or red light and low temperature is much lower than *CHS* induction following B/UVA/cold treatment.

6.15 *CHS* Transcript Accumulation in the *Arabidopsis hy4* Mutant Following Exposure to UVA, Blue or Blue/UVA Radiation at Low Temperature

The *Arabidopsis hy4* mutant lacks the CRY1 photoreceptor resulting in an impaired response to blue and UVA light (Cashmore, 1997). This mutant shows a much reduced induction of *CHS* transcripts in these light qualities (Jackson & Jenkins, 1995; Fuglevand *et al.*, 1996). However, *hy4* retains the synergistic interactions between UVA and blue light with UVB (Fuglevand *et al.*, 1996). The *hy4* mutant was employed in this study in order to determine whether the synergistic interaction between low temperature and blue/UVA involves a signal transduction mechanism originating from the CRY1 photoreceptor. *hy4-2.23N*, a *hy4* null mutant (Lin *et al.*, 1998) was used in this study.

Figure 6.9

CHS transcript accumulation in response to red light and cold in *Arabidopsis thaliana*. Wild type *Arabidopsis* seedlings were grown in conditions of non-inductive white light (20 μmol/m^2/s) for three weeks at 20 °C before being transferred for 24 hours to (A) white light (20 μmol/m^2/s) at 20 °C (lane 1), red light (85 μmol/^{2}/s) at 20 °C (lane 2), red light (85 μmol/m^2/s) at 10 °C (lane 3), blue/UVA light (50/20 μmol/m^2/s) at 10 °C (lane 4).

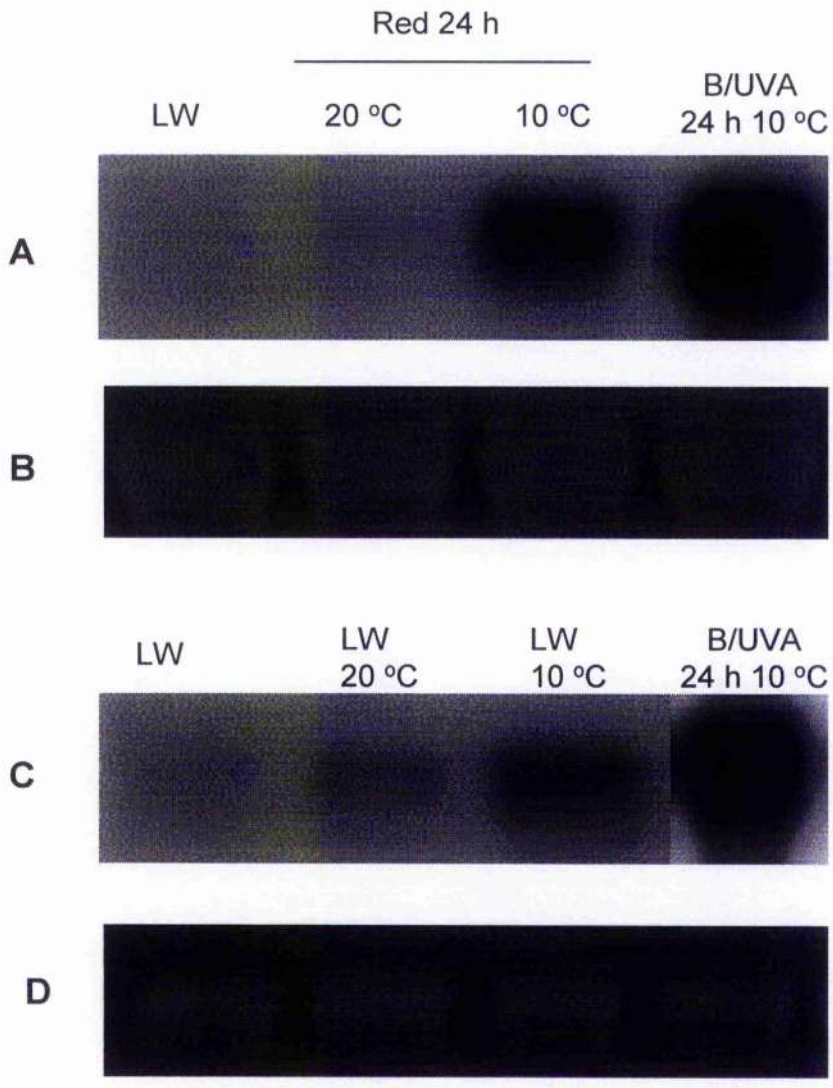
(B) total rRNA corresponding to lanes shown in (A) detected by UV fluorescence following reaction with ethidium bromide

(C) white light (20μmol/m^2/s) (lane 1), LW at 20 °C (lane 2), LW 10 °C (lane 3) or blue/UVA light (50/20 μmol/m^2/s) at 10 °C (lane 4).

(D) total rRNA corresponding to lanes shown in (C) detected as described in (B).

CHS transcript levels in total leaf RNA (5 μg per lane) were measured by hybridisation of a *CHS* probe to RNA gel blots (A and C).

