Isolation and characterisation of carotenoid biosynthetic genes from *Vitis vinifera*

by

Kerry Lyn Taylor
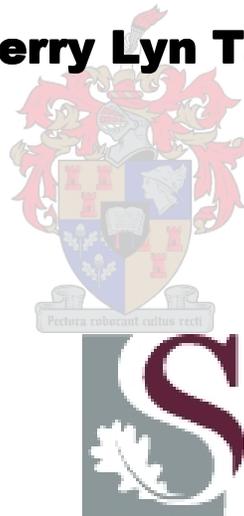
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| Promoter:	Co-promoter:
Prof. M.A. Vivier	Prof. V. R. Smith

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Kerry Taylor   9 March 2007

Kerry Lyn Taylor   **Date**
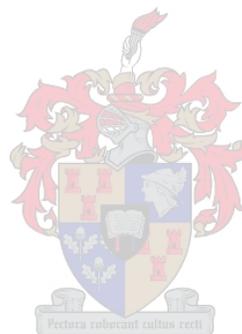
SUMMARY

Plants are constantly exposed to adverse environmental conditions including variations in light intensity and the availability of water resources. These abiotic factors are expected to worsen as the changing global climate places additional daily and seasonal demands on plant growth and productivity. As plants are incapable of avoiding stress they have developed a number of mechanisms to manage and adapt to the unfavourable conditions. Carotenoids represent one of these mechanisms; with the xanthophylls (oxygenated carotenes) playing an essential role in photoprotection following exposure to excess light energy. They are also precursors to the plant hormone abscisic acid (ABA) which plays a known role in stomatal regulation and thus drought tolerance. Carotenoids have been identified as potential targets for genetic manipulation to meet the existing nutritional demands (particularly vitamin A) and to enable plants to survive the climatic variations predicted. Thorough investigations into the regulation and functioning of each carotenoid biosynthetic gene *in vivo* as well as the roles of their encoded proteins are prerequisite.

Within the Grapevine Biotechnology Programme, a number of isoprenoid biosynthetic genes have been isolated from *Vitis vinifera* L. cv. Pinotage. From this vast resource two genes were chosen; namely a *lycopene β-cyclase (β-LCY)* and *9-cis epoxycarotenoid dioxygenase* (*NCED*) for detailed *in planta* analyses to address knowledge gaps in our current understanding of carotenoid biosynthesis in general, its regulation and the roles of the two target genes in these processes. Currently, the role of *β*-LCY within the chloroplasts is not well known. Although the relationship between *NCED* overexpression, ABA levels, reduced stomatal conductance and increased tolerance to water stress has been well-established, comprehensive physiological analysis of the resulting mutants during conditions of both water availability and shortage is not well documented. To assess their *in planta* role, functional copies of both genes were isolated from *Vitis vinifera* (cv. Pinotage), characterised and independently transformed into the genome of the model plant, *Arabidopsis thaliana*, in the sense orientation under a constitutive promoter.

In order to investigate these pertinent scientific questions and thus to evaluate the physiological role of each gene *in vivo*, a number of technologies were developed and/or adopted. These included a high-performance liquid chromatography method for profiling the major plant pigments in leaf tissue, a combination vapour phase extraction and electron impact-gas chromatography/mass spectrometry method for the phytohormone profiling as well as various physiological analyses including the use of chlorophyll *a* fluorescence to assess the photosynthetic and non-photochemical quenching (NPQ) capacities of the plants.

Overexpression of grapevine *β-LCY* (*Vvβ-LCY*) decreased lutein levels due to preferential partitioning of lycopene into the *β*-branch. This decrease was not met by an increase in either *β*-carotene or the xanthophyll cycle pigments implying that *Vvβ-LCY* is not able to regulate the flow of carbon through the pathway and provides additional evidence to the fluidity of this pathway whereby pigment levels are continually balanced. The decreased lutein levels observed under low light (LL) did not compromise the plants' ability to induce and maintain NPQ over a wide actinic light range. *Vvβ-LCY* transgenics also had lower

neoxanthin levels (and specifically the *cis*-isomer) under both LL and following exposure to high light (HL), which could be correlated to an increase in malondialdehyde. Although not corroborated, a novel and unexpected finding was an essential role for neoxanthin, and potentially lutein, in preventing or at least reducing lipid peroxidation under HL stress. The lower neoxanthin amounts may be due to silencing of the *Arabidopsis β-LCY* by the *Vvβ-LCY*, as the former may function as a NSY paralog as NSY is not encoded for in the *Arabidopsis* genome. Clearly, this study has confirmed that *Vvβ-LCY* partitions the carbon flux between the α- and β-branches, however, the catalytic action of this enzyme is dependent on the amount of substrate available and is thus not a regulatory step directing the flux within the pathway. Enzyme kinetic and detailed transcriptional analyses would confirm the above findings.

Overexpression of grapevine *NCED1 (VvNCED1)* increased ABA concentrations, delayed seed germination and resulted in a slight to severe reduction in the overall plant growth rate. NCED cleaves the 9-*cis* xanthophylls regulating ABA synthesis. However, contrary to expectations, constitutive levels of this regulatory enzyme did not deplete the total and individual chlorophylls and carotenoids in well-watered plants. Instead the *VvNCED1* transgenics simply exhibited a lower chloroplastic pigment complement with no concomitant effects on their photosynthetic capacity. Of particular interest, well-watered plants overexpressing the *VvNCED1* gene had an increased NPQ capacity of which the thermal energy dissipation component (qE) was the most significant. It has been speculated that this NPQ is associated with the phenotype conferred by *VvNCED1* overexpression and occurs independently of the xanthophyll cycle, and specifically zeaxanthin. This study confirmed that *VvNCED1* functions during drought tolerance via ABA regulation of stomatal conductance. A detailed study was done to understand the plants' response during water deficit. Typically, decreases in total and individual carotenoids and the maximum efficiency of photochemistry (F_v/F_m) as well as the relative water and soil moisture content were recorded. No changes were recorded in salicylic acid (SA) levels, while indole acetic acid (IAA) was positively correlated to ABA or vice versa. In contrast, the physiology of *VvNCED1* overexpressing lines was largely unaffected, indicating that a reduced stomatal conductance protects the plants against water stress.

This study has resulted in the isolation and characterisation of a carotenoid biosynthetic gene (*β-LCY*) and an abscisic acid synthesising gene (*NCED*). Significant advancements in our existing knowledge of the *in planta* role of both genes have been achieved. We have also reaffirmed that strict regulatory control and fluidity exists within the carotenoid biosynthetic pathway whereby individual pigment levels are constantly brought back into balance despite constitutive expression of one of the pathway gene members. These analyses provide valuable baseline information about individual genes which can be extended upon with other *omic* technologies in order to comprehend the full complexity involved in carotenogenesis.

OPSOMMING

Plante word voortdurend aan ongunstige omgewingstoestande, insluitend veranderinge in ligintensiteit en die beskikbaarheid van water, blootgestel. Daar word verwag dat hierdie abiotiese faktore meer omvattende negatiewe impakte sal hê soos veranderinge in die wêreldwye klimaat addisionele daaglikse en seisoenale eise aan die plantgroei- en ontwikkeling stel. Aangesien plante nie hierdie stresfaktore kan vermy nie, moes dit 'n aantal meganismes ontwikkel om by die ongunstige toestande aan te pas. Daarbenewens word landbou wêreldwyd met die verhoogde voedingseise van 'n konstant groeiende menslike populasie gekonfronteer. Karotenoïede is sentraal tot plante se weerstandsmeganismes teen abiotiese faktore, met die xantofiele (geoksideerde karotene) wat 'n kritiese rol speel in fotobeskerming na oorblootstelling aan ligenergie as goeie voorbeeld. Karotenoïedbiosintese vorm ook die voorlopers tot die planthormoon, absisiensuur (ABA), wat daarvoor bekend is dat dit huidmondjieregulering en droogtestresweerstand beïnvloed. Karotenoïede word dus geteiken in genetiese manipulasiestrategieë om in die bestaande voedingsbehoeftes te voorsien (veral vitamien A), asook om plante in staat te stel om by die klimaatsveranderinge wat voorspel word, aan te pas. In-diepte studies om die regulering en funksionering van elke karotenoïedbiosintesegeen, sowel as die funksies van die ge-enkodeerde proteïene te bepaal vorm 'n kritieke deel van die ondersteunende navorsing in die verband.

'n Verskeidenheid isoprenoïedbiosintetiese gene is reeds vanuit *Vitis vinifera* L. cv. Pinotage geïsoleer binne die Wingerdbiotegnologieprogram van die Instituut vir Wynbiotegnologie. Twee gene is gekies vir gedetaileerde *in planta* analises om sodoende bepaalde kennisgapings rakende die algemene begrip van karotenoïedbiosintese, die regulering daarvan, maar ook die rol van die twee gene in hierdie prosesse, te vul. Daar is ondersoek ingestel na die rol van 'n *likopeen-β-siklase* (*β-LCY*) binne die chloroplast, aangesien die rol van β-LCY in dié organel minder bekend is. Die rol van 'n *9-cis-epoksikarotenoïeddioksigenase* (*NCED*) onder waterstres en die gevolglike plantfisiologiese effekte is ook bestudeer. Die verwantskappe tussen *NCED* ooruitdrukking, ABA-vlakke, verminderde huidmondjiegeleiding en die gevolglike verhoogde droogteweerstand is reeds goed bekend, maar geen inligting rakende die verdere fisiologiese effekte in respons tot ooruitdrukking van die geen is beskikbaar nie. In hierdie studie is funksionele kopieë van beide wingerdgene onafhanklik ooruitgedruk in die genoom van die modelplant, *Arabidopsis thaliana*, deur gebruik te maak van 'n konstitutiewe promotor.

Die bestudering van die gene en veral hul *in vivo* rolle het genoodsaak dat sekere tegnologieë ontwikkel en/of geïmplementeer moes word vir hierdie studie. Dit sluit die volgende in: 'n HPLC-metode om profiele van plantpigmente in blaarweefsel te evalueer, 'n GC-MS analise vir die daarstelling van fitohormoonprofiele, sowel as verskeie fisiologiese analises, soos die gebruik van chlorofiel *a* fluoressensie om die fotosintetiese and nie-fotchemiese blussing (NPQ) vermoëns van die plante te bepaal. Ooruitdrukking van die wingerd *β-LCY* (*Vvβ-LCY*) het gelei tot verminderde luteïenvlakke, aangesien likopeen eerder in die β-vertakking van die pad beland het. Die vermindering het nie gelei tot 'n

gevolglike toename in β-karoteen of xantofielsikluspigmente nie, wat impliseer dat die geen nie die vloei van koolstof deur die pad kon reguleer nie. Dit bewys weereens dat die pad baie vloeibaar is deurdat pigmentvlakke voortdurend gebalanseer word. Met die uitsondering van neoxantien (en spesifiek die 9-*cis*-isomeer), was die pigmentvlakke (totale en individuele pigmente) van die *Vvβ-LCY* transgeniese lyne soortgelyk aan dié van die wildetipe (WT) na blootstelling aan hoë lig (HL). 'n Nuwe en onverwagte bevinding was dat neoxantien, asook luteïen, 'n potensiële rol in die regulering van lipiedperoksidase kan speel. Manipulering van die karotenoïedbiosintese by die punt van likopeensiklisering het nie die salisielsuur-(SA), indoolasynsuur- (IAA) of ABA-planthormoonprofiele beïnvloed nie. Daarby het β-LCY- ooruitdrukking nie die fotosintetiese en nie-fotochemiese blussingsvermoë (NPQ) verander nie. Hierdie studie het bevestig dat β-LCY die koolstofstroming tussen die β- en α-takke verdeel. Die katalitiese aksie van hierdie ensiem is egter afhanklik van die hoeveelheid substraat wat beskikbaar is en dit is dus nie 'n regulerende stap wat die stroming binne die pad bepaal nie.

Ooruitdrukking van *V. vinifera NCED1* (*VvNCED1*) het die ABA-vlakke verhoog, gelei tot vertraagde saadontkieming en 'n geringe tot ernstige vermindering in groeitempo veroorsaak. NCED splits die 9-*cis*-xantofiele wat ABA-opbou reguleer. In teenstelling met wat verwag is, het konstante hoë vlakke van hierdie regulerende ensiem nie die totale en individuele chlorofiel- en karotenoïedpoele uitgeput in plante sonder waterstres nie. Die *VvNCED1* transgeniese lyne het wel 'n laer chloroplastiese pigmentkompliment vertoon, met geen nadelige effekte op hul fotosintetiese vermoëns nie. Wat insiggewend was, is dat plante wat die *VvNCED1*-geen ooruitdruk 'n toename in NPQ-kapasiteit toon, waarvan die termiese energieverkwistingskomponent (qE) die beduidendste was. Daar word vermoed dat hierdie NPQ verbind kan word met die fenotipes wat deur *Vv*NCED1-ooruitdrukking veroorsaak word en dat dit onafhanklik van die xantofielsiklus voorkom, spesifiek ten opsigte van zeaxantien. Hierdie studie het ook bevestig dat *Vv*NCED1 tydens droogtestres en -weerstand *via* ABA- regulering van huidmondjiegeleiding funksioneer. 'n Omvattende studie is gedoen om die fisiologiese effek van watertekort op die plant te ondersoek. Tipiese aspekte wat waargeneem is, is die afnames in totale en individuele karotenoïede, die maksimum effektiwiteit van fotochemie (F_v/F_m) en die relatiewe inhoud van die water en grondvog. Geen veranderinge in SA-vlakke is waargeneem nie, terwyl IAA-positief met ABA gekorreleer het, en omgekeerd. In teenstelling hiermee, was die fisiologie van *Vv*NCED1-ooruitdrukkingslyne meestal nie beïnvloed nie, wat aandui dat verlaagde huidmondjiegeleiding die plante teen waterstres beskerm het.

Hierdie studie het tot die isolasie en karakterisering van 'n karotenoïedbiosintese- (*β-LCY*) en ABA-sintesegeen (*NCED*) gelei. Beduidende vooruitgang is gemaak in die begrip van die *in planta*-rol van albei gene. Die studie bevestig dat streng regulatoriese beheer en vloeibaarheid binne die karotenoïedbiosintetiese pad bestaan sodat individuele pigmentvlakke voortdurend in balans gebring word, self wanneer van die bydraende gene in die pad ooruitgedruk word. Die analises in dié studie verskaf waardevolle basisinligting per individuele geen waarop uitgebrei kan word met profieltegnologieë, soos *omics*-tegnologieë om uiteindelik die volle kompleksiteit van karotenogenese te verstaan.

This dissertation is dedicated to my family and friends!

BIOGRAPHICAL SKETCH

Kerry Lyn Taylor was born in Pietermaritzburg, South Africa on the 21st February 1974. She matriculated at Pietermaritzburg High School for Girls' in 1991. Kerry enrolled at the University of Natal (Pietermaritzburg) in 1992 and obtained a BSc degree (Biochemistry and Chemistry) in December 1994. A BSc Honours degree (Biochemistry, *cum laude*) was awarded in December 1995 and a MSc degree (Biochemistry; *cum laude*) in April 1998. In January 2001 she enrolled at Stellenbosch University for a PhD degree in Wine Biotechnology.
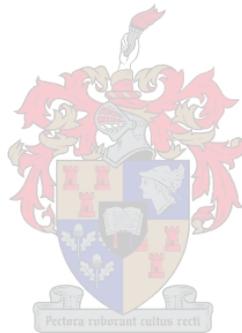
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PREFACE

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of Transgenic Research and Plant Biotechnology Journal to which Chapter 4 and 5 shall be submitted for publication. Chapter 3 has been published in the Journal of Chromatography A.

Chapter 1   **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2   **LITERATURE REVIEW**
Carotenoids: A role in light stress management and drought tolerance.

Chapter 3   **RESEARCH RESULTS**
High-performance liquid chromatography profiling of the major carotenoids in *Arabidopsis thaliana* leaf tissue.

Chapter 4   **RESEARCH RESULTS**
Investigations into the *in planta* role of grapevine β-LCY in *Arabidopsis*

Chapter 5   **RESEARCH RESULTS**
In planta functional analysis of *Vv*NCED1 from grapevine in *Arabidopsis*
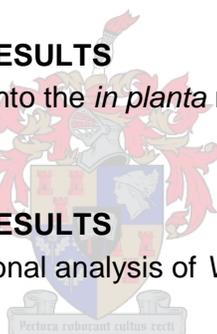
Chapter 6   **GENERAL DISCUSSIONS AND CONCLUSIONS**

I hereby declare that I was a co-contributor to a joined article by Me AE Brackenridge, a MSc student at the Institute for Wine Biotechnology, with respect to experiment planning and execution, data analysis, interpretation and problem-solving, and was principally responsible for writing up the published article (Chapter 3; with the exception of the Materials and Methods section). I was the primary contributor with respect to the experimental data presented on the multi-author manuscripts presented in Chapters 4 and 5.

Dr PR Young was involved in the development and execution of a "PCR/subgenomic approach" strategy for isolation of the gene encoding 9-*cis* Epoxycarotenoid dioxygenase from grapevine. Furthermore, he was solely responsible for the expression analyses reported in Chapter 5.