Figure 6.9 *CHS* Transcript Accumulation in Response to Red Light and Cold in *Arabidopsis thaliana*



Arabidopsis hy4 seedlings were exposed to UVA or blue radiation for 24 hours at 10 °C. In addition seedlings were exposed to blue/UVA radiation for 24 hours at 10 or 20 °C. Exposure of *hy4* seedlings to blue radiation produced the greatest induction of *CHS* transcripts (Figure 6.10). This level of induction was not observed with blue/UVA light perhaps indicating some form of communication between a blue light receptor and CRY1. However, induction of *CHS* transcripts in the *hy4* mutant following exposure to blue radiation was significantly lower than the response of wild type *Arabidopsis* seedlings to blue/UVA light at low temperature. Synergistic induction of *CHS* transcripts by blue/UVA radiation combined with low temperature was not observed. Therefore, this response is mediated by the CRY1 photoreceptor.

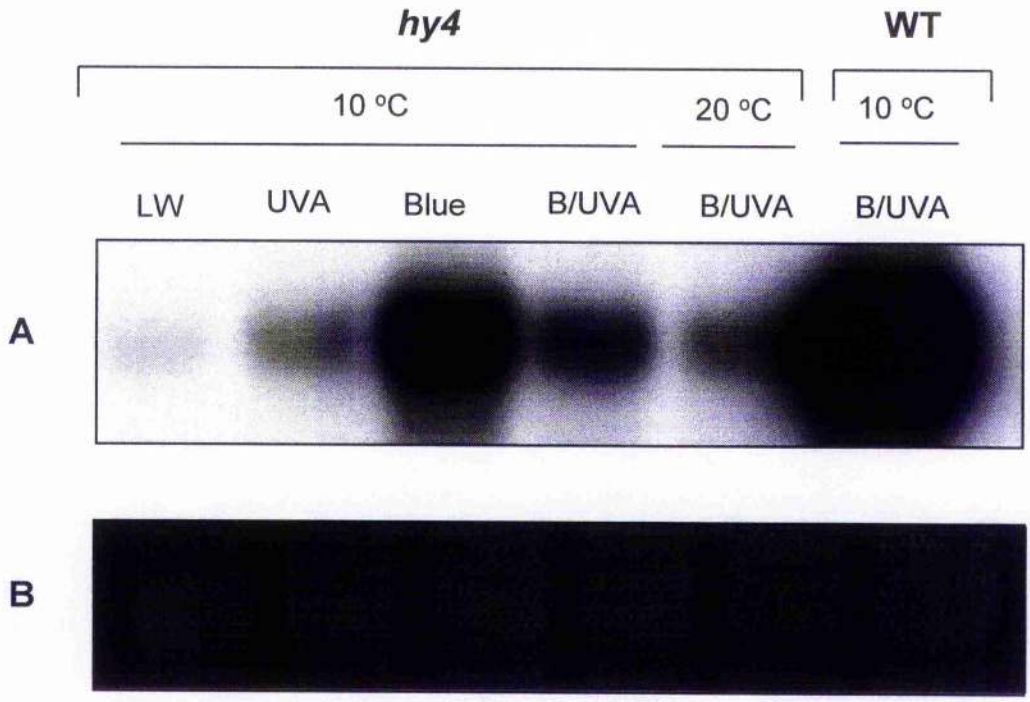
6.16 Induction of *CHS-GUS* Promoter Activity Following Exposure to Combinations of Blue, UVA and UVB Radiation at 10 °C

Light treatments known to induce *CHS-GUS* activity were combined to determine whether the synergistic increase in activity stimulated by blue/UVA/cold could be further increased by the addition of UVB radiation. It was previously shown that UVB is synergistic with UVA and blue light (Fuglevand *et al.*, 1996) and it was therefore interesting to see whether this synergism, when combined with UVA/blue/cold synergism

Figure 6.10

CHS transcript levels in the *Arabidopsis hy4* mutant following exposure to UVA, blue or B/UVA light at low temperature. *hy4 Arabidopsis* seedlings were grown in non-inductive conditions of white light (20 μmol/m^2/s) at 20 °C for 3 weeks before being transferred for 24 hours to white light (20 μmol/m^2/s) at 10 °C (lane 1), UVA light (20 μmol/m^2/s) at 10 °C (lane 2), blue light (50 μmol/m^2/s) at 10 °C (lane 3), blue/UVA light (50/20 μmol/m^2/s) at 10 °C (lane 4), blue/UVA light at 20 °C (lane 5). As a control, WT *Arabidopsis* seedlings grown in non-inductive white light at 20 °C for 3 weeks were exposed to blue/UVA light (50/20 μmol/m^2/s) at 10 °C for 24 hours (lane 6). (A) *CHS* transcript levels in total leaf RNA (5 μg per lane) were measured by hybridisation of a *CHS* probe to RNA gel blots. (B) total rRNA detected by UV fluorescence following reaction with ethidium bromide.