My supervisors Prof MA Vivier and Prof VR Smith were involved in the conceptual development of this study, and the continuous critical evaluation of the research taking place and of the resulting manuscript.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION
AND
SPECIFIC PROJECT AIMS

1.1   INTRODUCTION

Adverse environmental pressures, in the form of biotic and abiotic stress factors, have a negative effect on plant growth and productivity. Plants are exposed to daily and seasonal variations in temperature, light, salt and water availability in addition to challenges from a range of pathogens (Mahajan and Tuteja, 2005). To exacerbate this situation, world agriculture needs to get to grips with the challenges presented by the changing global climate and the mounting demands for increased, sustainable food production to satisfy the ever-growing human population (Climate Change: Synthesis Report, 2001; Guy *et al.*, 2006).

Plants have evolved a number of mechanisms to ensure survival and sustained growth under unfavourable growth conditions. Carotenoids represent one of these mechanisms; with the xanthophylls (oxygenated carotenes) playing an essential role in photoprotection (Young, 1991) and as precursors to the plant hormone abscisic acid (ABA; Seo and Koshiba, 2002). As such, carotenoids have been identified as potential targets for genetic manipulation to enhance the nutritional composition of food crops and to enable plants to cope with some of the extreme climatic variations predicted. These potential biotechnological benefits rely on fundamental knowledge of the biosynthesis and roles of carotenoids in plants. To this end detailed investigations into the regulation and functioning of carotenoid biosynthetic genes, as well as the evaluation of the *in vivo* roles of the encoding proteins are prerequisite and extremely valuable.

1.2   THE STUDY OF CAROTENOID BIOSYNTHESIS

The carotenoid biosynthetic pathway in photosynthetically active organisms has largely been elucidated with a clear understanding of the committed steps and responsible enzymes in this pathway. Several of the individual enzymes have been studied in detail, revealing substrate and co-factor specificities (reviewed by Cunningham and Gantt, 1998; Sandmann, 2001, 2006). The metabolites formed within this pathway have also been studied and functionally assigned to key metabolic and physiological processes in plants (Laule *et al.*, 2003), confirming this pathway to be of central importance in plant metabolism. Some of the functions of carotenoid pigments and down-stream products have been mentioned previously (section 1.1) and an ever increasing number of publications on these topics are rapidly expanding our knowledge on carotenoids in general, but most notably the contribution of individual pathway members towards these functions (Arvidsson et al., 1996; Cunningham *et al.*, 1996; Marin *et al.*, 1996; Sun *et al.*, 1996; Bartley *et al.*, 1999; Welsch *et al.*, 2000; Schwartz *et al.*, 2001; Fraser and Bramley, 2004).

A number of studies have been conducted whereby carotenoid biosynthetic genes isolated from various plant species have been homologously overexpressed in the host plant or heterologously introduced into a model plant (Fray *et al.*, 1995; Rosati *et al.*, 2000; Estévez *et al.*, 2001; Dharmapuri *et al.*, 2002; Fraser *et al.*, 2002; Römer *et al.*, 2002; Ravanello *et al.*, 2003; Paine *et al.*, 2005). These investigations and

others, along with the availability of several characterised *Arabidopsis* mutant lines, have provided additional invaluable insight into the regulation and functioning of some of the genes within the pathway. Most notably, carotenoid biosynthesis was revealed to be a fluid pathway with multiple checks and balances to the extent that alteration of a single enzyme frequently does not amount in the accumulation of its reaction product (reviewed by Fraser and Bramley, 2004). Instead considerable interplay within the pathway, due to multi-enzyme aggregates (Cunningham and Gantt, 1998), and between related pathways (Laule *et al.*, 2003) exists with unpredictable results (reviewed by Sandmann *et al.*, 2006).

Within the Grapevine Biotechnology Programme at the Institute for Wine Biotechnology, we have isolated 31 carotenoid biosynthetic genes from *Vitis vinifera* L. cv Pinotage and are currently analysing various aspects of this important pathway in grapevine. In addition, the *in planta* role of individual pathway members are being determined in model plants. The current study forms part of this research programme and focuses on two specific genes from the isoprenoid pathway of grapevine; namely lycopene β-cyclase (β-LCY) and *9-cis epoxycarotenoid dioxygenase (NCED)*. *Vitis vinifera* L. is a woody fruit-bearing perennial with a structure and morphology which differs considerably from that of model plants such as *Lycopersicon esculentum* (tomato) and *A. thaliana* (Driesel *et al.*, 2003). As such, the overriding goal of this study is to elucidate the *in planta* role of these genes and the strategy involves constitutive overexpression in the model plant *A. thaliana*, with subsequent and comprehensive analyses of the transgenic populations. These analyses are aimed at addressing knowledge gaps in our current understanding of carotenoid biosynthesis in general, its regulation and the roles of the two target genes in these processes. These gaps and the specific aims identified to address them are outlined in the following section.

1.3   SPECIFIC PROJECT AIMS

With respect to the regulation and roles of both grapevine β-LCY and *NCED* as well as the effects of overexpressing these genes on general metabolism and overall plant physiology, the following gaps exist in our understanding:

- The role of chromoplastic β-LCY is well established and has been successfully manipulated to increase the nutritional content of fruit-bearing species due to the accumulation of β-carotene. Yet, the role of β-LCY within the chloroplasts is not well known. Although β-LCY has been shown to regulate the partitioning of substrate at the lycopene bifurcation point, its ability to regulate the flux into the β-branch during optimal plant functioning and during light stress is unknown; and

- A number of *NCEDs* have been isolated from a wide range of plant species including tomato (Burbidge *et al.*, 1997), *Arabidopsis* (Tan *et al.*, 2003) and peanut (Wan and Li, 2006). Frequently, overexpression of this gene in model plants has rendered the resulting mutant lines to be drought tolerant, but

comprehensive physiological analyses of these transgenics during conditions of both water availability and shortage are not well documented.

To achieve these shortfalls in our current knowledge the following specific project objectives were formulated:

i. The independent cloning of the isolated full length sequences of both *β-LCY* and *NCED*, in the sense orientation, into the plant transformation vector pART27 under the constitutive cauliflower mosaic virus (CaMV) promoter and the octopine synthase terminator. The independent introduction of each of the heterologous grapevine genes into the genome of the model plant (*Arabidopsis*) via floral dipping will follow;

ii. The establishment of stable independent transgenic populations (T_4 generation) for each introduced transgene following integration (Southern hybridisation) and expression (northern hybridisation) analyses;

iii. The development, validation and implementation of a number of techniques for analyses of the physiological effects of the introduced grapevine gene(s). This will include a high performance liquid chromatography (HPLC) profiling method for quantification of the major carotenoids in the leaf tissue (Chapter 3), a combination vapour phase extraction (VPE) protocol and electron impact-gas chromatography/mass spectrometry (EI-GC/MS) separation and quantification method for profiling three plant phytohormones (Chapter 4 and 5), as well as a number of chlorophyll *a* fluorescence protocols for measuring the photosynthetic capacity and non-photochemical quenching (NPQ) abilities of the transgenic plants (Chapter 4 and 5);

iv. The effects of *β-LCY* transgene overexpression on plant phenotype, and the carotenoid, chlorophyll and phytohormone profiles of plantlets grown under ambient growth conditions (low light; LL) and after being challenged by light available in excess (high light; HL) will be established. Under these conditions the degree of lipid peroxidation indicative of damage to the thylakoid membranes will also be quantified. In addition, the photosynthetic and NPQ of chlorophyll *a* fluorescence capacities will be determined over a range of light intensities.

v. Similarly, the effects of *NCED* transgene overexpression on general plant morphology, and the carotenoid and chlorophyll profiles of plantlets grown under LL and following exposure to HL will be determined as well as the influence of the transgene on the plantlets ability to use light energy for photosynthesis and dissipate any excess energy non-radiatively;

vi. The *NCED* transgenics will also be monitored under conditions where water is available or is limiting. A detailed physiological examination of the chlorophyll, carotenoid and phytohormone profiles, the resulting stomatal conductance, as well as any effects on lipid peroxidation and the maximum quantum yield of

photosystem II (PSII) will be conducted in those plants displaying potential drought tolerance and/or susceptibility; and

vii. In all instances WT control plantlets will be included and used as a benchmark to evaluate the transgenic populations.

The data acquired from these investigations will serve invaluable in contributing to our fundamental knowledge currently available regarding the regulation and function(s) of *β-LCY* and *NCED in vivo.* Additionally, information regarding the metabolites synthesised under the action of these gene products will be made available, bearing in mind that carotenoid metabolism is a complex fluid pathway. Furthermore, this study will contribute to the functional analysis of the two genes from grapevine and aid in evaluating them as potential targets which ultimately may play a role in our long-term objective of genetic improvement of grapevine.

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CHAPTER 2

LITERATURE REVIEW

CAROTENOIDS: A ROLE IN LIGHT STRESS MANAGEMENT AND DROUGHT TOLERANCE

2.1 INTRODUCTION – PLANTS, CAROTENOIDS AND THE CHANGING GLOBAL CLIMATE

Extremities in light irradiation and variations in water accessibility and availability have been identified as the most important ecological constraints influencing plant growth, survival and productivity globally (Türkan *et al.*, 2005). Plants have evolved a number of enzymatic and non-enzymatic protective mechanisms to avoid or tolerate these environmental stress factors on a day-to-day basis (Jung, 2004). Combined with these existing challenges, global climate change presents a dilemma of intangible proportions. The following statement was released by the U.S. Geological Survey and the U.S. Fish and Wildlife Service (Future Challenges Project, 2004). "Changes in atmospheric composition (especially increased concentrations of greenhouse gases) have the potential to alter the radiative balances of the earth's atmosphere, so changing regional and global climates and affecting natural flows of energy and materials underpinning ecosystem processes". The World Wildlife Foundation (WWF) (www.panda.com) has forecast that the most threatening changes range in extremes from drought to severe floods and altered seasons. Amongst the envisaged consequences of these changes, modifications to vegetation type and structure and a severe reduction in plant distribution and biodiversity have been highlighted (Inouye, 2000). This situation is further exacerbated when the nutritional demands of an ever-growing human population are considered (Guy *et al.*, 2006).

Concern has been expressed as to whether the existing plant defensive mechanisms are adequate to survive the additional stresses posed by accelerated global warming. Within the timescale predicted for climatic change, an evolutionary adaptive response is not a feasible option. Although controversial, genetic engineering has been recognised as the most practical alternative, in which a plants natural ability to cope with unfavourable environmental conditions is enhanced.

Carotenoids have been selected as potential targets to generate plants that are tolerant to environmental stresses and have an increased nutritional content (Sandmann *et al.*, 2006). This has necessitated detailed investigations into the functioning and regulation of each gene within the carotenoid biosynthetic pathway and interactions with other pathways. This review will make mention of the role(s) of lycopene β-cyclase (β-LCY), due to its integral position in the pathway catalysing β-carotene formation (the precursor to vitamin A and the photoprotective xanthophylls), and 9-*cis* epoxycarotenoid dioxygenase (NCED), the rate-limiting step in the synthesis of the phytohormone abscisic acid (ABA) which plays a known role in mediating drought tolerance. Within the framework of photosynthesis and carotenoid biosynthesis, current knowledge regarding the functioning and regulation of each gene and in particular β-LCY and NCED and their reaction products will be briefly discussed, specifically in the context of light stress management and drought tolerance.

2.2 PHOTOSYNTHESIS, A MEANS OF GENERATING CARBON ENERGY

2.2.1 Photosynthetic apparatus

In photosynthetic eukaryotes, photosynthesis takes place within the chloroplasts, with the light reactions proceeding in the thylakoid membranes. These membranes contain two reaction centres (RCs), namely photosystem I (PSI) and photosystem II (PSII), and their associated antenna pigment-protein complexes. The PSII core comprises six chlorophyll a's (chl a), two pheophytins, (Phe) two quinines (Q_A and Q_B) two β-carotenes and one nonheme iron bound to a pair of hydrophobic D1 (psbA) and D2 (psbD) membrane polypeptides (Green and Durnford, 1996; Horton *et al.*, 1996). Assembly of the transmembrane α-helices of, at least, the D1 polypeptide requires β-carotene (Tracewell *et al.*, 2001) which strategically plays a role as a potent antioxidant (Frank and Brudvig, 2004). The crystal structure of the antenna pigment-protein complexes, also know as the light-harvesting complexes (LHCs), has been resolved at 2.72 Å (Liu *et al.*, 2004). Within this complex the associated pigments are chl a and b (Jansson, 1994) as well as the xanthophylls, lutein, neoxanthin and violaxanthin (Young, 1991; 1993) which are responsible for harvesting light energy and delivering it rapidly, efficiently and irreversibly, via electron exchange (Mimuro and Katoh, 1991) or resonance transfer (Förster, 1967), to the RCs for photochemistry. These pigments are bound non-covalently to two strongly hydrophobic polypeptides, CP43 (psbC) and CP47 (psbB), which are tightly associated with the D1 and D2 proteins, respectively (Boekema *et al.*, 2000; Gómez and Chitnis, 2000). These polypeptides are typically hydrophobic and are responsible for preserving the strict positional requirements of these pigments ensuring optimal performance efficiency (Tracewell *et al.*, 2001). Lutein and β-carotene are reported to be unevenly distributed between the PS's as they are preferentially ubiquitously present in PSII and PSI, respectively (Demmig-Adams *et al.*, 1996). Although they carry out the antagonistic function of excess light dissipation, zeaxanthin and antheraxanthin are also found associated with the LHC's. The cytochrome b_6f complex is evenly distributed across the thylakoid membranes connecting the two RCs, PSII and PSI (Govindjee, 2000).

2.2.2 Photosynthesis overview

Incident light is harvested by an array of antennae pigments with the chlorophylls and carotenoids absorbing strongly in the red and green-blue visible regions of the spectrum, respectively (Liu *et al.*, 2004). This energy is rapidly transferred in a multiple step energy transfer to the photosynthetic RCs. In the PSII RC, oxidation of chlorophyll proceeds and an electron is pulled from a nearby tyrosine residue of the D1 polypeptide and a further electron is generated via the water-splitting complex (Tracewell *et al.*, 2001). Electrons are then transported along a cascade from PSII to Phe and subsequently to Q_A and Q_B which in turn transfers the electrons to the cytochrome b_6f complex via plastoquinone. Similarly, light is harvested by the antenna pigments in PSI, the light energy is transferred to chlorophyll and the

generated electrons are targeted for reduction of $NADP^+$ (Vermaas, 1998). Subsequently a proton gradient is generated across the thylakoid membrane and is used for the synthesis of ATP. It is quite clear that photosynthesis depends on rapid, efficient energy transfer and maintenance of a redox state where the various acceptors are in the correct conformation for receiving electrons (Frank and Brudvig, 2004).

Environmental stress is known to reduce the overall rate and efficiency of photochemistry and hence the amount of carbon energy generated which is required for general plant functioning, growth and overall productivity. Carotenoids are integral components of the photosynthetic membranes, to the extent that the inability of a plant to synthesise these pigments has potentially lethal effects (Cunningham and Gantt, 1998). Within any living system, strict control mechanisms are in place to guarantee sustained synthesis and regulation of any vital components. The carotenoid biosynthetic pathway and its individual members are no exception.

2.3   CAROTENOID BIOSYNTHESIS AND REGULATION

2.3.1 Carotenoid biosynthesis: structure confers function

2.3.1.1   *Carotenoid biosynthetic enzymes*

The first carotene or hydrocarbon carotenoid was isolated from carrot roots in 1831 by Wachenroder and, in 1837, Berzelius reported the first xanthophyll or oxygen-containing carotene from senescent leaves (Armstrong and Hearst, 1996). Over the years, carotenoids have become recognised as being the most diverse and widespread group of natural pigments found in nature (Bartley and Scolnik, 1994).