Figure 6.10 *CHS* Transcript Levels in the *Arabidopsis hy4* Mutant Following Exposure to UVA, Blue or B/UVA Light at Low Temperature



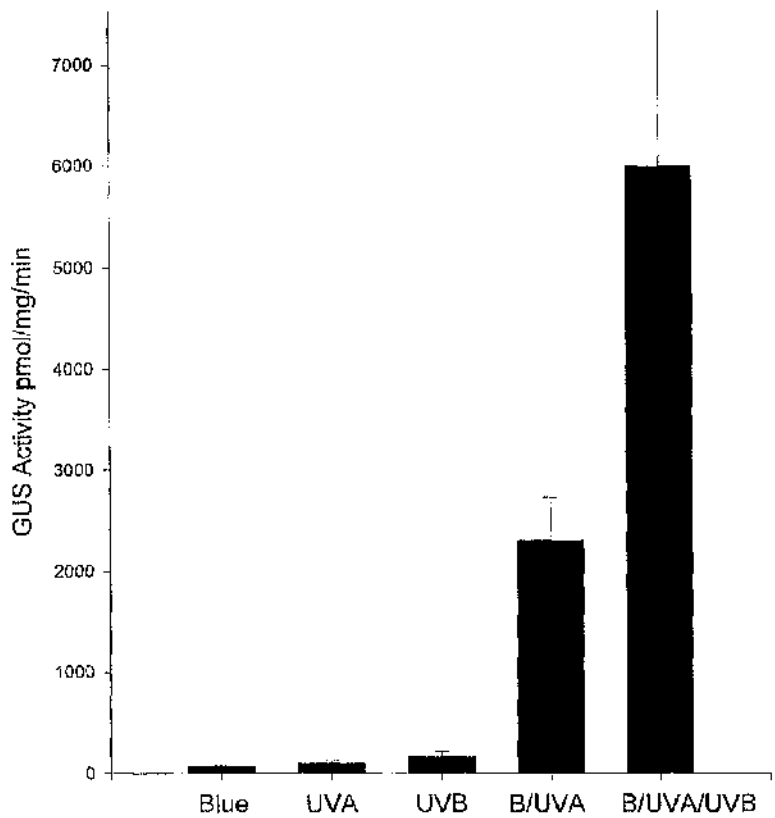
would stimulate a further increase in *CHS* promoter activity.

Arabidopsis seedlings were exposed to 24 hours of blue, UVA, UVB, B/UVA or B/UVA/UVB radiation at 10 °C (Figure 6.11). GUS activity of seedlings prior to light treatments was 38.9 ± 13.6. This was observed to increase to 63.9 ± 12.0, 97.5 ± 23.5 and 164.6 ± 54.9 pmol 4-MU/mg/min following exposure to blue, UVA and UVB radiation respectively. More substantial increases were observed following exposure to B/UVA radiation, a GUS activity of 2295.0 ± 430.0 pmol/mg/min after 24 hours. However B/UVA/UVB irradiation produced GUS activity ~2.6 fold higher than blue/UVA alone, reaching 5996.0 ± 1545.8 pmol/mg/min. Combining the synergistic induction of *CHS-GUS* promoter activity by blue/UVA radiation in the presence of cold with B/UVA/UVB synergism caused a large increase in *CHS* promoter activity.

Figure 6.11

Induction of *CHS-GUS* promoter activity following exposure to combinations of blue, UVA and UVB light at 10 °C. *Arabidopsis* NM4 plants were grown in conditions of non-inductive white light (20 μmol/m^2/s) at 20 °C for 3 weeks before being transferred for 24 hours to blue light (50 μmol/m^2/s), UVA light (20 μmol/m^2/s), UVB radiation (3.5 μmol/m^2/s), blue/UVA (50/20 μmol/m^2/s) or blue/UVA/UVB (50/20/3.5 μmol/m^2/s) at 10 °C. GUS activity was determined and is expressed as pmol 4-MU/mg/min. Bars indicate standard errors where n=10.

Figure 6.11 Induction of *CHS-GUS* Promoter Activity Following Exposure to Combinations of Blue, UVA and UVB Light at 10 °C



6.17 Discussion

In mature leaves of *Arabidopsis thaliana*, *CHS* gene transcription is regulated by UV and blue light (Jackson & Jenkins, 1995; Fuglevand *et al.*, 1996). Regulation by UVB and UVA/blue light is believed to occur via separate but interacting signal transduction mechanisms (Fuglevand *et al.*, 1996). Pharmacological studies using inhibitors and antagonists of known signal transduction mechanisms provide further evidence for the presence of distinct UVA/blue and UVB signalling pathways. There is a requirement for calmodulin in UVB signal transduction but not for blue/UVA signal transduction (Christie & Jenkins, 1996).

Studies by Fuglevand *et al* (1996) demonstrated that both blue and UVA light, when given separately induce *CHS-GUS* expression and *CHS* transcript accumulation in *Arabidopsis*. When blue and UVA radiation are given together the effects on *CHS-GUS* expression are additive. However, UVA and blue light produce synergistic responses when given together with UVB.

In the present study, exposure of *Arabidopsis* NM4 seedlings to blue or UVA light clearly induced *CHS-GUS* expression and *CHS* transcript accumulation. This is less apparent in some of the autoradiographs because of the exposure time employed. As expected, combining blue and UVA light produced an approximately additive increase in

CHS-GUS expression. Exposing NM4 seedlings to blue or UVA radiation at low temperature (10 °C) produced a small increase in *CHS-GUS* expression. However, combining blue/UVA light with low temperature induced a synergistic increase in *CHS-GUS* expression and *CHS* transcript accumulation. Levels of *CHS-GUS* expression were found to be more than 10 fold higher than the activity expected if effects of UVA/blue and cold were additive. Combining blue/UVA light with low temperature produced a strong synergistic response. No such response has been reported previously.