Carotenoids are lipid-soluble pigments which comprise a vast number of products within the isoprenoid biosynthetic pathways, all of which originate with the universal biological precursor, isopentenyl diphosphate (IPP; Spurgeon and Porter, 1983). Two distinct isoprenoid biosynthetic routes have been identified: the acetate/mevalonate (MVA) pathway responsible for the formation of cytoplasmic sterols and the glyceraldehyde phosphate/pyruvate phosphate, also known as the MVA-independent or deoxyxylulose phosphate (DXP; Lange *et al.*, 2000) pathway resulting in the chloroplast-bound isoprenoids (Lichtenthaler *et al.*, 1997). Despite considerable crosstalk between the two pathways (Eisenreich *et al.*, 2001; Laule *et al.*, 2003), it is the latter which is of greater interest to us as it results in, amongst others, the chlorophylls, carotenoids and ultimately the phytohormone abscisic acid (Fig. 2.1).
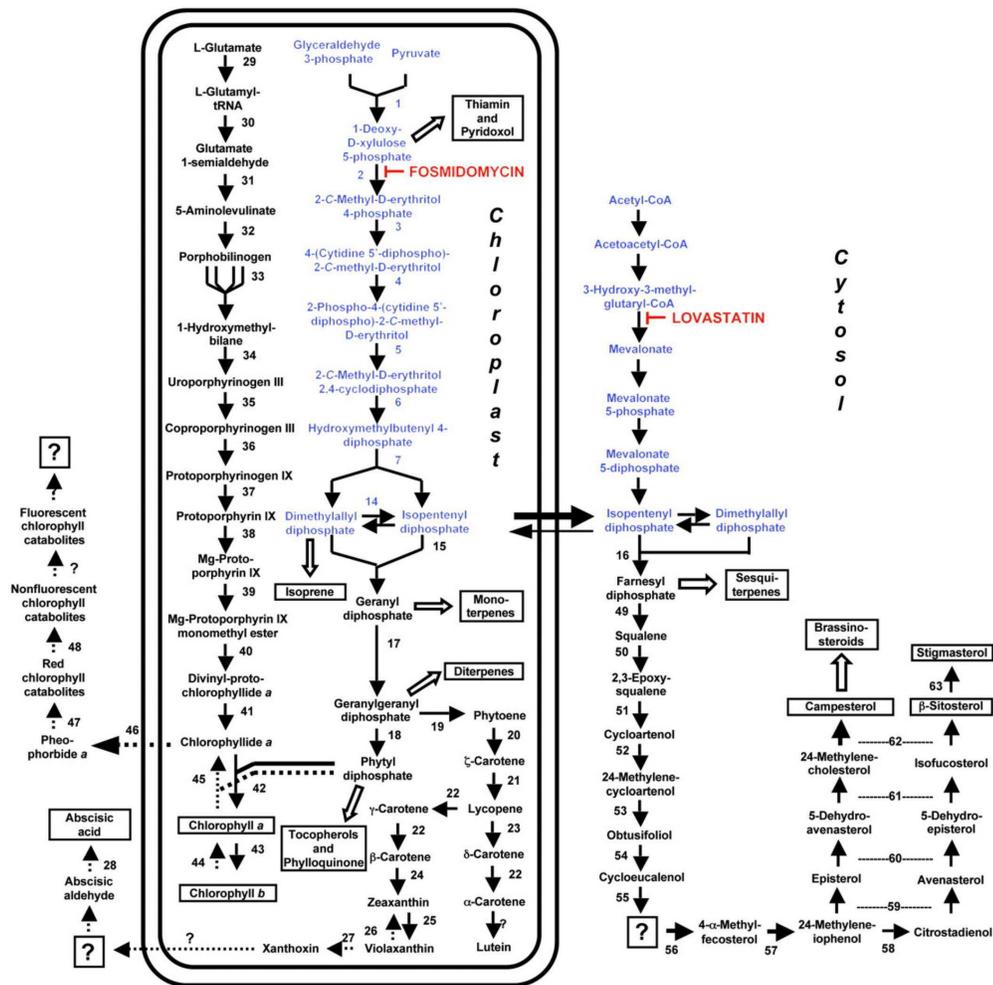
Figure 2.1. Schematic representation of the crosstalk within the chloroplasts and in the cytosol during isoprenoid metabolism.

Numbers corresponding to those enzymes directly involved in carotenoid metabolism are explained. 14 – IPP isomerase; 15 – geranyl pyrophosphate synthase; 16 – farnesyl pyrophosphate synthase; 17 – geranylgeranyl pyrophosphate synthase; 19 – phytoene synthase; 20 - phytoene desaturase; 21 – ζ-carotene desaturase; 22 – lycopene β-cyclase; 23 – lycopene ε-cyclase; 24 – β-carotene hydroxylase; 25- zeaxanthin epoxidase; 26 – violaxanthin deepoxidase; 27 – 9-*cis* epoxycarotenoid dioxygenase; 28 – abscisic acid aldehyde (adapted from Laule *et al.*, 2003).

Carotenoid biosynthetic enzymes are nuclear-encoded, translated as precursors and imported post-translationally into the plastids (Bartley and Scolnik, 1994). There they are responsible for directing the assembly of the C_{40} carbon skeleton following tail-to-tail condensation of two molecules of the C_{20} compound geranylgeranyl pyrophosphate (GGPP), which is composed of four IPP units (Cunningham and Gantt, 1998). Formation of the final GGPP product is sequentially under the control of an IPP isomerase (IPI), a geranyl pyrophosphate synthase (GPS), a farnesyl pyrophosphate synthase (FPS) and finally a geranyl geranyl pyrophosphate synthase (GGPS). Typically, the C_{40} carbon backbone of carotenoids has between 3-15 conjugated double bonds. The number and position of these double bonds within the

chromophore are responsible for the characteristic colour and the photochemical properties of the individual carotenoid (Britton, 1988).

The colourless, symmetrical C_{40} carotene phytoene is formed in a two-step reaction regulated by phytoene synthase (PSY; Schmidhauser *et al.*, 1994). Phytoene production is recognised as the first committed step in carotenogenesis and is a key regulatory point in carotenoid synthesis (Cunningham, 2002). All PSY's are membrane-associated to enable delivery of phytoene to the chloroplast membranes and require ATP and Mn^{2+} or Mg^{2+} for activity (Fraser and Bramley, 2004). PSY is typically encoded by a single gene, however, two *PSY*s were found in tobacco (Busch *et al.*, 2002) and tomato (Bartley and Scolnik, 1993).

Colourless phytoene undergoes a series of desaturation reactions catalysed by phytoene desaturase (PDS; Bartley *et al.*, 1991) and ζ-carotene desaturase (ZDS; Linden *et al.*, 1994) thereby lengthening the carbon double-bonded series yielding the final red-coloured lycopene product. Desaturation is connected to the photosynthetic electron transport chain via plastoquinone which accepts the removed hydrogen molecule (Sandmann *et al.*, 2006). All PDS and ZDS genes contain a conserved dinucleotide (FAD/NADP) binding site domain, however, homology on an amino acid level is only in the range of 33-35% (Fraser and Bramley, 2004).

The subsequent cyclisation of the linear lycopene is an important branch point in the carotenoid biosynthetic pathway yielding two types of carotenes: α-carotene, which has one β-ring and one ε-ring, and orange β-carotene, where the introduction of two β-rings is regulated by β-LCY (Cunningham *et al.*, 1996; Fig 2.1). As mentioned previously, β-carotene is a precursor for vitamin A synthesis and functions as a potent antioxidant protecting chlorophyll in the RCs. Typically β-LCY and ε-LCY are each present as single copy genes, are 30% homologous on an amino acid level and both have a characteristic dinucleotide (FAD/NADP) binding sequence motif.

Hydroxylation of the hydrocarbons α-carotene and β-carotene by β-carotene hydroxylase (BCH) produces the xanthophylls lutein and zeaxanthin (Hundle *et al.*, 1993), respectively. This enzyme is integrally situated within the thylakoid membranes and contains a number of conserved histidine residues.

The epoxidation of yellow-coloured zeaxanthin to violaxanthin proceeds under the action of zeaxanthin epoxidase (ZEP) via an antheraxanthin intermediate. This reaction is reversible under high light resulting in the phenomenon known as xanthophyll cycling following the induction of violaxanthin deepoxidase (VDE). The xanthophyll cycle pigments are integrally involved in the dissipation of excess light energy and hence play a central role in light stress management. Single copies of both ZEP and VDE have been found in the genome of a number of plant species and have been shown to require ferredoxin (Bouvier *et al.*, 1996) and ascorbate (Rockholm and Yamamoto, 1996), respectively, for activity.

Finally, neoxanthin synthase (NSY) converts violaxanthin into neoxanthin, the 9-*cis* geometrical isomers of which are cleaved by NCED to form xanthoxin, the immediate precursor to ABA (Schwartz *et al.*, 1997; Seo and Koshiba, 2002). Five

NCED members have been identified in *Arabidopsis* along with four closely related carotenoid cleavage dioxygenases (CCDs). NCED has been unquestioningly established to be the rate-limiting step in ABA formation. As ABA has been shown to play a role in mediating a plant's response to water stress, a positive correlation has been found between *NCED* expression and subsequent protein activity and drought tolerance. It is clear that most reactions within the carotenoid biosynthetic pathway have been conclusively established, however, thus far *NSY* has only been found in the genome of two plant species, potato and tomato. It is postulated that a bi-functional β-LCY instead operates in lieu of NSY under certain conditions (Fraser and Bramley, 2004). Furthermore, it is believed, though not yet proven, that a *cis*-isomerase converts the *trans*-forms of violaxanthin and neoxanthin into their *cis*-isomers.

2.3.1.2 Carotenoids in chloroplasts

Carotenoids have three primary essential *in vivo* functions. Firstly, they are important structural and light-harvesting components of the RCs and LHCs anchoring integral membrane proteins and extending the light absorption spectrum into the blue-green region. This function is mediated by their extended carbon backbone which is stabilised by the conjugated double bond system. Secondly, they quench excess light energy via a myriad of mechanisms, thereby protecting the components of the thylakoid membrane from lipid peroxidation. The conformation of β-carotene makes it a powerful antioxidant while zeaxanthin's structural arrangement makes it optimal for excess light energy dissipation (see section 2.4.5). Finally, they are precursors to the plant hormone abscisic acid (Rock and Zeevaart, 1991; Chernys and Zeevaart, 2000), which plays a role in co-ordinating plant responses to external stress factors (Finkelstein and Rock, 2002; Seo and Koshiba, 2002; Xiong and Zhu, 2003), regulating seed maturation and primary dormancy as well as influencing fruit development (Zeevaart and Creelman, 1988).

2.3.1.3 Carotenoids in chromoplasts

In chromoplasts, carotenoids are considered to be secondary metabolites contributing towards many of the bright colours of fruits and flowers (Bartley and Scolnik, 1995), responsible for attracting insects and birds for pollination. These brilliant red, yellow and orange colours are due to the absorption of light by chromophores of seven or more conjugated double bonds (Bartley and Scolnik, 1994) and can primarily be attributed to the accumulation of lycopene, β-carotene and zeaxanthin, amongst others. The brilliant colouring of some birds, insects and marine invertebrates are also due to accumulated carotenoids derived from carotenoids in their diet.

2.3.2 Regulation of carotenoid biosynthesis

Spatial, temporal, and environmental regulation of the carotenoid biosynthetic pathway is a stringently controlled process and as such is still not completely understood on either a genetic or enzymatic level (Sandmann *et al.*, 2006). From research into genetically modified increases or decreases of a specific intermediate it is clear, however, that robust feedback control mechanisms exist which compensate for these engineered changes ensuring that the total carotenoid content remains largely unchanged (Pogson *et al.*, 1996, 1998; DellaPenna, 1999; Pogson and Rissler, 2000; Rissler and Pogson, 2001). Regulation within the chloroplasts and the chromoplasts is distinctly different. Within the chromoplasts, developmental regulation is strongly controlled on a transcriptional level with minimal flexibility. In contrast, chloroplastidic regulation of carotenogenesis is primarily light-dependent with regulation proceeding largely on both a transcriptional and translational level and to a lesser extent via end product feedback.

2.3.2.1 *Regulation in chloroplasts*

Light plays a central role during the regulation of carotenoid synthesis in photosynthetic tissues. During de-etiolation of seedlings a light-induced, phytochrome-mediated response up-regulates chlorophyll biosynthesis (Matters and Beale, 1995; Von Lintig *et al.*, 1997) and induces the transcription of *PSY* causing carotenoid accumulation (Bohne and Linden, 2002). *PDS* and *GGPS* transcript levels remain constant. During the lifetime of an individual plant, changes in incident light intensity have the potential to cause photooxidative damage to the extent that carotenoid degradation may exceed biosynthesis. To circumvent this problem, basal levels of carotenoids are maintained. Furthermore, the relative amounts/activities of the lycopene cyclases (β-LCY and ε-LCY) have been shown to account for the partitioning of substrate between the α- and β-branches (Fig. 2.1; Cunningham and Gantt, 1996); thereby selectively producing the photoprotective xanthophylls (specifically zeaxanthin) under a stress condition (Demmig-Adams and Adams, 1992). It remains to be shown whether β-LCY regulates a rate-limiting step controlling the amount of lycopene available for cyclisation. However, some experimental evidence is available that lycopene levels are mediated upstream by PSY and/or PDS (Misawa *et al.*, 1994; Romer *et al.*, 2002).

Xanthophyll synthesis (through *BCH* and *ZEP* expression) has been shown to be coordinated with the formation of the antenna complexes while *VDE* regulation appears to be under post-translational control (Woitsch and Römer, 2003). Expression levels of these three genes are also phytochrome regulated. End product regulation of the *PDS* promoter has been eluded towards most likely by β-carotene, the xanthophylls or ABA (Corona *et al.*, 1996).

Regulation is also exerted by metabolic interaction within and between pathways. Overexpression of *PSY*, which has been shown to control the precursor flux into the carotenoid pathway, depleted the GGPP pool thereby decreasing the availability of

gibberellins (Hemmerlin *et al.*, 2003; Laule *et al.*, 2003). It is thus clear that if the precursor pool is increased, the total amount of carotenoids will increase. Similarly deoxyxylulose phosphate synthase (*DXS*) overexpression increased the precursor pool (Estévez *et al.*, 2001) whereas the combined up-regulation of *DXS* and 3-hydroxy-3-methylglutaryl-CoA-synthase (*HMGR-CoA)* depleted the precursor and hence total carotenoid pool (Enfissi *et al.*, 2005).

2.3.2.2 *Regulation in chromoplasts*

Chromoplastic regulation of carotenoid biosynthesis differs from that in the chloroplasts, with transcriptional regulation of gene expression and the presence of sequestering storage structures accounting for the carotenoid accumulation which occurs (Howitt and Pogson, 2006). Tomato ripening is typically used as a model system to describe plastid differentiation and the associated increase in pigment levels. During the green stages of fruit development, the carotenoid complement closely resembles that of green leaf tissue. However, at the 'breaker' stage of ripening, *PSY* and *PDS* are upregulated while transcript levels of β-*LCY* and ε-*LCY* decrease drastically. This results in an accumulation of lycopene and a concomitant colour change from green to orange (Giuliano *et al.*, 1993; Bramley, 1997, 2002). It has recently been established that these alterations in expression observed are controlled by phytochrome following a light signal and that these light receptors are simultaneously capable of post-translational modification of the levels of enzyme activity (Scholfield and Paliyath, 2005). This phenomenon has also been observed in other fruit-bearing species (Römer *et al.,* 1993; Aggelis *et al.*, 1997; Ikoma *et al.*, 2001) and it is reportedly the same mechanism that operates in flowers (Pecker *et al.*, 1996; Schledz *et al.*, 1996; Moehs *et al.*, 2001) and anthers (Wiermann and Gubatz, 1992). Hence carotenoid accumulation in chromoplasts is for the most part developmentally regulated at the level of transcription with activation of the functional enzyme and feedback inhibition playing a minor role (Ronen *et al.*, 2000). Once the increased levels of carotenoids have been achieved, an extensive network of lipoprotein structures (Vishnevetsky *et al.*, 1999a and b) exist which bind carotenoids hydrophobically serving as a vast storage pool.

2.4 CAROTENOIDS: A PARADOXICAL ROLE IN LIGHT HARVESTING AND EXCESS LIGHT DISSIPATION

The PSII machinery faces two conflicting demands. On the one hand the efficient harvesting of light energy is required to generate electrons to drive photochemistry, but on the other hand carotenoids play a central role in protecting the photosynthetic apparatus from harm imposed by adverse environmental factors, predominantly via zeaxanthin, antheraxanthin and β-carotene (Horton and Ruban, 2005).

2.4.1 Harvested light energy has one of three fates

Plants are constantly subjected to light of erratic intensity over several orders of magnitude with changes taking place within seconds and between seasons. A number of biochemical and developmental responses exist in order to optimise plant growth and photosynthesis. These include regulation of the size of the LHC's through a combination of gene expression and proteolysis and adjustment of the leaf position and chloroplast movements to maximise or minimise the light harvesting capacity (Brugnoli and Björkman, 1992).

It is quite common that light energy is available and is absorbed in excess of what can be utilised for photosynthesis, hence the plant needs a rapid protective mechanism to avoid potential irreversible damage. Typically harvested light energy is transferred to the chlorophylls within the RC's promoting them from the ground state to a single excitation state (^{1}Chl*). This energy subsequently faces one of three fates where each process is in competition with the others: it may be used for photochemistry; it may be re-emitted as light (chlorophyll fluorescence); and it may be non-photochemically quenched (NPQ) as heat (Maxwell and Johnston, 2000; Muller et al., 2001).

2.4.2 Oxidant and antioxidant signalling in plants

Triplet excited state chlorophyll (^{3}Chl*) can combine with molecular oxygen, forming reactive singlet oxygen species (ROS) with the capacity to cause irreversible damage to the component proteins, lipids and pigments of the thylakoid membranes (Demmig-Adams et al., 1996; Horton and Ruban, 2005). The ability of carotenoids (and in particular β-carotene) to quench ^{3}Chl*, thereby circumventing any likely danger makes them indispensable in photosynthetic membranes (Frank and Brudvig, 2004). Quenching takes places via an electron exchange (Kühlbrandt et al., 1994) or charge transfer mechanism (Dreuw et al., 2003). It has been estimated that between 4% and 25% of photons absorbed can be dissipated from ^{3}Chl* (Niyogi, 2000) in a mechanism that is classified as an unregulated, constitutive process. Regardless of the efficient mechanisms in place, it is inevitable that damage to the photosynthetic RCs will occur, resulting in inactivation and necessitating protein turnover and RC repair (Aro et al., 1993).