The response of the *CHS* promoter to low temperature and light appears to be specific for UVA/blue light. UVB irradiation produced only a slight increase in *CHS-GUS* promoter activity in plants simultaneously exposed to low temperature. In addition, exposure of plants to red light at low temperature did not increase *CHS-GUS* expression significantly more than non-inductive white light at low temperature.

Previous studies found that combining UVB induction of *CHS* with either UVA or blue radiation produced a synergistic increase in *CHS-GUS* expression. In addition, maximum *CHS-GUS* expression was observed when blue light was given before UVB/UVA radiation. It was hypothesised that the blue light treatment may activate a signal transduction mechanism that enhances the subsequent response to UVB (Fuglevand *et al.*, 1996). It was therefore conceivable that exposure of NM4 seedlings to blue/UVA light prior to the addition of cold may prime the signal transduction mechanism so as to enhance the subsequent response to cold stress. In contrast, exposure

of seedlings to blue/UVA light prior to the addition of cold greatly reduced the subsequent response to low temperature. The highest GUS activities were detected when blue/UVA treatments were given simultaneously with cold treatments.

Under the same rationale, it was conceivable that pre-treating plants with blue or UVA light at 20 °C may enhance the subsequent response to blue/UVA/cold treatment. However, blue or UVA pre-treatment did not produce any further induction of *CHS-GUS* promoter activity than blue/UVA/cold treatment alone. Indeed, pre-treatment appeared to impair the subsequent response to blue/UVA light and low temperature.

CHS transcript levels were analysed in plants exposed to blue, UVA or blue/UVA light prior to the addition of low temperature. Prior exposure to UVA, blue or blue/UVA light did not stimulate *CHS* transcript accumulation further than exposure to blue/UVA light for 8 hours at 10 °C.

The effect of exposing seedlings to blue or UVA light prior to blue/UVA/cold treatment on *CHS* transcript accumulation was assessed. As previously discussed, it was conceivable that exposure of plants to UVA or blue light prior to the highly inductive blue/UVA/cold treatment may enhance the *CHS* response to light and cold stress. Transcript accumulation was found to be greater following blue or UVA pre-treatment than blue/UVA pre-treatment at 20 °C. This result differs from that suggested by *CHS-GUS* expression. Although blue or UVA pre-treatment induced higher levels of *CHS* transcripts this pattern is not represented in product accumulation (GUS). This may

indicate post-transcriptional regulation and possibly effects of low temperature on synthesis of the GUS product. In addition, differences between *CHS-GUS* expression data and *CHS* transcript accumulation may indicate developmental differences between the samples collected for each analysis. All leaves were harvested for RNA analysis whereas the expanding pair of true leaves were harvested for *CHS-GUS* analyses. It is also possible that there may be regulatory differences between the *Sinapis alba CHS* promoter transformed into NM4 and the endogenous *Arabidopsis thaliana CHS* promoter in wild type seedlings employed for RNA analyses.

Combining blue/UVA and UVB treatments at low temperature induced the greatest *CHS-GUS* activity measured in this study. Induction was found to be almost three fold higher than that of seedlings exposed to blue/UVA and cold treatment. It is likely that the UVB/blue/UVA synergistic response can combine with the blue/UVA/cold synergism to greatly increase *CHS-GUS* expression. Clearly the level of accumulation was much greater than would be expected from an additive effect of blue/UVA/cold and UVB induction.

Previous studies with the *Arabidopsis hy4* mutant have indicated a reduced induction of genes relating to flavonoid biosynthesis including *DFR, CHI* and *CHS* in response to white and blue light (Jackson & Jenkins, 1995). It was later observed that *CHS* transcription in *hy4* is reduced as compared to wild type plants in response to blue and UVA radiation. Responses to UVB, including the synergistic response to blue and

UVA with UVB are retained in the *hy4* mutant (Fuglevand *et al.*, 1996).

In this study the response of the *hy4* mutant was assessed in conditions of blue, UVA and blue/UVA light at 20 °C and 10 °C. Although *hy4* is a blue light response mutant, some induction of *CHS* transcript accumulation was observed following exposure to blue light. This result was also observed by Jackson & Jenkins (1995) and Fuglevand *et al* (1996). It is possible that the phytochrome photoreceptor may be responsible for this response to blue light. Alternatively a separate blue light photoreceptor, such as CRY2, may be involved (Fuglevand *et al.*, 1996).

As expected, the *hy4* mutant showed a very limited response to UVA or blue/UVA light. In addition, the synergistic interaction of blue/UVA radiation at low temperature was not observed. The response of the *hy4* mutant to blue, UVA or blue/UVA treatment was significantly less than that of wild type *Arabidopsis* plants to blue/UVA treatment at 10 °C. Therefore CRY1 is required for the interaction between UVA/blue light and low temperature.

The information gained in this study can be used to build upon the phototransduction model proposed by Fuglevand *et al* (1996), see Figure 6.12. Cold interacts in a synergistic manner with blue/UVA light but not with UVB radiation. It is therefore likely that the signal transduction mechanism originating from cold perception is interacting with the blue/UVA phototransduction pathway originating from the CRY1 photoreceptor.