Extremely high levels of ROS are deadly, however it has recently become clear that sublethal oxidant levels have a crucial role to play in redox sensing and signalling in combination with antioxidants (Foyer and Noctor, 2003, 2005; Ledford and Niyogi, 2005). Although oxidation of integral membrane components contributes to reduced plant vigour, oxidation of signal molecules is essential during plant perception and induction of a defense response to a variety of environmental and developmental elicitors. Amongst the diverse processes activated by ROS include gene expression, stomatal closure, root growth and programmed cell death (Wagner et al., 2004) especially during a hypersensitive response. During these processes a strong interaction exists between the causative oxidant and the responding

antioxidant, typically glutathione, ascorbate, α-tocopherol and, of course, carotenoids (Arora *et al.*, 2002).

2.4.3 Overview of NPQ or thermal energy dissipation mechanisms

As an alternative to the formation of ROS, excess energy can be safely dissipated as heat during rapidly-inducible NPQ or thermal energy dissipation. Basically, this process involves transfer of excess energy from ^{3}Chl* following an interaction with zeaxanthin (Demmig *et al.*, 1987; Niyogi *et al.*, 1998; Njyogi, 2000; Ma *et al.*, 2003; Holt *et al.*, 2005; Niyogi *et al.*, 2005). However, this mechanism has proven to be far more complex than initially anticipated and the actual role of zeaxanthin in NPQ has not been unequivocally established. This is dealt with in section 2.4.5.

NPQ has a number of components, classified according to their speed of response and their state of prolonged response. Firstly, flexible thermal energy dissipation or qE is activated within minutes and is directly related to the conversion of violaxanthin to zeaxanthin within the xanthophyll cycle (Gilmore and Yamamoto, 1992; Horton and Ruban, 1992; Pfundel and Bilger, 1994; Demmig-Adams and Adams, 2006). This comprises the most prevalent component of NPQ especially in wild type (WT) *Arabidopsis* (Li *et al.*, 2000). Secondly, under prolonged environmental stress conditions a pH-independent sustained thermal energy dissipation (qI) kicks into action involving zeaxanthin but not dependent on its formation. Although, qI is mechanistically similar to qE, it is associated with photoinhibition of photosynthesis and is independent of the lumen pH (Niyogi, 2000). Finally, a third related form of NPQ has been discovered recently which is sustained for long periods in the dark and is discussed in section 2.4.6.

2.4.4 *Arabidopsis* mutants: insight into flexible thermal energy dissipation

The creation of a number of xanthophyll or lutein deficient mutants has provided an invaluable *in vivo* tool for gaining a greater insight into the physiology, biochemistry and structural arrangement of PSII and the associated photosynthetic and photoprotective processes.

2.4.4.1 *npq1*

This mutant is incapable of de-epoxidating violaxanthin to zeaxanthin and hence is devoid of the *VDE* gene. Interestingly, this mutant is only partially defective in NPQ, establishing a firm requirement for deepoxidation of violaxanthin to antheraxanthin and zeaxanthin, but indicating that there is a component of NPQ which is independent of the xanthophyll cycle (Niyogi *et al.*, 1997; 1998). Two theories exist for the apparent xanthophyll cycle-independent NPQ observed. Residual amounts of zeaxanthin and antheraxanthin may have accumulated as a result of incomplete violaxanthin synthesis. Small amounts of zeaxanthin and antheraxanthin have been shown to be sufficient to induce significant NPQ (Gilmore, 1997). Alternatively, lutein may be responsible for the NPQ observed (Niyogi *et al.*, 1997; Pogson *et al.*, 1996).

2.4.4.2 *npq2 (aba1)*

This mutant is defective in violaxanthin de-epoxidase and thus accumulates zeaxanthin constitutively. Consequently, NPQ is activated to the same extent as WT however for longer periods of time and is more slowly reversible which has deleterious effects on the photosynthetic capacity of the plants (Niyogi *et al.*, 1997, 1998; DellaPenna, 1999) largely due to structural rearrangement(s) and hence reduced light-harvesting efficiency.

2.4.4.3 *npq4*

A critical role for PsbS, a PSII subunit also known as CP22, as the site for proton-binding during the initiation of flexible thermal energy dissipation was eluded towards following the construction of a PsbS-deficient mutant, *npq4* (Peterson and Havir, 2000). This mutant was capable of harvesting light energy but was defective in the qE component of NPQ (Li *et al.*, 2000). The findings from this mutant clearly reveal a positive correlation between PsbS protein levels and qE capacity.

2.4.4.4 *lut1 and lut2*

These mutants are defective in the production of lutein synthesis, the most predominant plant carotenoid. The *lut1* mutant is characterised as a deletion of the gene encoding ε-ring hydroxylase and as such these plants accumulate zeinoxanthin, the immediate precursor to lutein (Pogson *et al.*, 1996). Plants classified as *lut2* mutants lack a ε-LCY. In both cases, no phenotypical aberrations were visible in that normal plant growth and development was observed with a normal leaf chlorophyll complement under moderate light. This indicates that the xanthophyll cycle pigments can functionally and structurally complement lutein (Gilmore, 2001). However, these mutants had delayed NPQ which was also induced to a lower extent than the WT control. This work eludes towards a direct or indirect role for lutein in NPQ *in vivo* (Pogson *et al.*, 1998; Lokstein *et al.*, 2002).

2.4.5 A working model for flexible thermal energy dissipation

The chlorophylls in the LHCs are positioned for maximum efficiency of light harvesting. Any excess harvested energy may be rapidly dissipated (typically 1 ps) following transfer from the chlorophylls to the pigment with the lowest energy level. Zeaxanthin has an excited S_1 state lower than that of chlorophyll, allowing it to accept energy readily. Furthermore, zeaxanthin is more hydrophobic than violaxanthin due to its extended double bond system and the absence of terminal epoxy groups and as such replaces violaxanthin within the hydrophobic thylakoid membrane readily. As the photosynthetic rate increases, the lumenal pH drops and expression of the pH-dependent VDE is induced (Hager *et al.*, 1969; Demmig-Adams, 1990; Pfundel and Bilger, 1994; Hieber *et al.*, 2000). Violaxanthin is then converted to zeaxanthin which then binds to the violaxanthin site in the LHC. Instead of energy being transferred to the RC's, the increasing zeaxanthin content receives it from the

chlorophylls within the LHC (Fig. 2.2). Subsequently, photosynthesis decreases, the luminal pH increases and the de-epoxidase is inactivated. This cycle is initiated again following the absorption of excess light.

Two theories exist about the mechanism of flexible thermal energy dissipation. Both are xanthophyll-dependent but differ according to the conformational requirements for quenching. It has been established that protonation of eight conserved acidic amino acids of the PsbS protein (Li *et al.*, 2000, 2002a and b) are essential during quenching, however researchers are unsure as to whether a concomitant conformational re-arrangement is initiated. As early as 1989, a structural change required for NPQ was alluded towards following light-induced spectral absorbance measurements at 535 nm (Bilger and Björkman, 1990, 1994). The necessity for a structural change is still supported by Ruban *et al.* (1999) and Liu *et al.* (2004) and is believed to be essential to bring the orbitals of the donors and acceptors of excess excitation energy closer together (Horton *et al.*, 1996). However, most recently Strandfuss *et al.* (2005) have put forward a conformation-independent mechanism. They determined the crystal structure of pea LHCII at pH 5.5 and drew comparisons to the structure of the spinach complex which was grown at pH 7.5 (Liu *et al.*, 2004). No differences were recorded.
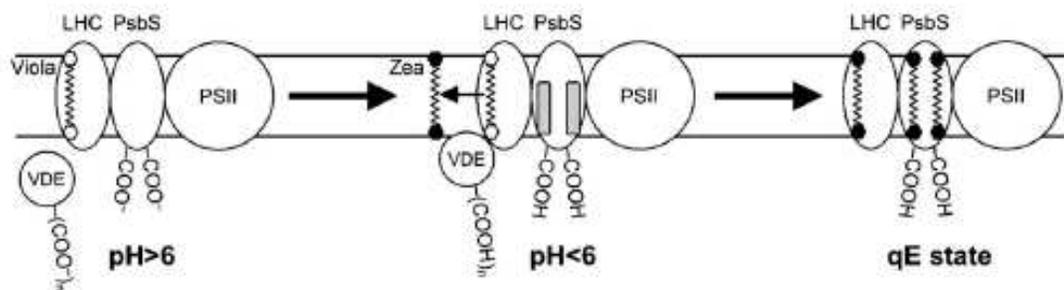
Figure 2.2. A schematic representation 0f the Mechanism for flexible thermal energy dissipation in plants (Niyogi *et al.*, 2005).

The thylakoid lumen pH is typically greater than 6 and violaxanthin is bound in the LHCs. As light energy becomes available in excess, the thylakoid lumen pH falls below 6 bringing about protonation of the carboxylate side chains of VDE and PsbS. VDE protonation activates the enzyme and it binds to the thylakoid membrane catalysing the conversion of violaxanthin (Viola) to zeaxanthin (Zea). The binding of Zea to protonated sites in PsbS facilitates dissipation of excess light energy.

2.4.6 Photoprotection by sustained thermal energy dissipation

During prolonged environmental stress exposure when photosynthesis has been significantly down-regulated, the flexible, ΔpH-dependent mechanism of dissipation is replaced by an efficient but relatively inflexible and long-lived process. In fact, sustained ΔpH-independent dissipation may be maintained for prolonged periods in the dark, implying that continued exposure to excess light is not necessary for continuance of the state required for thermal dissipation. During this process, the xanthophyll cycle is static with continuously high concentrations of zeaxanthin and antheraxanthin. Attempts to identify any additional role players, other than

zeaxanthin, which are pivotal during sustained dissipation are currently underway (Demmig-Adams and Adams, 2006). Sustained reductions in maximal light-harvesting efficiency (calculated as F_v/F_m) in conjunction with a degradation of the PSII cores or change in the PSII arrangement to a photochemical inactive state is often associated with photoinhibitory damage to the PSII reaction centres (Demmig *et al.*, 1987). This component of NPQ is either irreversible or more slowly reversible and may be due to a combination of photoprotection and photodamage (Müller *et al.*, 2001).

Two mechanisms of pH-independent sustained dissipation have been reported with differing kinetics and triggering signals. It seems that sustained thermal dissipation is maintained due to a continuous structural rearrangement of PSII or due to the complete degradation of some of the PSII core proteins. Retained zeaxanthin and antheraxanthin levels have been associated with the upregulation of PsbS-related proteins but not necessarily with the PsbS protein itself (Demmig-Adams *et al.*, 2006; Zarter *et al.*, 2006a, b and c). These proteins have been identified as members of the light-harvesting, chlorophyll *a/b*-binding proteins (Jansson, 1999; Heddad and Adamska, 2002; Andersson *et al.*, 2003). In addition to mediating NPQ, zeaxanthin plays an additional crucial role in suppressing ROS-elicited lipid peroxidation (Havaux and Niyogi, 1999; Havaux *et al.*, 2000; Baroli *et al.*, 2004). The exact mechanism(s) awaits elucidation.

Summary

Clearly, carotenoids are integrally responsible for maintaining the integrity of the photosynthetic apparatus, aiding light harvesting during photosynthesis and facilitating the harmless dissipation of excess light energy via a number of complex mechanisms. Consequently, tight regulation of the genes encoding the enzymes controlling carotenoid biosynthesis is crucial. During carotenogenesis, developmental transcriptional regulation accounts for the changes taking place during fruit and flower development and is typically associated with up-regulation of PDS and PSY while β-LCY is down-regulated. In contrast, carotenoid metabolism in the photosynthetically active tissues is largely light-dependent with a considerable amount of metabolite interaction between and within pathways. Due to the complexity of carotenoid biosynthesis formation within the chloroplasts and the regulation thereof, a number of gaps still exist in our existing knowledge. This includes a more detailed understanding of a β-LCY within the pathway and following high light exposure. Although it is clear that β-LCY apportions the substrate between the α- and β-branches of the pathway, it is not known whether this enzyme is a rate-limiting step. Furthermore, end product regulation of the PDS promoter via β-carotene and/or the xanthophylls has been suggested but not proven.

2.5 CAROTENOIDS: PRECURSORS FOR ABA SYNTHESIS

ABA biosynthesis proceeds via two different pathways, of which the cleavage of 9-*cis*-isomers of violaxanthin and neoxanthin formed during carotenogenesis comprises the so-called "indirect pathway" (Zeevaart and Creelman, 1988). The epoxidation of zeaxanthin into violaxanthin has been recognised as the first direct step in ABA production. As mentioned previously, the synthesis of neoxanthin from violaxanthin has not been completely elucidated but is believed to involve an NSY or a bifunctional β-LCY (Nambara and Marion-Poll, 2005). Furthermore, no *trans-cis* isomerase has been isolated (Strand *et al.*, 2000). NCED has been recognised as a rate-determining step in ABA synthesis and is responsible for cleaving the 9-*cis*-xanthophylls into xanthoxin (Schwartz *et al.*, 1997). Although only established in *Arabidopsis*, it is believed that xanthoxin is converted into ABA in a two-step reaction via abscisic aldehyde (González-Guzmán *et al.*, 2002) and abscisic carboxylic acid (Seo *et al.*, 2000).

2.5.1 Biological functions of ABA

ABA is normally present at basal levels however, during seed maturation and in response to environmental stress factors this phytohormone accumulates.

2.5.1.1 Seed development

Although not solely responsible, ABA initiates embryo maturation, the synthesis of storage reserves and late embryogenesis abundant (LEA) proteins, and seed dormancy (Bentsink and Koornneef, 2002; Nambara and Marion-Poll, 2003).

2.5.1.2 Adverse environmental conditions

Particularly during water stress, ABA promotes the closure of stomata to reduce water loss via transpiration. Furthermore, increased ABA activates a number of stress-responsive genes which aid in mediating the effects of the perceived stress (Bray, 2002).

These diverse roles in a number of plant cellular processes require stringent, complex regulatory control mechanisms; an understanding of which will enable manipulation of the existing processes to engineer increased tolerance to adverse environmental conditions (Xiong and Zhu, 2003).

2.5.2 ABA regulation

Characterisation of the *viviparous 14* (*vp14*) gene locus in maize revealed that the open reading frame encoded a *NCED*; whose expression was positively correlated to ABA levels (Tan *et al.*, 1997).

2.5.2.1 Developmental regulation

Within the seeds, ABA is either derived maternally (in which case it directs the production of storage proteins) or it is synthesised *de novo* in the embryo, initiating seed dormancy (Bentsink and Koornneef, 2002; Finkelstein and Rock., 2002).

2.5.2.2 Abiotic stress regulation - correlation between ABA, NCED and drought tolerance

Increased *de novo* biosynthesis through upregulation of ABA biosynthetic genes, results in the accumulation of ABA under adverse environmental conditions, particularly water stress, salinity and light (Xiong and Zhu, 2003).

ZEP does not appear to limit ABA synthesis and transcripts are present ubiquitously in photosynthetic tissue. Furthermore, ZEP was not upregulated during drought stress but expression followed the circadian rhythm and hence higher transcript levels were detected during the day. In contrast, within the roots ZEP is noticeably regulated by water stress (Audran *et al.*, 1998; Thompson *et al.*, 2000).

The expression pattern of *NCED* is diurnal, reaching a peak value immediately prior to the onset of darkness (Thompson *et al.*, 2000). However, most notably is the correlation in the increase in *NCED* transcripts, the corresponding NCED protein levels and the accumulation of ABA in roots, xylem sap and leaves during drought stress. This has been observed for *NCED1* of *Phaseolus vulgaris* (Qin and Zeevaart, 1999), *Vigna unguiculata* (Iuchi *et al.*, 2000), *Persea americana* (Chernys and Zeevaart, 2002), *Citrus sinensis* (Rodrigo *et al.*, 2006) and *NCED 3* of *Citrus clementina* (Agustí *et al.*, 2006). Further research established that *NCED* overexpression is correlated with increased drought tolerance. An increased tolerance to water-limiting conditions was observed following constitutive homologous overexpression of *Gentiana lutea NCED2* (Zhu *et al.*, 2006), *Arachis hypogaea NCED1* (Wan and Li, 2006) and *A. thaliana NCED3* (Iuchi *et al.*, 2001) as well as *P. vulgaris NCED1* in tobacco (Qin and Zeevaart, 2002). The identification of the perceived environmental stress conditions and the initiation of the signal transduction pathway catalysing NCED overexpression await elucidation (Schwartz *et al.*, 2003).

Summary

Only a brief account of the biological functions and regulation of ABA has been presented here, but the importance of this phytohormone is well documented and accepted. It is also known that its regulation is complex. Through control of the stomatal aperture, ABA has been repeatedly shown to mediate drought tolerance. Furthermore, as NCED regulates ABA formation in the chloroplasts, this gene plays a key role during water deficit. However, a detailed account of the signal transduction pathways initiated during water stress awaits elucidation. The ability to overexpress genes of interest in model plants has yielded interesting drought-resistant phenotypes when this approach was applied to *NCEDs*, but comprehensive physiological analyses on these mutant lines rarely occurred.

2.6 ISOLATION AND *IN PLANTA* CHARACTERISATION OF CAROTENOID BIOSYNTHETIC GENES IN *VITIS VINIFERA*

Within the Grapevine Biotechnology Programme at the Institute for Wine Biotechnology, several genes either directly or indirectly involved in carotenoid biosynthesis were isolated from *Vitis vinifera* L. cv Pinotage (Table 2.1). These isolated genes serve as useful resources to elucidate their regulatory role within the pathway, to establish the physiological function(s) of their gene products and to identify genes which ultimately might be useful in playing a role independently in genetic improvement strategies.

Currently two of the 31 isolated genes have been overexpressed in a heterologous environment (*Arabidopsis thaliana*) and characterised according to their pigment (carotenoid and chlorophyll) complement and their photosynthetic and non-photochemical quenching capacities (Brackenridge, 2006). The two genes chosen encode for a β-carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*). *In planta* analyses found *VvBCH* overexpressing lines to have elevated xanthophyll cycle pigments (particularly violaxanthin) which were more readily available for conversion to zeaxanthin following exposure to excess light. However, no difference in the final magnitude of NPQ was found. In strong contrast, the *VvZEP* lines had reduced zeaxanthin levels which resulted in a decreased induction and final magnitude of NPQ (in comparison to WT) which was associated with photodamage (Brackenridge, 2006).

In order to extend our fundamental knowledge of grapevine-derived carotenoid pathway genes and to elucidate their possible roles, detailed *in planta* analyses of some of the remaining 29 isolated genes are currently underway. The analyses of two of these genes, β-*LCY* and *NCED* are outlined in chapters 4 and 5 of this dissertation, respectively.

Table 2.1: Full length isoprenoid biosynthetic genes isolated from *Vitis vinifera* cv. Pinotage.

Pathway	Gene
MVA	*Acetoacetyl CoA thiolase (AACT)*
	3-hydroxy-3-methylglutaryl-CoA synthase (HMGS)
	3-hydroxy-3-methylglutaryl-CoA reductase1 (HMGR1)
	3-hydroxy-3-methylglutaryl-CoA reductase2 (HMGR2)
	MVA kinase (MK)
	Phospho-MVA kinase (PMK)
	Diphospho-MVA decarboxylase (DPMD)
DXP	*1-Deoxy-$_D$-xylulose 5-phosphate synthase（DXS)*
	1-Deoxy-$_D$-xylulose 5-phosphate reductoisomerase(DXR)
	4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase (ispE)
	2-C-methylerythritol-2,4-cyclodiphosphate synthase (ispF)
	1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (ispG)
	4-hydroxy-3-methylbut-2-enyl diphosphate reductase (ispH)
General	*Isopentenyl diphosphate isomerase (ipi)*
	Farnesyl diphosphate synthase (FPS)
	Geranyl diphosphate synthase1 (GPS1)
	Geranyl diphosphate synthase2 (GPS2)
	Geranyl geranyl pyrophosphate synthase (GGPS)
Carotenoid	*Phytoene synthase (PSY)*
	Phytoene desaturase (PDS)
	ζ-carotene desaturase (ZDS)
	cis-Isomerase (c-iso)
	Lycopene ε-cyclase (ε-LCY)
	Lycopene β-cyclase (β-LCY)
	β–carotene hydroxylase (BCH)
	Zeaxanthin epoxidase (ZEP)
	Violanthin deepoxidase (VDE)
	Capsanthin capsorubin synthase (CCS)
	9-cis Epoxycarotenoid dioxygenase (NCED)
	Carotenoid cleavage dioxygenase (CCD)

2.7   CONCLUSION

In summary, carotenoids play a central role in general plant metabolism and specifically photosynthesis; serving as precursors to nutritional compounds and the general stress phytohormone abscisic acid, and synthesising the photoprotective xanthophylls within their biosynthetic pathway. The Future Challenges Project (2004) has described biotechnology and bioengineering as "tools used in managing and conserving plants, animals and their habitats". Carotenoids have been identified as the ideal targets for genetic manipulation of plant species to confer abiotic stress tolerance facilitating sustained growth and productivity and to meet the additional challenges posed by the changing global climate. However, we need to extend the existing knowledge of the pathway itself as well as the *in planta* role of each gene including expression profiles, enzymatic activities, precursor pool sizes, regulation within the pathway and interaction with other pathways (Fraser and Bramley, 2004) in order to make an informed choice regarding the gene(s) to manipulate.

In the context of light stress management the role for zeaxanthin (and antheraxanthin) has been clearly resolved. However, the concomitant effects of light stress and the existing protective mechanisms on the rest of the pathway as well as a potential interaction with related pathways are unclear. β-carotene also plays a critical role as an antioxidant removing harmful reactive oxygen species. However, a number of questions remain regarding its synthesis and the regulatory role of β-LCY within the pathway and potentially in photosynthesis. Furthermore, although β-LCY controls the partitioning of substrate between the α- and β-branches and the formation of β-carotene, other regulatory roles and functions of this enzyme has not been fully elucidated yet and merits investigation, specifically in relation to chloroplastic carotenogenesis. Similarly, NCED has been shown to regulate ABA formation which in turn regulates stomatal conductance thereby conferring a drought tolerant phenotype during water stress. However, the exact physiological consequences and the pigment and hormone profiles of plants with these phenotypes are still lacking and will help to understand the role of NCED in the mechanisms conferring drought tolerance.

It is clear from literature and a wealth of recent and topical publications that the study of the carotenoid biosynthetic pathway is providing insight into a central and highly regulated metabolic pathway of plants. The ability to upregulate or silence individual pathway members in model plants and study the subsequent effects on genetic, metabolic, biochemical, as well as physiological levels, is yielding valuable data on these carotenoid biosynthetic genes.

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CHAPTER 3

RESEARCH RESULTS

High-performance liquid chromatography profiling of the major carotenoids in *Arabidopsis thaliana* leaf tissue

High-performance liquid chromatography profiling of the major carotenoids in *Arabidopsis thaliana* leaf tissue

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Abstract

Carotenoids are extremely sensitive to a variety of physico-chemical attacks which may have a profound effect on their characteristic properties, thereby influencing the accurate identification and quantification of individual compounds. In this light, a comprehensive summary of the pitfalls encountered and precautions to be administered during handling and storage of authentic standards and samples was found to be incomplete. Furthermore, acceptable baseline separation of *trans*-lutein from *trans*-zeaxanthin and between the *cis*- and *trans*-forms of neoxanthin and violaxanthin has not been satisfactorily demonstrated. Hence the most optimal sample preparation and analytical steps were determined and a sensitive and reproducible method for the quantitative HPLC profiling of the principal carotenoids found in plant leaf tissue was developed. A reverse-phase C_{30} column with a binary mobile solvent system was used for the baseline separation of eight of the major carotenoids and the two chlorophylls (*a* and *b*) within 18 min. These compounds were identified via the use of authentic standards, their spectral characteristics and HPLC-atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) confirmation. This method has been successfully applied for the quantification of plant pigments in *Arabidopsis thaliana* wild-type (WT) leaf tissue and in two *A. thaliana* non-photochemical mutants, namely *npq1* and *npq2*. These mutants have previously been well-characterised and provided valuable reference data as well as acting as internal controls for the assessment of our new method.
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Keywords: HPLC; C_{30}; Authentic standards; Carotenoids; Quantification; Plants; *Arabidopsis*

1. Introduction

Carotenoids belong to a group of deeply coloured yellow, orange or red pigments that occur abundantly in plants, and to a lesser degree in animals and humans, where they fulfil an array of tasks [1,2]. They consist of long, aliphatic, conjugated double bonded systems, which serve as light-absorbing chromophores, and are usually composed of eight isoprene units with the molecular formula $C_{40}H_{56}$ [3]. The majority of natural carotenes have double bonds in the all-*trans* position, however, some exhibit a *cis*–*trans* configuration [4]. These carotenes may become oxygenated to form the xanthophylls. The primary functions of carotenoids are associated with chlorophyll to aid photosynthesis and phototaxis by operating as accessory light harvesting pigments in the spectral range of 450–570 nm. This role is mainly carried out by the xanthophylls lutein, violaxanthin, neoxanthin and, to a lesser extent, β-carotene [5]. Alternatively, carotenoids function, non-radiatively, as potent free radical quenchers, singlet oxygen scavengers and lipid antioxidants [6–8] thereby acting as photoprotectants under excess light conditions. The xanthophyll zeaxanthin is primarily responsible for the safe dissipation of excess light energy as heat, whereas β-carotene, amongst others, is a potent antioxidant [9].

The reliable and reproducible quantification of plant carotenoids is a complex task. Extensive research has been carried out detailing the precautions and steps to be taken during pigment extraction from plant tissues [4,10–14], however there is no standard procedure due to the wide variety of sample types and compositions. Physico-chemical attacks especially by light, temperature, and oxygen [11] have profound effects on the structure and configuration of these lipophilic pigments. This influences their physico-chemical properties and specifically their solubility, their absorption spectra and their chromatographic tendencies [15]. Hence, it is crucial that steps are taken

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to ensure that these properties, characteristic for the individual chromophores, remain intact as they are integral in effective carotenoid identification and quantitative analyses.

Pigments have been extracted using an array of solvents [16–21], revealing chloroform as the extraction solvent of choice for pigment isolation from plant leaf tissue [22]. This solvent was found to provide a 10-fold higher carotenoid extraction capability from the plant tissue and eliminated unwanted UV interference during online photodiode array (PDA) analysis following HPLC. This extraction protocol has found use for an array of plant tissues and plant types, including tobacco and tomato leaves and nectary tissues [22,23], as well as *Arabidopsis* leaves [22] and seeds [24,25].

Following extraction, carotenoids were classically quantified using open-column and thin layer chromatography, with a range of stationary phases [11] and solvents [14,26]. However, the proceeding spectrophotometric quantification was found to result in an approximately 50% underestimation in comparison to quantification using HPLC [22]. Additionally, HPLC offers the advantages of rapidity, relative simplicity, ease of automation, sensitivity, precision, sample preservation, and on-line detection over the full spectral range (photodiode array detection, DAD) [11,27].

HPLC methods use isocratic or gradient mobile phases in either a normal-phase or reverse-phase (RP) mode. A wide range of stationary phases and mobile phases have been investigated to achieve maximal separation and resolution of the major carotenoids present in the tissue of interest (refer to review articles [27,28]). Typically a C_{18} reverse-phase column (monomeric or polymeric) has been used [29,30]. However, more recently, a triacontyl (C_{30}) chemically-bonded phase column was developed at the National Institute of Standards and Technology (Gaithersburg, MD, USA) [31]. This matrix has reproducibly been found to give the highest separation selectivity for a range of carotenoids [32,33] due, in part, to the increased thickness of the stationary phase [34].

An important quality parameter of a carotenoid separation system is its ability to resolve mixtures of lutein/zeaxanthin and the *cis*- and *trans*-forms of violaxanthin/neoxanthin [35]. The authors felt that a suitable system was not available for accurate identification and quantification of the above-mentioned carotenoids that specifically play a role during plant stress, particularly excess light. A study was thus initiated to provide an improved and reproducible method for the accurate step-by-step quantitative profiling of the principal carotenoids found in plant tissues. The ability of the developed system to quantify plant pigments was evaluated relative to published data on *Arabidopsis thaliana* WT plantlets and the non-photochemical quenching mutants, *npq1* and *npq2*, with known shifts in carotenoid profiles and contents compared to WTs [36].

2. Experimental

2.1. Plant material and growth conditions

The *A. thaliana* ecotype Columbia WT and two *Arabidopsis* mutants, *npq1* and *npq2* (obtained from the *Arabidopsis*

Biological Resource Centre, Ohio State University, CO, USA), were grown in peat (Jiffy Products International, Norway) and vermiculite in a 3:1 ratio within a controlled growth room. Growth room conditions were maintained at 23 °C, 55% relative humidity, 16 h-light:8 h-dark cycle and a uniform photon flux density of 43 μmol photons m^{-2} s^{-1}. Plants were watered by sub-irrigation twice weekly and a standard nutrient solution (Nitrosol, Fleuron, Universal Selected Services, Braamfontein, South Africa) was applied fortnightly.

2.2. Analytical materials

The following solvents were purchased from Sigma–Aldrich (Steinheim, Germany): TBME, ethyl acetate, chloroform and hexane. Methanol and butylated hydroxytoluene (BHT) were acquired from Fluka Chemie (Buchs, Switzerland), triethylamine from Merck (Hohenbrunn, Germany) and ammonium acetate from BHD (VWR International, Poole, UK). All solvents and chemicals used during the sample preparation and HPLC analysis were of HPLC grade, with the exception of sodium chloride (Fluka Chemie) and Tris base (Roche Diagnostics, Mannheim, Germany) which were of analytical grade. The authentic standards *trans*-β-carotene (purity ≥ 97%), *trans*-lycopene (purity ≥ 90%) and β-apo-caroten-8-al (purity ≥ 96%; Fluka Chemie), astaxanthin (purity ≥ 98%; Sigma–Aldrich) and *trans*-zeaxanthin (purity guaranteed as per thin layer chromatography; Carl Roth, Karlsruhe, Germany) were obtained. All the ratios and percentages of solvents are indicated as volume per volume (v/v), unless otherwise stated.

2.3. Preparation of standards

The authentic standards detailed in Table 1 were dissolved in their respective solvents with the addition of 0.1% (w/v) BHT and stored immediately, as recommended, at −20 °C [30]. The *trans*-zeaxanthin and *trans*-lycopene standards, however, were divided into 500 μL aliquots in amber HPLC vials and dried under a stream of nitrogen gas prior to storage in order to prevent isomerisation [2]. These standards were re-dissolved in the appropriate solvent prior to use. The concentrations of the stock solutions and the working dilutions for standard curve construction and method validation are listed in Table 1. All dilutions were made in ethyl acetate–methanol (1:4).

2.4. Sample preparation

Rosette leaves of 4-week-old WT, *npq1* and *npq2* mutant plants were harvested, frozen in liquid nitrogen, freeze dried overnight and ground to a fine powder. In all instances, care was taken to ensure three biological populations, typically made up of at least three individual plantlets, were represented and were analysed independently in triplicate. Two milligrams of freeze dried material was extracted according to Fraser et al. [22] in micro-centrifuge tubes. The material was gently mixed with 100 μL of methanol containing 0.4 μg of each of the internal standards astaxanthin and β-apo-caroten-8-al. After 5 min, 100 μL of 50 mM Tris–HCl (pH 8.0) containing 1 M NaCl was

Table 1
The authentic standards used and their preparation for standard curve construction and method validation

Authentic standard	Solvent for stock solution	Stock concentration ($\mu g\,mL^{-1}$)	Working concentration ($\mu g\,mL^{-1}$)
Astaxanthin	Chloroform–hexane (1:9)	100	10
β-Apo-caroten-8-al	Ethylacetate–methanol (1:4)	100	10
trans-Zeaxanthin	Chloroform	100	10
trans-β-Carotene	Chloroform–hexane (1:9)	100	10
trans-Lycopene	Chloroform	100	2

added and mixed by inversion for 5 min. Chloroform (400 μL) was added for partitioning of the carotenoids. The mixture was incubated for 10 min and centrifuged at $3000 \times g$ for 5 min at 4 °C. After centrifugation, the lower chloroform phase was collected and the upper aqueous phase re-extracted with 400 μL chloroform. The chloroform lower phases were pooled and dried under a stream of nitrogen. Dried samples were stored under an atmosphere of nitrogen at −20 °C. Prior to HPLC analysis, samples were dissolved in 200 μL of a 1:4 ethyl acetate, methanol solution containing 0.1% (w/v) BHT and centrifuged at RT for 5 min at $12\,000 \times g$. Samples were shielded from strong light and kept on ice during all procedures.

2.5. Chromatographic conditions

The carotenoid pigments were separated by RP-HPLC on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD system. A YMC30 column (100 mm × 2.1 mm) and YMC30 guard cartridge (10 mm × 2.1 mm, particle size 3 μm) both from YMC Europe (Schermbeck, Germany) were used. Chemstation software for LC3D (Rev.A.10.01[1635]; Hewlett-Packard, Waldbronn, Germany) was used for data processing. Various temperatures, flow rates, modifiers and binary solvent systems in either isocratic or gradient mode were experimented with, to compare the separation efficiency of the major carotenoids in plant leaf tissue. After a number of investigations, the binary mobile phase consisting of 3% ddH$_2$O in methanol containing 0.05 M ammonium acetate (solvent A) and 100% TBME (solvent B) was found to be the most effective. Both solvents contained 0.1% (w/v) BHT and 0.05% triethylamine. A flow rate of 500 μL min^{-1} at 25 °C with an injection volume of 10 μL was used. Elution was carried out according to the following program: isocratic at 3% B for 12 min followed by a linear gradient from 3% to 38% B in 1 min, isocratic at 38% B for 2 min, a linear increase to 68% B in 1 min, isocratic at 68% for 5 min followed by a linear decrease to 3% B in 4 min. The column was equilibrated for 10 min at the starting conditions before each injection.