Figure 6.12 Model of UVA/Blue/UVB/Cold Regulation of *CHS* Expression in *Arabidopsis thaliana*

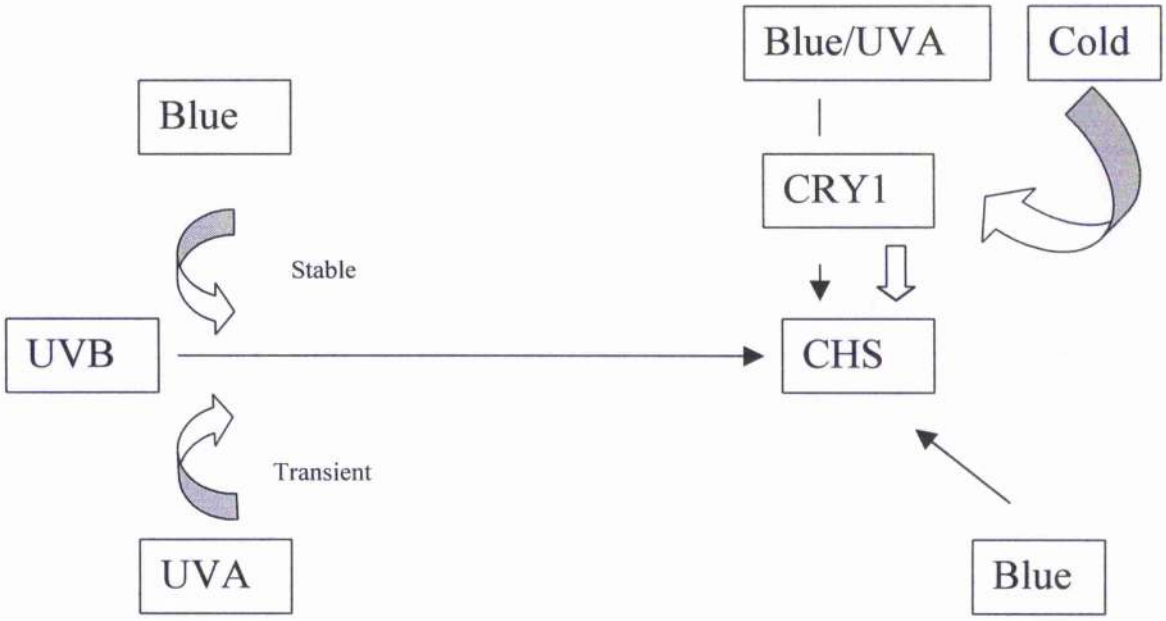


Figure 6.12

Model of UVA/Blue/UVB regulation of *CHS* expression in *Arabidopsis thaliana*, adapted from Fuglevand *et al* (1996). UVB when combined with blue light induces a synergistic increase in *CHS* expression; induction can be increased by exposing plants to blue light prior to UVB. It was hypothesised that exposure to blue light produced a signal capable of enhancing any subsequent response to UVB. This effect was observed even when the blue and UVB treatments were separated by several hours of darkness. Combining UVB with UVA light also produced a synergistic increase in *CHS* expression. However this effect was only observed when UVA and UVB light treatments were given simultaneously, any signal generated by prior UVA treatment was deemed to be transient (Fuglevand *et al.*, 1996). Cold interacts in a synergistic manner with blue/UVA light but not with UVB radiation. It is therefore proposed that the signal transduction mechanism originating from cold perception is interacting with the blue/UVA phototransduction pathway originating from the CRY1 photoreceptor

6.18 Conclusion

Combining cold treatment with blue/UVA light produces a synergistic increase in *CHS*-promoter activity in wild-type seedlings of *Arabidopsis thaliana*. No equivalent response is observed with red or UVB light. The *hy4* blue light response mutant of *Arabidopsis* did not display this light/cold synergism suggesting that the inductive effects of low temperature on *CHS* act in concert with the CRY1 UVA/blue phototransduction pathway. The blue/UVA/cold synergistic response appears to combine with the blue/UVA/UVB synergistic response to further stimulate *CHS* expression.

Chapter 7 Final Discussion

7.1 Introduction

A wide range of commonly consumed fruits and vegetables have been shown to contain flavonols (Hertog *et al.*, 1992, Crozier *et al.*, 1997). Flavonols are believed to exert an antioxidative effect within the human body, which may protect against coronary heart disease (Hertog *et al.*, 1993) and some cancers (Knekt *et al.*, 1997). It is therefore important to understand how these potentially important compounds are regulated within plant tissues.

The work presented in this study demonstrates that the flavonol content of fruits and vegetative plant tissue depends on plant variety and method of cultivation. In addition the flavonol content of plant tissues is regulated by the environmental factors light, nutrient availability and low temperature.

7.2 Identification of 'High Flavonol' Tomato Fruit Varieties

Different fruits and vegetables have been shown to contain vastly different concentrations of flavonols. Within a single species different varieties have also been shown to contain very different flavonol concentrations. For example, in a study by Crozier *et al.* (1997) the red leafed Lollo Rosso lettuce was found to contain 911 μg quercetin/g compared to only 11 μg quercetin/g in the round lettuce variety Cortina. It is