LC–MS was performed using a Waters Quattro Micro mass spectrophotometer connected to an Alliance 2695 HPLC system and PDA detector (Waters 996, Milford, MA, USA). Carotenoids were detected with APCI in the negative mode. A cone voltage of 35 V and capillary voltage of 2.8 kV was used. The corona pin was set at 7 μA. The capillary and vaporisation temperatures, the gas flow rates and all other parameters were optimised for maximum sensitivity using all of the authentic standards available (with the exception of lycopene).

2.6. Identification and quantification of carotenoids

Identification of carotenoids was achieved by comparing their retention times and visible spectra with literature data [11,37], and with that of the authentic standards. LC–MS analyses confirmed identification.

The elution of the various carotenoid pigments was followed at 450 nm and 470 nm (lycopene) with a constant reference wavelength at 800 nm.

Standard curves for the quantification of carotenoids were obtained by plotting amount (ng) against area, which was obtained from triplicate injections. The correlation co-efficient (r^2) and regression equations were obtained using Statistica 7 software (Statsoft, Tulsa, UK). Astaxanthin, β-apo-caroten-8-al, trans-zeaxanthin, trans-β-carotene and trans-lycopene were quantified according to their respective authentic standards. The response factor of the trans-zeaxanthin authentic standard was also used to quantify the xanthophyll pigments; namely trans- and cis-neoxanthin, trans-violaxanthin, trans-antheraxanthin and trans-lutein, while the response factor of the authentic trans-β-carotene standard was also used for its cis-isomer. The chlorophyll concentration of the extracts was determined spectrophotometrically using the extinction coefficients in 100% methanol as described by Lichtenthaler and Wellburn [38]. The absorbance measured at 666 nm and 653 nm was used in the following equations: chl $a = 15.65 \times A_{666} - 7.34 \times A_{653}$ and chl $b = 27.05 \times A_{653} - 11.21 \times A_{666}$. The carotenoid concentration was determined from the standard curves and expressed as millimole pigment per mole chlorophyll a.

2.7. Determination of limits of detection (LOD) and quantification (LOQ)

The detection and quantification limits were determined for all the standards based on a method described by the International Conference on Harmonisation [39]. The LOD were determined as the amount that resulted in a peak with a height two to three times that of the baseline noise. The LOQ were determined as the lowest injected amount which could be reproducibly quantified (RSD ≤ 3%).

2.8. Accuracy and recovery

Recovery of individual carotenoids from the sample matrix was determined according to the amounts extracted from the matrix after a blank matrix was spiked with known concentrations of the available standards. Care was taken to mimic sample

preparation and standards were added at levels expected to be found in the samples.

To determine recovery from the column, the authentic standards were injected separately with and without the column and their response areas were determined. Due to the varying polarity and hence retention differential of each standard employed, the mobile phase composition was manipulated to mimic the normal run conditions as closely as possible. Astaxanthin and β-apo-caroten-8-al were eluted using 3:97 (v/v) TBME–methanol–water (97:3), *trans*-zeaxanthin and *trans*-β-carotene were eluted using 38:62 (v/v) TBME–methanol–water (97:3) and, finally, *trans*-lycopene was eluted using 68:32 (v/v) TBME–methanol–water (97:3). The percentage recovery from the column was expressed as a percentage of the total response (peak area) obtained with the column against that obtained without the column (which was taken to represent 100% recovery). Values determined were the average of three injections.

3. Results and discussion

3.1. HPLC method development, evaluation, validation and handling of the authentic standards

Separation of the various pigment constituents extracted from plant leaf tissues, on the C_{30} column, was evaluated under a range of mobile phase conditions. Methanol and TBME were chosen as the two primary mobile phases and were modified accordingly. Selectivity has been reported to be strongly dependent on the choice of mobile phase modifier. Furthermore, the separation of the polar carotenoids is influenced by the presence of water, whereas non-polar carotenoids are relatively insensitive to this parameter [40]. For this reason, a range of final percentages of water (0–10%) in the methanol mobile phase was investigated in combination with a variety of starting mobile phase conditions, from 0 to 16% TBME, and an array of running conditions (data not shown).

Several investigations have been conducted comparing column selectivity with temperature. The retention of some of the α- and β-carotene isomers was found to increase with increasing temperature (up to ∼35 °C) due to increased solute stationary phase interactions [41,42], while the optimal separation of lutein from zeaxanthin was obtained at lower temperature (−13 °C) due to increased rigidity of the column matrix [35]. Despite these conflicting reports it is clear that close column tempera-

ture regulation is essential for a reproducible elution profile of all the major carotenoids of interest [43]. As such several column temperatures were investigated in this study ranging from 25 to 50 °C (data not shown), with room temperature (25 °C) eventually chosen as optimal in combination with the aqueous environment, mobile phase modifiers and multistage gradient elution conditions selected (as detailed in Section 2). In summary, after various optimisations were investigated a final HPLC analytical procedure was agreed upon that provided optimal, reproducible separation of the principal carotenoids in plant leaf tissue within a period of 18 min.

Carotenoids are insoluble in water and have limited solubility in methanol and acetonitrile, hence the choice of solvent and its purity is of utmost importance [10,44]. Chloroform, dichloromethane and tetrahydrofuran are the favoured solvents for carotenoids; where solubility in the range of 1000–10 000 μg mL^{-1} has been reported [10]. The stock solutions used for each authentic standard are illustrated in Table 1 and were found to be stable in darkness at −20 °C, in their respective solvent(s) containing the antioxidant BHT, for a period of ∼2 months. At a concentration of 100 μg mL^{-1} the astaxanthin stock solution was near its solubility limit and required gentle warming at 37 °C prior to use. The working solutions (also detailed in Table 1) were diluted in ethyl acetate–methanol (1:4) because pigment solubility is high in this medium and it mimics the mobile phase closely thereby minimizing aberrant peak tailing and the production of artefacts due to injection solvent and mobile phase incompatibilities. These working solution concentrations were maintained at 10 μg mL^{-1} and were found to be stable for a period of up to 1 month at −20 °C. The exception was lycopene with a working solution concentration of 2 μg mL^{-1}. Furthermore, its stability deteriorated rapidly between consecutive HPLC analyses after a period of ∼36 h, despite dissolution in its preferred solvent, chloroform. This finding is in agreement with previous work [43] where *trans*-lycopene, dissolved in chloroform, degraded on standing into several of its isomers. However, our findings disagree with those published in that stability was not restored following the addition of 0.1% (w/v) BHT. More recently, a similar phenomenon of lycopene instability (a maximum of 3 days at −20 °C) has been reported resulting in great variation in its calibration and measurement [45]. Nevertheless, the HPLC method was set up for the detection and quantification of this hydrocarbon which is strongly retained, requiring

Table 2
Parameters determined during validation of the developed methodology

Compound	Recovery from column (%)	Validation parameter				
		Regression equation	r^2	Standards error	LOQ (ng)	Recovery during extraction (%)
Astaxanthin	98.1	$y = (28.6240 \pm 0.2534)x + (-1.2259 \pm 3.9389)$	0.9999	1.7917	1.5	89
β-Apo-caroten-8-al	97.6	$y = (54.2684 \pm 0.3352)x + (30.4864 \pm 5.2110)$	1.0000	2.3703	1.0	86
trans-Zeaxanthin	99.3	$y = (33.5090 \pm 0.1736)x + 48.5440 \pm 21.3561)$	0.9999	34.1421	1.0	82
trans-β-Carotene	98.0	$y = (37.1497 \pm 0.1494)x + (13.3756 \pm 9.1728)$	1.0000	10.9067	3.0	99
trans-Lycopene	86.6	$y = (171.6713 \pm 0.4480)x + (-6.1315 \pm 6.4297)$	1.0000	10.6878	0.5	ND[a]

[a] Not determined.

a TBME gradient with a final concentration of up to 68% for elution.

To ensure that maximum column recovery of the plant carotenoids under investigation was possible, low concentrations of the solvent modifiers ammonium acetate (0.05 M to the methanol mobile phase) and triethylamine (0.05% to both the methanol and TBME mobile phases) were added [46,47]. It is postulated that the mode of action of these salts facilitates buffering of the acidity of the mobile phase or of the acidity of the free silanol groups in the stationary phase [30]. Percentage recovery of astaxanthin, β-apo-caroten-8-al, *trans*-zeaxanthin, *trans*-β-carotene and *trans*-lycopene, in the presence of both salts, is indicated (Table 2). The antioxidant BHT was also included in the mobile phases at a final concentration of 0.1% (w/v) to minimise on-column degradation. It is postulated that the somewhat lower recovery obtained for *trans*-lycopene is due to the increased retention of this slightly more hydrophobic compound following interaction with the hydrophobic stationary phase.

The regression equations and the limits of quantification for all the authentic standards used are detailed (Table 2). The response of astaxanthin, β-apo-caroten-8-al, *trans*-zeaxanthin, *trans*-β-carotene and *trans*-lycopene was linear over the range used for the standard curve and the relative standard deviation of the regression was well within published acceptable limits at a maximum of 1.4% [48].

The recovery during the extraction procedures was estimated during a mock extraction (without plant tissue) with only four of the standards available. After HPLC analysis, the recovery of each carotenoid was calculated based on the ratio of the amount determined by HPLC to the known amount added prior to commencing extraction (Table 2).

The reproducibility of the whole method, including pigment extraction from plant tissue and subsequent HPLC analysis, was thus determined for three of the individual carotenoids (*trans*-violaxanthin, *trans*-lutein and *trans*-β-carotene) using three biological populations of *A. thaliana* WT tissue, with three technical repeats performed per population. The percentage standard deviation for *trans*-violaxanthin, *trans*-lutein and *trans*-β-carotene (following recovery correction against β-apo-caroten-8-al) was found to be 6.6, 5.1 and 6.0, respectively. This lies well within the acceptable range of 10–15%. It should be noted that the reproducibility of this method represents a single experiment and was not re-evaluated after a period of time.

3.2. Extraction of major carotenoids from plant tissue

The extraction procedure recommended by Fraser et al. [22] was used with care being taken at all times to avoid or minimise contact with heat and artificial light. Furthermore, pigment stability was enhanced by immediately flash freezing the harvested sample in liquid nitrogen and then freeze drying the plant tissue prior to storage at −80 °C [49], thereby avoiding loss due to degradation. Furthermore, the extracted products were dried under a stream of nitrogen gas to minimise the effects of oxidation and were stored at −80 °C prior to HPLC analysis. Care was taken to evaporate just to dryness to prevent degradation of carotenoids (especially lycopene [50]) and preferential adhering

of some of the carotenoids to the walls of the container [46]. It has been suggested that the degree of chlorophyll degradation [37] and the ratio of *cis*- and *trans*-isomers of β-carotene (where a smaller ratio indicates that greater care was taken to prevent isomerisation of the *trans* form to its *cis* counterpart [2]) may be influenced by the manner is which samples were handled prior to and during the extraction process. Regardless of precautionary steps taken during the extraction procedure a small 9-*cis*-β-carotene peak was observed in all samples, however, the extent of chlorophyll degradation, specifically chlorophyll *a*, was considerably reduced following the addition of the antioxidant to the mobile phases, as well as to the injection solvents. We have established that two milligrams of freeze dried tissue was optimal to ensure complete extraction of the plant pigments by the combination of methanol–chloroform (1:4) and was sufficient for the accurate quantification of all the principal pigments of interest. A mixture of ethyl acetate–methanol (1:4) was used as the injection solvent for compatibility with the mobile phases.

Two internal standards were employed during sample extraction to monitor any losses during the extraction procedure. As such it was critical that the running conditions allowed for accurate and reproducible quantification of both standards. Previously, difficulties had been reported in using β-apo-caroten-8-al as the internal standard of choice due to co-elution with chlorophyll *b* [30]. However, under the current HPLC analytical conditions, the β-apo-caroten-8-al peak was 100% pure, while astaxanthin co-eluted with an unidentified xanthophyll. As these two standards showed similar recoveries during the extraction procedure (see Section 3.1 above), only the single internal standard, β-apo-caroten-8-al, was used in all subsequent experiments.

3.3. HPLC system for the profiling of the major plant carotenoids

Due to the complexity of carotenoids in plant tissues, a gradient solvent system comprising of 97% methanol-distilled water and TBME (as described in Section 2) was developed on a RP-C$_{30}$ stationary phase. Using this method, effective baseline or near baseline resolution was achieved for the three classes of components found in plant leaf tissue: the xanthophylls (*cis*- and *trans*-neoxanthin, *trans*-violaxanthin, *trans*-lutein and *trans*-zeaxanthin); the chlorophylls (*a* and *b*) and the hydrocarbon carotenoids (9-*cis*- and *trans*-β-carotene). In agreement with previous investigations [51], lycopene was not detected in leaf tissue due to the tight chloroplast light-induced regulation of the gene encoding its conversion to β-carotene or lutein. A representative chromatogram of the major carotenoids in an *A. thaliana* WT leaf tissue sample is illustrated in Fig. 1A. The α-values for all peaks were found to be greater than one (Table 3), indicating that a good selectivity of mobile phase to sample components was achieved.

Initial identification of the individual components is based on their chromatographic behaviour (i.e. order of elution) and their visible absorption spectra; where the wavelength of maximum absorption and the spectral fine structure are characteristic of each chromophore, in comparison to available authentic
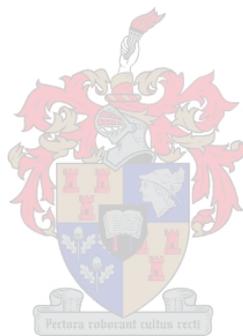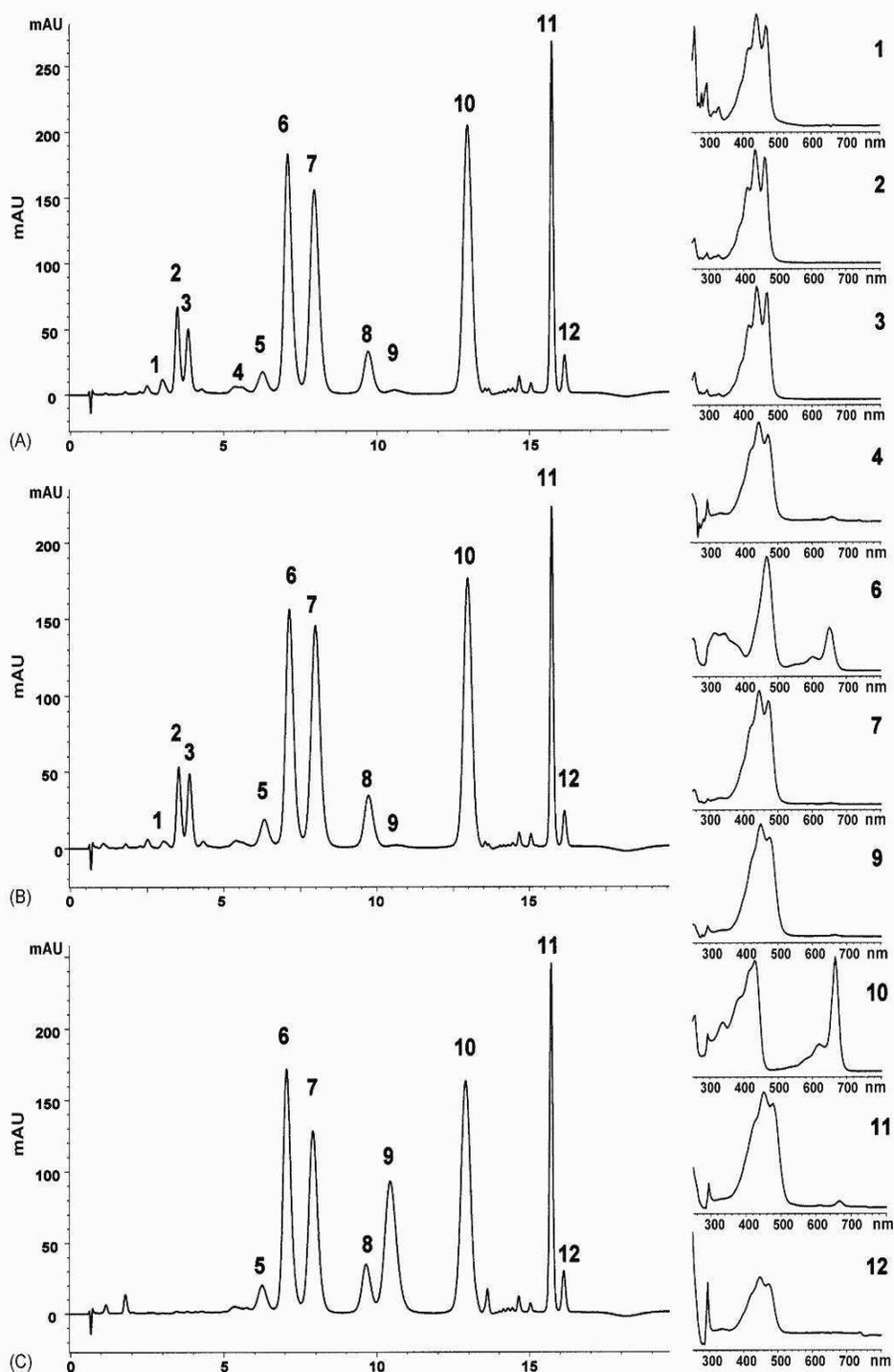
Fig. 1. HPLC profiles of the major carotenoids in: (A) *A. thaliana* WT; (B) *A. thaliana npq1*; and (C) *A. thaliana npq2* leaf tissue. Peak identification: (1) *trans*-neoxanthin (6.21, 2.68, <LOQ); (2) *trans*-violaxanthin (28.73, 24.22, <LOQ); (3) *cis*-neoxanthin (24.34, 24.49, <LOQ); (4) *trans*-antheraxanthin (<LOQ, <LOQ, <LOQ); (5) astaxanthin (N/A); (6) chlorophyll *b* (N/A); (7) *trans*-lutein (135.64, 130.36, 90.16); (8) β-Apo-caroten-8-al (12.95, 17.59, 13.33); (9) *trans*-zeaxanthin (4.43, 2.55, 88.03); (10) chlorophyll *a* (N/A); (11) *trans*-β-carotene (73.84, 64.95, 55.40); and (12) 9-*cis*-β-carotene (12.40, 11.52, 9.77). The amounts indicated in brackets refer to the corresponding amounts in nanograms per 10 μL injected for peaks in chromatogram A, B and C, respectively. N/A denotes not applicable and <LOQ, below the quantification limit. The visible absorbance spectrum of each major carotenoid is indicated on the right-hand side.