therefore of interest to identify those crops or particular varieties that are especially high in flavonols and may have increased nutritional value. In this study tomato fruit varieties from six different countries were analysed for flavonol content. Fruit variety was identified as an important factor influencing the flavonol content of tomatoes. Varieties Bond and Havanera were both normal sized, field grown Spanish tomatoes obtained from plants cultivated along-side each other on the same plot near Valencia (Spain). None-the-less the total flavonol content of Bond fruit was 10.9 μg as compared to 6.6 μg in Havanera (Table 3.5). The flavonol content of tomato fruits with deep red or purple skins was investigated. It was hypothesised that the skins of such varieties appear to contain substantial amounts of anthocyanins, and as flavonols originate from the same branch of the phenylpropanoid pathway as anthocyanins (Holton & Cornish, 1995; Duthie & Crozier, 2000) the skins might also contain elevated levels of flavonols. Red leafed, anthocyanin rich varieties of lettuce have been shown to contain very high levels of conjugated quercetin compared to many green leafed varieties (Crozier *et al.*, 1997). The skin of the darkly pigmented tomato variety Noire Charbonneuse was found to be particularly rich in flavonols (440 μg/g/fwt). However, not all of the darkly pigmented varieties were found to be high in flavonols; skin from variety Aubergine, a variety characterised by purple striations, contained only 108 μg/flavonol/g.

It was clear from analysis of Spanish tomatoes that the highest flavonol concentrations were present in cherry tomatoes, with lower levels in normal sized and beefsteak fruits. One factor which may explain the elevated levels of flavonols in cherry tomatoes compared to normal varieties, first reported by Crozier *et al* (1997), is the higher skin: volume ratio of small tomatoes compared to larger varieties.

Cherry tomato varieties and some darkly pigmented fruit varieties may be identified as being particularly rich in flavonols and may allow for the production of high flavonol fruits without having to resort to genetic modification.

7.3 Effect of Country of Origin on Flavonol Content

Tomato fruits grown in warm sunny climates such as Spain and S.Africa were found to contain far higher concentrations of flavonols than British fruits. An understanding of the effect of fruit variety and environmental conditions on the flavonol content of tomato fruits may allow British tomato fruit growers to optimise these conditions in order to increase the flavonol content of their produce.

Commercial tomato fruit growing in Scotland and England requires the use of glasshouses in order to maintain the temperatures required for fruit set and also to control pests and disease. Due to the costs involved in building and maintaining these glasshouses, tomato growing is of high intensity with plants tightly packed together and temperature and feeding regimes maximised to obtain the highest possible yield. Tomato plants are grown in strictly regimented rows with sufficient space between the rows only to allow staff to hand pick the fruits. In addition, each tomato plant is connected by a wire to the roof of the greenhouse; as the plant grows the wire is lowered to allow more room for growth upwards, a consequence of this is that the tomato fruits are always found at the bottom of the plants. These developing fruits receive little direct sunlight, with light being filtered first through glass and then through the foliage of surrounding plants.

Fruit from warmer climates such as Spain are usually field grown and if necessary they are shielded from the elements using plastic rather than glass. The developing fruits would receive more sunlight and would be exposed to UVB light.

Light, particularly UVB light, is known to be an important factor in the induction of flavonoids in many plant species (Lois, 1994; Brandt *et al.*, 1995; Price *et al.*, 1995). Where light is the main limiting factor to flavonol induction other influences such as tomato fruit variety may have less impact. The flavonol content of British fruits may be increased primarily by providing greater exposure of developing tomato fruits to sunlight. This may require a change in cultivation methods to physically alter the manner in which tomato plants are cultivated to avoid shading of the fruit, e.g. trellising. Alternatively, supplementary lighting could be provided at the level of the fruits, although this would increase production costs and may not be commercially viable.

In addition to increasing the light induction of flavonols within tomato fruits, the adoption of cherry tomato varieties believed to have a greater capacity to synthesise flavonols may also allow for a greater flavonol content of British produce. Alternatively, growing heavily pigmented tomato varieties such as Noire Charbonneuse, which appear genetically predisposed to synthesise higher levels of flavonoids including flavonols, may allow for the production of high flavonol fruits in Britain.

7.4 Screening Processed Tomato Products for Flavonol Content

Tomato flavonols were able to survive industrial processing methods and could be detected in a wide range of tomato based food products. Tomato juice was found to be a rich source of flavonols with a total flavonol content of 14-16 μg/ml, comparable with that of red wine which can vary from 4.6- 41.6 μg/ml (McDonald *et al.*, 1998). Safeway tomato puree was also identified as a particularly rich source of flavonols containing 70 μg/g. In contrast to tomato fruit, which contains almost exclusively conjugated quercetin, up to 30 % of the quercetin in processed produce was in the free form. The accumulation of free quercetin may be a result of enzymatic hydrolysis of rutin and other quercetin conjugates during pasteurisation and processing procedures.

7.5 Absorption and Excretion of Flavonols from Tomato

Identification of flavonol rich foods is clearly important with respect to their potential nutritional value. However it is also necessary to determine whether these flavonols are absorbed by the human body during digestion. Following the consumption of Spanish cherry tomatoes c.v Paloma or tomato juice (Del Monte) flavonols were detected in human plasma. Quercetin was detected in plasma, mainly in the conjugated form believed to be derived from rutin, with kaempferol appearing not to be absorbed or absorbed at very low levels. Conjugated flavonols were also detected unchanged in urine following consumption of tomato fruits or tomato juice. This evidence suggests that flavonols from tomatoes are absorbed and are available in the body to exert biological activity.

7.6 Environmental Regulation of Flavonol Accumulation in Plant Tissues

7.6.1 The Effect of Nutrient Deficiency on Flavonol Accumulation in Plant Tissues

Previous studies indicated a link between nutrient deficiency and flavonoid accumulation in plant tissues (Carpena *et al.,* 1982; Zornoza & Esteban, 1984). Information on the exact nature of these flavonoids is lacking. In addition, although tomato was commonly used as a model system in which to study the effects of nutrient deprivation there is little information on the effect of nutrient stress on tomato fruits.

The effect of nitrogen or phosphorus deprivation on the flavonol content of plant tissues was studied initially using *Arabidopsis thaliana* seedlings as a test system and then on tomato seedlings, mature vegetative tissue and fruit tissue. The flavonol content of *Arabidopsis* seedlings was found to be far higher than that of tomato, 25 fold higher in conditions of zero nitrogen and 16 fold higher in conditions of zero phosphate. *Arabidopsis* was therefore an excellent system in which to quickly determine the flavonol response of plant tissues to nutrient stress. Exposure of *Arabidopsis* or tomato seedlings to conditions of reduced nitrogen or phosphorus demonstrated a clear inverse relationship between nitrogen and phosphorus nutrition and flavonol content. On the basis of this observation a trial was established under commercial conditions to determine the effect of nutrient stress on the flavonol content of mature leaf tissue and tomato fruit tissue. Consistent with previous work (Carpena *et al.,* 1982; Bongue-Bartelsman & Phillips., 1995), reduced nitrogen availability caused an increase in flavonol content in the leaves of tomato plants, reduced phosphorus nutrition did not elicit this response.

The effect of nitrogen and phosphate deprivation on the flavonol content of tomato fruit tissue was also assessed. To the best of the author's knowledge, this is the first study to assess the effect of nutrient stress on the individual flavonols of tomato fruits in a commercial setting. The skins of red, breaker and green fruits were analysed for flavonol content. Exposure to the low nitrogen or phosphate treatments caused an increase in the flavonol content of tomato fruit skins early in the ripening process (mature green stage). Any effect of nutrient stress on the flavonol content of fruits was lost as ripening progressed. It is possible that green fruits may have to compete with other plant sinks for available nutrients and may therefore suffer from a lack of available nutrients. During ripening the sink strength of the fruit is likely to increase such that the nutrient deficiency no longer has any effect on flavonol accumulation. Alternatively, induction of flavonols in the skins of green fruits may be important to protect the fruit tissues and developing seeds from penetration by potentially damaging radiation. As ripening is a process of dying during which the tomato seeds within the fruit reach maturity, the induction of such protective compounds may carry no further advantage.

The present study provides clear evidence that the flavonol content of plant tissues is influenced by their nutritional status. Manipulation of nutrient availability does not stimulate increased levels of flavonols within tomato fruits. However, nutrient stress could be used to manipulate the flavonol content of vegetative crops, although nutrient stress may have implications for disease resistance and reduction in yield.

7.6.2 Effect of Light Quality and Low Temperature on *CHS* Expression in *Arabidopsis thaliana*

As information on signal transduction mechanisms and interactions between regulatory systems becomes available, signal transduction models can begin to be constructed, not just of individual pathways but of complex networks (Jenkins, 1999). Such a network would be required to decipher the range of environmental information regulating *CHS* expression in order to produce an appropriate response.

Early in *Arabidopsis* seedling development, red light induces *CHS* expression via the well-characterised phytochrome photoreceptor. *CHS* induction by red/far red light during photomorphogenesis allows photoprotectants to be accumulated before photosynthetic apparatus sensitive to the damaging components of sunlight are produced. Phytochrome signal transduction regulating *CHS* transcript accumulation has been shown to require G-protein activation (Roux, 1994) and cGMP (Bowler *et al.*, 1994).

CHS expression in mature *Arabidopsis* leaf tissue is regulated by UVB, UVA and blue light (Jackson & Jenkins, 1995; Fuglevand *et al.*, 1996). Regulation by UVB and UVA/blue light is believed to occur via separate but interacting signal transduction mechanisms (Fuglevand *et al.*, 1996). Pharmacological studies using inhibitors and antagonists of known signal transduction components provide further evidence for the presence of distinct UVA/blue and UVB signalling pathways (Christie & Jenkins, 1996). There is a requirement for calmodulin in UVB signal transduction but not for UVA/blue signal transduction. Both UVB and UVA/blue signalling require an increase in cytosolic

calcium, protein kinase and phosphatase activity was also required. Such studies show that both UVB and UVA/blue signalling pathways are distinct from phytochrome signal transduction.

Signal transduction originating from UVB can interact with blue and UVA light to increase *CHS* transcript accumulation in a synergistic manner. In addition, *CHS* induction is increased further if the blue light treatment precedes the UVB/UVA treatment (Fuglevand *et al.*, 1996). It was hypothesised that exposure to blue light produced a signal that could enhance the subsequent response to UVB light. Studies with the *Arabidopsis hy4* mutant revealed that blue/UVA light regulation of *CHS* transcript accumulation is mediated by the CRY1 photoreceptor. Although the *hy4* mutant lacks the CRY1 photoreceptor and shows a reduced inductive response to blue and UVA light it retains the synergistic increase in *CHS* transcript accumulation when blue or UVA light is combined with UVB.