Table 3
Peak identification of plant carotenoids separated on a C_{30} HPLC column and their spectral characteristics (in the eluting solvent) following DAD

Peak number	Compound	Molecular weight (average)[a]	Retention factor (κ)	Separation factor (α)	Spectral characteristics (nm)						%III/II[b]		APCI-MS (negative) m/z
					Observed[c]			Reported			Observed[c]	Reported	
1	trans-Neoxanthin	600.9	3.55	1.00	418	438	466	417	440	470[d]	66	–[i]	ND[j]
2	trans-Violaxanthin	600.9	4.29	1.16	414	438	470	415	436	466[e]	80	90[e]	600.0
3	cis-Neoxanthin	600.9	4.82	1.10	414	434	462	413	435	464[d]	81	80[f]	600.0
4	trans-Antheraxanthin	584.9	7.45	1.45	422	442	470	422	444	472[f]	41	55[f]	ND[j]
5	Astaxanthin	596.4	8.49	1.12		470			478[f]		0	0	596.0
6	Chlorophyll b	907.5	9.73	1.13		466	650		469	652[e]	0	0	906.0
7	trans-Lutein	568.9	11.04	1.12	(422)	446	470	422	443	470[e]	56	62[e]	568.0
8	β-Apo-caroten-8-al	416.3	13.69	1.22		462			462[g]		0	0	416.0
9	trans-Zeaxanthin	568.9	15.00	1.09	(426)	450	474	(429)	448	473[e]	18	22[e]	568.1
10	Chlorophyll a	893.5	18.58	1.22		430	666		431	665[e]	0	0	893.0
11	trans-β-Carotene	536.9	22.72	1.21	(426)	450	478	(429)	449	475[e]	17	25[e]	536.0
12	9-cis-β-Carotene	536.9	23.53	1.03	(422)	446	470	–[i]	447	472[h]	18	–[i]	536.0

[a] www.lipidbase.jp.

[b] Ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between two peaks as baseline, multiplied by 100, in ethanol [15].

[c] A gradient mobile phase of methanol containing 3% water, 0.05% triethylamine and 0.05 M ammonium acetate (solvent A) and 100% TBME containing 0.05% triethylamine (solvent B) (from 97:3 to 32:68, v/v) was used.

[d] Measured in methanol–water (9:1).

[e] Measured in methanol.

[f] Measured in ethanol.

[g] Measured in methanol–TBME (~1:1).

[h] Measured in methanol–TBME (8:2).

[i] Data not available.

[j] Not Determined.

Table 4
Compounds quantified by C_{30} RP-HPLC of WT *Arabidopsis* and the *npq1* and *npq2 Arabidopsis* mutants

Peak number	Compound	WT[a]	npq1[a]	npq2[a]
1	*trans*-Neoxanthin[b]	6.95 ± 0.03	4.05 ± 0.20	<LOQ[c]
2	*trans*-Violaxanthin[b]	31.57 ± 0.41	31.43 ± 2.78	<LOQ[c]
3	*cis*-Neoxanthin[b]	29.22 ± 2.84	32.12 ± 2.54	<LOQ[c]
4	*trans*-Antheraxanthin[b]	<LOQ[c]	<LOQ[c]	<LOQ[c]
7	*trans*-Lutein[b]	154.88 ± 1.22	183.06 ± 13.50	117.11 ± 11.54
9	*trans*-Zeaxanthin[b]	5.52 ± 0.13	4.60 ± 0.19	114.29 ± 11.30
11	*trans*-β-Carotene[b]	89.47 ± 0.84	96.20 ± 7.22	76.32 ± 7.58
12	9-*cis*-β-Carotene[b]	15.15 ± 0.11	15.89 ± 1.49	14.09 ± 1.24
	Total carotenoids[b]	332.77 ± 5.29	367.35 ± 27.59	321.81 ± 31.94
10	Chlorophyll *a*[d]	13.90 ± 0.84	10.83 ± 0.66	12.14 ± 1.08
6	Chlorophyll *b*[d]	7.60 ± 0.49	5.89 ± 0.37	6.62 ± 0.59
	Chlorophyll *a/b*	1.83 ± 0.03	1.83 ± 0.02	1.83 ± 0.01

[a] Average of nine analyses (three biological populations, each with three technical repeats) ± standards error.
[b] Expressed as millimoles carotenoid per mole chlorophyll *a*.
[c] Below the quantification limit.
[d] Expressed as micromoles chlorophyll per gram freeze dried tissue.

standards. When a standard was not commercially available, peaks were identified according to previously published criteria [37] and HPLC-APCI-MS. Typically carotenoids exhibit three absorption maxima, with the position of λ_{max} being directly related to the number of conjugated double bonds. *cis*-Isomerisation of the double-bonded system gives the compound a slightly lighter colour and brings about both a hypsochromic shift (normally between 2 and 6 nm at lower wavelengths) and a hypochromic effect (decrease in absorbance due to the appearance of a "*cis*" peak approximately 142 nm below the λ_{max} of its *trans*-isomer) [37]. The absorption maxima and the %III/II of each of the major plant pigments studied, along with the previously published data obtained using a similar solvent system, is shown in Table 3. Overall, the spectra are in good agreement with published data. Small differences are expected due to the fact that the spectra are taken in mixed solvents during HPLC elution [45]. The absorption spectra obtained for each individual compound in the current binary mobile phase system is illustrated in Fig. 1. Recently, emphasis has been placed on the use of MS and/or nuclear magnetic resonance (NMR) spectroscopy to avoid peak misidentification and accurate quantification [1,15,40,52].

Peaks 6 and 10 were identified as chlorophyll *b* and *a*, respectively. Peaks 5 and 8 were recognised as the two internal standards astaxanthin and β-apo-caroten-8-al, respectively, due to their characteristic one peak maxima. However, the astaxanthin peak co-eluted with an unidentified xanthophyll making accurate quantification impossible. Peaks 2 and 3 were successfully identified as *trans*-violaxanthin and *cis*-neoxanthin, respectively, due to their similar spectral characteristics, significantly different %III/II ratio and the shift observed in the former compound following stress application (especially light) as *trans*-violaxanthin is rapidly converted to zeaxanthin. Peak 1 was tentatively assigned to *trans*-neoxanthin due to the hypsochromic shift of 4 nm observed relative to its *cis*-isomer and due to its alignment with results obtained in previous experiments [53,54]. Peaks 4, 7, 9, 11 and 12 were found to be *trans*-

antheraxanthin, *trans*-lutein, *trans*-zeaxanthin, *trans*-β-carotene and its 9-*cis*-isomer, respectively, due to their characteristically similar absorption spectra and their retention times, in comparison to authentic standards and published data obtained on C_{30} columns.

3.4. Application of carotenoid profiling to A. thaliana WT and npq mutants

The quantitative distribution of the xanthophylls, chlorophylls and the carotenes in the leaves of *A. thaliana* WT and the *npq1* and *npq2* mutants is detailed in Table 4. The *npq1* mutant lacks violaxanthin de-epoxidase and hence is unable to convert *trans*-violaxanthin to *trans*-zeaxanthin under high light conditions, whereas the *npq2* mutant does not have the zeaxanthin epoxidase gene and thus accumulates *trans*-zeaxanthin constitutively [36]. This phenomenon is reflected in the results obtained in that the levels of *trans*-zeaxanthin in the *npq1* mutants are basal while the levels of *trans*-violaxanthin and *cis*-neoxanthin reflect those of the WT. In contrast, the *npq2* mutant has considerably higher levels of *trans*-zeaxanthin than the WT whereas the levels of *cis*-neoxanthin, *trans*-violaxanthin and *trans*-antheraxanthin are negligible. The results obtained are in agreement with values and trends reported previously [36,52]; where *cis*-neoxanthin (~1–5%), *trans*-violaxanthin (~10–28%), *trans*-lutein (~30–60%) and *trans*-β-carotene (~12–25%) were the predominant carotenoids present in unstressed WT plant leaf tissue.

4. Conclusion

As mentioned previously, the reliable and reproducible quantification of carotenoids is a complex task. To date a range of different methodologies have been used for the extraction, storage and HPLC analysis of carotenoids from a wide range of tissue types, with no specific method being preferred. Furthermore, the specific physico-chemical properties of each compound, which

varies considerably between individual carotenoids, makes use, handling and storage a precise science. We have outlined all problems encountered during authentic standard and sample preparation, handling and subsequent storage and have developed a clear step-by-step procedure for the quantitative profiling of the principal carotenoids in plant leaf tissue. This method has been demonstrated to effectively distinguish *A. thaliana* WT plantlets from two non-photochemical quenching mutants (*npq1* and *npq2*) and it is postulated that it might find application for all green leafy tissue samples. Although an evaluation of the scope of this method did not form part of the current study, it has recently found successful application for *Nicotiana tabacum* in our environment while *Vitis vinifera* and indigenous fynbos vegetation (Western Cape) will shortly be investigated.

Acknowledgements

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CHAPTER 4

RESEARCH RESULTS

**Investigations into the *In Planta* Role of
Grapevine β-LCY in *Arabidopsis***

Investigations into the *In Planta* Role of Grapevine β-LCY in *Arabidopsis*

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ABSTRACT

Carotenoids are targets for genetic engineering to increase the nutritional content of a number of crops and enhance the plant's innate protective mechanisms against daily and seasonal abiotic stress factors. This has lead to investigations into the *in planta* regulation and functioning of the genes involved in carotenogenesis as well as the *in vivo* functions of their encoded products. To learn more about the role of β-LCY within the carotenoid biosynthetic pathway, specifically in photosynthetically active tissues, the *Vitis vinifera β-LCY* was overexpressed in the non-fruit bearing model plant *Arabidopsis thaliana*. Four homozygous populations stably overexpressing the *Vvβ-LCY* were obtained containing between one and five copies of the transgene. All populations had a general plant morphology and phytohormone profile resembling that of the wild type (WT). High performance liquid chromatography (HPLC) analysis for quantification of the leaf pigments and carotenoids revealed that overexpression had decreased the neoxanthin and lutein content in the transgenics. The decreased lutein levels were not met by a specific increase in any intermediate within the β-branch (including β-carotene and the xanthophylls). Hence *Vvβ-LCY* was able to apportion the flux at the bifurcation point but was not able to specifically increase the carbon flow into this leg of the pathway, probably due to control points further upstream. A direct or indirect role for lutein in non-photochemical chlorophyll *a* fluorescence quenching (NPQ) of excess light energy is known. The decreased lutein levels did not, however, impact on NPQ over a range of actinic light intensities. With the exception of neoxanthin where levels remained lower in the transgenics, high light (HL) exposure revealed a similar total and individual carotenoid content in the WT and transgenic lines. A potential correlation was drawn between decreased neoxanthin (and perhaps lutein) and the increased lipid peroxidation observed in the transgenics. It is possible that the reduced neoxanthin results from silencing of the *Arabidopsis β-LCY* by the *Vvβ-LCY*; as it may function as a cyclic paralog, specifically since *Arabidopsis* does not contain a neoxanthin synthase *(NSY)*. Cumulatively these results provide additional evidence as to the *in planta* regulation and role of a grapevine β-LCY and extends our knowledge on carotenogenesis in chloroplasts. Furthermore, this work has outlined the necessity for transcriptional regulation analyses of some of the probable upstream control points in the transgenics and WT to confirm the possible regulatory mechanisms used to balance the carotenoid pigment levels as was seen in this study. A strategy to also increase the precursor pool in combination with an overexpression of β-LCY could also further advance our understanding of carotenogenesis in chloroplasts and the role of β-LCY therein.

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4.1 INTRODUCTION

The carotenoids lycopene and β-carotene are members of the most widely distributed group of natural pigments. Collectively carotenoids are responsible for many of the brilliant colours found in flowers and fruits. The carotenoid biosynthetic pathway is central in general plant metabolism with phytoene being recognised as the first C_{40} hydrocarbon precursor. Phytoene undergoes a series of desaturations to form lycopene (Hirschberg, 2001; Fraser and Bramley, 2004). The pathway branches at this point, which is of particular interest in this study and is pivotal for xanthophyll biosynthesis. Lycopene is partitioned to the competing cyclases according to their relative activities (Cunningham and Gantt, 2001), resulting in carotenes with two types of rings (Cunningham *et al.*, 1996): α–carotene, the immediate precursor to lutein, and β-carotene. The latter reaction is controlled by the enzyme lycopene β-cyclase (β-LCY) and involves sequential cyclisation of lycopene to produce β-carotene via γ-carotene. β-carotene is cardinal to several key leaf functions. It is intimately associated with chlorophyll in the photosynthetic reaction centres of the chloroplasts (Young, 1993) where it is involved in the assembly of the D1 and D2 polypeptides and is responsible for quenching singlet oxygen, thereby minimising lipid peroxidation (Paiva and Russell, 1999; Tracewell *et al.*, 2001).

β-carotene is in turn hydroxylated to form the xanthophyll zeaxanthin. Zeaxanthin cycles between antheraxanthin and violaxanthin under the action of two antagonistic light-dependent enzymes, zeaxanthin epoxidase (ZEP) and violaxanthin deepoxidase (VDE). This is the so-called xanthophyll cycle that plays an integral role in the non-radiative dissipation of excess absorbed light energy (Demmig-Adams *et al.*, 1996). In addition, violaxanthin, lutein and neoxanthin function as antioxidants and light-harvesting pigments in the chlorophyll antennae system. Subsequent oxidative cleavage of violaxanthin results in products that form abscisic acid (Schwartz *et al.*, 2003), the hormone that controls seed dormancy and is also the major plant stress hormone that regulates, amongst other things, stomatal conductance (Bentsink and Koornneef, 2002; Chaves *et al.*, 2003).

A number of biotechnological approaches have been launched to enhance the levels of carotenoids in a range of microorganisms (e.g. *Candida utilis*; Miura *et al.*, 1998; Shimada *et al.*, 1998; Schmidt-Dannert, 2000) and plant species (Giuliano *et al.*, 2000, Sandmann, 2001a and b; Fraser and Bramley, 2004), with particular attention being given to β-carotene. Transgenic tomato fruits have been produced with a 5-fold increase, and canola seeds with a 50-fold increase, in β-carotene (Shewmaker *et al.*, 1999; Dharmapuri *et al.*, 2002; Römer *et al.*, 2002) following manipulation of transcript levels of *phytoene synthase* (*PSY*) and *β-LCY*. The greatest success was the production of "golden rice" (Ye *et al.*, 2000; Beyer *et al.*, 2002; Al-Babili and Beyer, 2005) where yellow β-carotene bearing rice endosperm was obtained, by introducing genes for the entire β-carotene biosynthetic pathway.

Despite success achieved in generating transgenic crops with higher levels of provitamin A, genetic engineering currently aims to also increase plant productivity and tolerance to a range of biotic and abiotic stress factors (Lassner and Bedbrook, 2001). Amongst others, carotenoid biosynthetic genes have been identified as targets for manipulation, necessitating detailed investigations into the function and regulation of each gene. In this study, *β-LCY* was selected due to its integral position within the carotenoid biosynthetic pathway, responsible for converting lycopene into *β*-carotene- the substrate for both vitamin A and the xanthophylls. Furthermore, although chromoplastic *β-LCY* has been well studied, little research has been conducted into the regulation and functioning of chloroplastic *β-LCY*.