Light regulation of *CHS* transcript accumulation is further complicated by interactions between the cryptochrome UVA/blue light receptor and phytochrome (Wade & Jenkins, unpublished). In conditions of limited light, cryptochrome and phytochrome signal transduction pathways interact to allow de-etiolation and synthesis of photoprotectants (Ahmad & Cashmore, 1997; Casal & Mazzella, 1998).

Previous studies have shown that plants synthesise photoprotectants in the presence of light in response to low temperature (Leyva *et al.*, 1995). Cold signal transduction has been shown to require increased cytosolic calcium, protein kinase and protein phosphatase activity (Knight *et al.*, 1996; Monroy & Dhindsa, 1995). Signal

transduction ultimately leads to changes in flavonoid gene transcript accumulation including *PAL, CHS* and to a lesser extent *4Cl* and *CHI* (Christie *et al.*, 1994).

In this study, the branch of the signal transduction network leading to regulation of *CHS* transcript accumulation in conditions of low temperature has been identified. Combining blue/UVA light with low temperature (10 °C) induced a synergistic increase in *CHS* expression. This *CHS* response appears to be specific for UVA/blue light. UVB irradiation produced only a slight increase in *CHS* expression in plants simultaneously exposed to low temperature. Similarly, exposure of plants to red light at low temperature did not increase *CHS* expression significantly more than non-inductive white light at low temperature.

The *Arabidopsis hy4* mutant lacking the CRY1 blue/UVA photoreceptor showed a very limited response to blue or UVA light at low temperature. In addition, the synergistic interaction of blue/UVA light at low temperature was not observed. It is therefore proposed that the signal transduction mechanism originating from cold perception is interacting with the blue/UVA phototransduction pathway originating from the CRY1 photoreceptor.

Combining blue/UVA and UVB light treatments at low temperature produced the greatest induction of *CHS* promoter activity observed in this study. This level of induction may result from an interaction between two synergistic responses, the blue/UVA/cold synergism and blue/UVA/UVB synergism.

The induction of flavonoids in conditions of low temperature in the presence of light suggests a photoprotective role. Simultaneous exposure to light and low temperature can cause severe inhibition of photosynthesis (Hodgson & Raison, 1989).

Reduced efficiency of the photosynthetic apparatus at low temperatures due to factors such as membrane disruption and reduced enzyme activity can lead to a build up of highly oxidative photosynthetic by-products in the presence of light. Such by-products would normally be eliminated by superoxide dismutase and catalase, however, at low temperatures the activity of these enzymes is inhibited (Graham & Patterson, 1982). Accumulation of anthocyanins and other photoprotective pigments such as flavonols in conditions of low temperature would reduce penetration of harmful levels of light thereby affording protection against the deleterious effects of cold on photosynthesis. In addition, it is possible that flavonols and anthocyanins may function as antioxidants in plant cells, although this has not been fully investigated.

7.7 Conclusions

Tomatoes and tomato-based products are a rich source of conjugated quercetin and kaempferol. Cherry tomatoes originating from warm, sunny climates were found to contain the highest flavonol concentrations. Tomato flavonols were able to withstand industrial processing methods allowing their detection in a wide variety of tomato based products. Tomato juice and tomato puree, were found to be particularly rich in flavonols.

Following consumption of tomato fruits and tomato juice, conjugated quercetin was detected unchanged in plasma and urine. Flavonols present in tomato fruits are therefore absorbable and bioavailable.

The flavonol content of plant tissues is influenced by their nutritional status. Nutrient deprivation caused an increase in flavonol accumulation in vegetative plant tissue and in fruit tissue during the early stages of development.

Flavonol biosynthesis is regulated by light and temperature. Cold treatment combined with blue/UVA light produces a synergistic increase in *CHS*-promoter activity in wild-type *Arabidopsis* seedlings. The *hy4* blue light response mutant of *Arabidopsis* did not display this light/cold synergism suggesting that the signalling pathway mediating *CHS* regulation interacts with the CRY1 UVA/blue phototransduction pathway.

7.8 Future Work

The flavonol content of tomato fruits grown in Britain is generally very low compared to imported fruits. It would be of interest to determine whether the flavonol content of British fruits could be increased by the adoption of tomato fruit varieties identified as being particularly rich in flavonols. Such varieties could be tested in a commercial setting. In addition, the effect of providing greater light exposure to developing tomato fruits could be tested by providing supplementary lighting at the level of the fruits or trellising the vines such that the fruits are directly exposed to sunlight.

It is clear that nutrient stress increases the flavonol content of seedling and vegetative plant tissues of *Arabidopsis* and tomato. The effect of nutrient stress on flavonol content could be tested on a vegetative crop plant such as cabbage or lettuce. It would be of interest to determine whether exposure to a short period of nutrient stress

could elevate flavonol levels without significantly reducing yield or resistance to pests and disease.

Studies with *Arabidopsis,* wild type and *hy4* mutant allowed a hypothesis relating to interactions between cold and UVA/blue light signal transduction to be proposed. These experiments could be extended to include other blue light mutants such as the related photoreceptor mutant *cry2,* or the double mutant *cry1/cry2.* In addition, an interaction was identified between the synergistic blue/UVA/cold reaction and blue/UVA/UVB synergistic induction of *CHS* transcript accumulation. It would be of interest to determine whether this interaction between two synergisms was additive or whether there was a further synergistic increase in *CHS* expression.

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