In this study, the functional *Vitis vinifera β-LCY* (*Vvβ-LCY*) was constitutively overexpressed in the non-fruit bearing model plant *Arabidopsis thaliana*. Levels of total and individual carotenoids in the *Vvβ-LCY* transgenic plants were quantified to ascertain whether the conversion of lycopene to *β*-carotene is a rate-limiting step in the synthesis of the downstream xanthophylls. The effects of *Vvβ-LCY* overexpression on the photosynthetic capacity of the plant including its ability to dissipate excess excitation energy were determined. The levels of several major plant hormones and the number of malondialdehyde equivalents were also measured. All analyses were conducted under low light (LL) and after a high light stress (HL) and were interpreted relative to untransformed wild type (WT) controls. The results obtained provide valuable insights towards the role of *Vvβ-LCY* in the chloroplasts.

4.2   MATERIALS AND METHODS

4.2.1 Plant material, growth conditions and induction of abiotic stress conditions

A. thaliana ecotype Columbia, obtained from the *Arabidopsis* Biological Resource Centre (Ohio State University, CO, USA), was used as WT and as the host for *Agrobacterium tumefaciens*-mediated transformations. Seeds of *A. thaliana* were sown by sprinkling them over the surface of a mixture of re-hydrated peat discs (Jiffy Products International, Norway) and vermiculite (3:1; w/w) in 5.5 cm diameter pots. The pots were covered with plastic lids to avoid seed desiccation and were cold stratificated for three days at 4°C. They were placed in a growth room at 23°C, 55% relative humidity with a 16h-light: 8h-dark cycle. Photon flux density in the light period was 43 µmol photons $m^{-2}.s^{-1}$. Plants were watered by irrigating the pots (~50 mL) twice weekly and a standard nutrient solution (Nitrosol, Fleuron, Universal Selected Services, Braamfontein, RSA) was applied fortnightly. The surface of the potting mixture was inspected regularly for fungal contamination. A 1% (v/v) solution of Funginex (Efecto, Silverton, RSA) was administered to prevent and control infection.

Transgenic populations of *Arabidopsis* were analysed for HL and drought stress. A statistically representative number of plantlets from each independent transgenic line and the WT control were grown under normal growth room conditions (LL) until approximately five weeks of age. Plants were arranged randomly in the growth room to normalise any variation in the growth conditions. For the application of a HL stress, plantlets were removed from the controlled growth room and subjected to full midday sunlight during mid winter for a period of 30 min at a light intensity of ~1400 µmol photons $m^{-2}.s^{-1}$ and a temperature of approximately 25°C. A drought stress was imposed by withholding water for a period of 18 days. Mature fully-expanded rosette leaves were harvested, frozen immediately in liquid nitrogen, freeze dried (FD) overnight and stored at -80°C prior to analysis as described in the relevant experimental section. Control samples were harvested two days prior to the application of the HL stress. However, for the drought tolerance experiment plants were divided into two groups; control plants subjected to a normal watering routine and stressed plants were watering was withheld.

4.2.2 Plasmids, bacterial strains and growth conditions

Unless otherwise stated, all *Escherichia coli* DH5α transformed constructs were inoculated in Luria Bertani (LB, Merck, NJ, USA) media, supplemented with the appropriate antibiotic(s) and incubated at 37°C, with shaking. *A. tumefaciens* strain GV3101 (carrying the helper plasmid pMP90; Koncz and Schell, 1986) containing the binary construct, pART27 (Gleave, 1992), was cultured at 30°C in LB media containing 0.1% (w/v) glucose, 150 µg.mL^{-1} rifampicin (chromosomal resistance), 15 µg.mL^{-1} gentamycin (helper plasmid resistance) and 25 µg.mL^{-1} kanamycin (binary plasmid resistance). In all cases an initial overnight culture (5 mL) was inoculated from a glycerol stock which was prepared from a single primary transformant colony and was stored at -80°C.

4.2.3 DNA manipulations and visualisation

All PCR amplifications were performed using ~10 ng plasmid DNA as template in a Biometra TRIO-Thermoblock (Westburg, Leusden, Netherlands) using the following basic program: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 35 s, annealing at 55°C for 35 s and elongation at 72°C for 1 min per kilobase, and a final elongation of 10 min at 72°C. All DNA fragments were separated in a 1% (w/v) agarose Tris-acetate-EDTA (TAE) gel and the fragments of interest were purified from the gel using the QIAquick Gel Extraction Kit as indicated by the manufacturer (Qiagen, Hilden, Germany). Unless otherwise indicated, all molecular biology techniques were performed as described by Sambrook *et al.* (1989). A digital camera (Alphaimager 1220; Alpha Innotech Corporation, San Leandro, CA, USA) supported by the AlphaEase v5.5 densitometry software (Alpha Innotech Corporation) was used to quantify the intensity of bands on ethidium bromide-stained gels and DIG-exposed chemiluminescent films (Hyperfilm ECL,

Amersham-Pharmacia Biotech, Buckinghamshire, UK). The full length cDNA clone, containing the *Vvβ-LCY* gene, was isolated as described previously (Young, 2004). The following primers were designed to amplify the coding sequence from cDNA: *β-LCY*ATG5': ATG gat act tta ctc aag act cat aat aag c-3' and *β-LCY*STOP5': gtt cca TCA tct taa tcc ttg tcc tg-3'. The start codon and the complement of the stop codon are indicated in capitals. The resulting full length cDNA PCR product (1515 bp) was cloned into the pGEM(T) Easy vector system (Promega Corporation, Madison, WI, USA) and sequenced using the M13 forward- and reverse-sequencing primers on an ABI Prism 3100 Genetic Analyser (Central Analytical Facility, Stellenbosch University, Stellenbosch, RSA).

4.2.4 Construction of plant transformation vector

The full length cDNA copy of *Vvβ-LCY* was excised from the pGEM(T) Easy construct and a two-step cloning strategy was followed to subclone the gene into the multiple cloning site of the primary cloning vector pART7 (Gleave, 1992). Firstly, a 261 bp *Eco*RI/*Spe*I fragment was cloned into the *Eco*RI/*Xba*I sites, followed by the larger 1266 bp *Eco*RI/*Eco*RI fragment into the *Eco*RI site. The resulting cDNA cassette was introduced into the dephosphorylated *Not*I sites of the binary plant transformation vector pART27 yielding *Vvβ-LCY*-pART27 (Gleave, 1992). The resulting clone was sequenced to confirm that cloning had not introduced a frame-shift. The recombinant binary construct was transferred from *E. coli* DH5α into *A. tumefaciens* strain GV3101 by electroporation (1100 V, 201 Ω, 25 μF, 5 ms; EasyJet, EquiBio, Kent UK).

4.2.5 Transformation of WT *A. thaliana* and selection of putative positive transformants

Six *A. thaliana* WT plantlets were selected as the target tissue for *A. tumefaciens*-mediated transformation of the *Vvβ-LCY*-pART27 construct via floral dipping as described by Clough and Bent (1998) using kanamycin resistance as the selection marker. Dipping was repeated six days later to increase transformation efficiency. An untransformed WT and transformed empty pART27 vector control were included. Seeds were harvested, with care being taken to ensure that the seed from transformation replicates remained separate to ensure independent progeny. Dried seeds were stored at room temperature (RT; for short term storage) or -20°C (for long term storage). T_0 refers to the original WT plants which underwent transformation. The plantlets which arise from the seed produced by these parental plants are denoted T_1 and all subsequent progeny are referred to as T_2, T_3 and T_4.

Putative *Vvβ-LCY* transformed seeds were vapour-phase sterilised in an aerobic chamber in the fume hood using a combination of 37% (v/v) HCl and bleach (calcium hypochlorite) granules for 4 h. The sterilised seeds were plated out at a density of approximately 1000 seeds per 50 cm^2 and grown in a controlled environment. Putative positive transformants were selected according to their ability to produce

green leaves and a well-established root system on half-strength Murashige and Skoog (MS) media (Murashige and Skoog, 1962) containing kanamycin (25 μg.mL^{-1}), hardened off and grown to maturity in the growth room. Homozygous plantlets stably overexpressing the *Vvβ-LCY* gene were identified by a 100% segregation of kanamycin-resistant plants in the T$_4$ progeny. Integration (Southern hybridisation) and expression (northern hybridisation) analyses were conducted to confirm these results.

4.2.6 Genetic analysis of *Vvβ-LCY* transgenic populations

For the transgene integration and expression analysis of the final T$_4$ population, 12 individuals from four independent *Vvβ-LCY* transgenic lines L7.8, L4.7, L15.5 and L18.10 were randomly chosen. In each case an *A. thaliana* WT was included as a negative control.

Genomic DNA (10 μg) was extracted from two small *A. thaliana* leaves according to the method of McGarvey and Kaper (1991) and digested with *Nco*I, which recognises two sites within the *Vvβ-LCY*-pART27 cassette. This produces a single hybridisation band of 1912 bp and a variable band of at least 2740 bp per integration event, depending on the point of integration in the model plant's genome. Restriction fragments were separated on a 0.8% (w/v) Tris-Borate-EDTA (TBE) agarose gel and blotted overnight onto a positively charged Hybond-N nylon membrane as described by the supplier (Amersham-Pharmacia Biotech) in 0.5x TAE. Pre-hybridisation took place in hybridisation buffer [50% (v/v) formamide, 7% (w/v) SDS, 5x SSC, 0.1% (w/v) *N*-lauroylsarcosine, 2% (w/v) casein and maleic acid buffer (pH 7.5)] at 42°C, with gentle shaking for a minimum of 2 h. The DIG labelled *Vvβ-LCY* cDNA probe was denatured for 15 min in a boiling water bath, cooled on ice (2 min) and added to fresh hybridisation solution (at a concentration of 25 ng.mL^{-1}). Hybridisation took place for at least 16 h, with gentle shaking, at 42°C. Blots were washed twice (for 20 min) in 2x SSC (containing 0.1% [w/v] SDS) at 42°C and 0.2x SSC (containing 0.1% [w/v] SDS) at 68°C. Detection was performed using high performance chemiluminescent film (Amersham-Pharmacia Biotech).

Total RNA (10 μg) was extracted from two small *A. thaliana* leaves in a buffer consisting of 200 mM Tris-HCl (pH 8.5), 1.5% (w/v) SDS, 300 mM LiCl, 10 mM Na$_2$EDTA, 1% (m/v) Na-deoxycholate, 1% (v/v) Nonidet P-40, 1% (w/v) meta-bisulphite and 5 mM thiourea (Joubert, 2004). 700 μL of this extraction buffer and 300 μL of phenol were utilised per extraction. Thereafter, sample preparation proceeded according to the method described for the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Samples were run on a 1.2% (w/v) agarose-formaldehyde gel and transferred overnight onto a positively charged Hybond-N nylon membrane as described by the supplier (Amersham-Pharmacia Biotech) in 0.5x TAE. Pre-hybridisation, hybridisation, post-hybridisation washes and detection took place as described above except that the hybridisation temperature was increased to 50°C.

4.2.7 Phenotypical analysis of WT and *Vvβ-LCY* transgenic populations

The morphology and growth of at least 20 T_4 generation plantlets per independent *A. thaliana Vvβ-LCY* line were compared with that of the WT. This included germination efficiency; overall growth rate; rosette formation; leaf size, number, colour and texture; bolt formation and length; and the total number of seeds formed. The root system was not inspected. To evaluate germination efficiency, 50-100 dried seeds of the homozygous T_4 generation transgenic lines and WT were spread on half-strength MS agar plates in triplicate. Seeds were monitored every 24 h for a total period of ten days and germination was scored when two fully expanded green cotyledons had developed.

4.2.8 Hormone analysis of WT and *Vvβ-LCY* transgenic populations

Phytohormone levels of 24 five-week-old T_4 generation plantlets per independent *A. thaliana Vvβ-LCY* line were measured under LL and immediately following the application of a HL stress. *A. thaliana* WT plantlets were included as control.

A preparative vapour phase extraction and electron impact-gas chromatography/mass spectrometry (EI-GC/MS)-based metabolic profiling approach of a number of acidic phytohormones was followed, as developed and described in detail by Schmelz *et al.* (2003, 2004). All solvents used during the sample preparation and EI-GC/MS analysis were of HPLC/GC grade. Plant tissue was treated (LL and HL exposure) and harvested, FD overnight, ground to a fine powder and 5 mg was aliquoted into a 1.9 mL eppendorf tube for extraction. Briefly, 300 µL 1-propanol:ddH_2O:conc. HCl (2:1:0.005) and 30 ng of the internal standard *o*-anisic acid (2-methoxybenzoic acid; Sigma-Aldrich, Steinheim, Germany) was added to the homogenised plant tissue, and vortexed thoroughly for 30 s. Dichloromethane (1 mL) was added per sample, mixed thoroughly for 5 s and centrifuged (12 500 g; 30 s, RT). The lower organic phase was removed and transferred to a 4 mL glass screw-cap vial (La-Pha-Pack, Werner Reifferscheidt, Langerwehe, Germany). Trimethylsilyldiazomethane (2 M in hexane; 4 µL; Fluka Chemie) was added, the vials were sealed with teflon-lined screw caps (La-Pha-Pack) and allowed to stand at RT for 30 min for complete conversion of all the phytohormone acids to their corresponding methylesters. The activity of the derivatisation reagent was subsequently quenched via the addition of acetic acid (2 M in hexane; 4 µL; Sigma-Aldrich). Extraction of the derivatised organic phase proceeded. An inert filter trap (containing ~20 mg SuperQ; Analytical Research Systems, Gainesville, Florida, USA) and a 22-gauge needle (delivering low pressure N_2 gas at a flow rate of 500 mL.min^{-1}) were both inserted into the septum. The apparatus set-up required for VPE is described in detail by Schmelz *et al.* (2004). Briefly, the sample vial was placed in an aluminium heating block (70°C) until all solvent had evaporated (~6 min), whereupon the vial was transferred to a second heating block set at 200°C for a period of 2 min. The trapped volatiles were then eluted with 150 µL dichloromethane and analysed by EI-GC/MS.

A Trace GC (ThermoFinningan, Milan, Italy) coupled to a Trace MS (Thermo MassLab, Manchester, UK) was used for all analyses. GC/MS conditions were amended from that described by Schmelz *et al.*, (2004). Briefly, 2 µL of the dichloromethane eluent was injected in the split/splitless injector of the GC, operated in the splitless mode (purge time 3.5 min, 50 mL.min^{-1}) at 280°C. Compounds were separated on a Factor Four VF5-MS capillary column (Varian, Palo Alto, CA, USA) with dimensions 30 m x 0.25 mm x 0.25 mm. Flow of the carrier gas (Helium) through the column was 0.7 mL.min^{-1}, in the constant flow mode. The oven program used was 40°C for 1 min, ramp 15°C.min^{-1} to 250°C (5 min), ramp 20°C.min^{-1} (2 min). In order to avoid carryover a post-run was performed after each analysis at 280°C under a head-pressure of 300 kPa. The temperature of the MS interface was kept at 280°C and the source at 200°C. The MS-detector was operated in EI mode at 70 eV and selected ion monitoring (SIM) mode. The electron multiplier voltage was set at 500 V. Three carboxylic acid methyl esters analytes were detected and quantified using SIM with retention times and ion mass to charge ratios (m/z) as follows: methyl salicylate (MeSA; 8.39 min, m/z 92, 120, 152); methyl indole-3-acetate (MeIAA; 13.81 min, m/z 130) and abscisic acid methyl ester (MeABA; 15.60 min, m/z 190). The internal standard, *o*-anisic acid methyl ester, was eluted at 9.70 min with m/z 92, 120 and 152.

External calibration curves were constructed for each analyte over the range from 8-800 pg. The regression equations and their correlation co-efficients in addition to the limit of quantification (LOQ) for each phytohormone investigated is detailed in Table 3.1. The hormone concentrations were determined from their respective standard curves and expressed as ng.g^{-1} FD tissue

Table 3.1: Calibration parameters determined for each phytohormone investigated

Phytohormone	Regression equation	r^2	LOQ (pg)
SA	y = 0.0133 x + 0.0009	0.9961	8
IAA	y = 0.0105 x - 0.2377	0.9952	8
ABA	y = 0.0030 x + 0.0515	0.9962	8

4.2.9 Pigment analysis of WT and *Vvβ-LCY* transgenic populations

Pigment concentrations were measured on 24 five-week-old T_4 generation plantlets per independent *A. thaliana Vvβ-LCY* line under LL and immediately following the application of a HL stress. *A. thaliana* WT plantlets were included as control.

All solvents used during the sample preparation and High Performance Liquid Chromatography (HPLC) analysis were of HPLC grade. Unless otherwise stated, we refer to the *trans*-form of each compound. *β*-carotene and neoxanthin are reported as the sum of their *cis*- (specifically 9-*cis* for *β*-carotene) and *trans*-forms. Plant tissue treated (LL and HL exposure) and harvested was FD overnight and ground to a fine powder. FD material was extracted according to Taylor *et al.* (2006). The

carotenoid pigments were separated by Reverse Phase (RP)-HPLC on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector (DAD) system as described in detail by Taylor *et al.* (2006). Identification of carotenoids was achieved by comparing their retention times and visible spectra with literature data (Oliver and Palou, 2000; Rodriguez-Amaya and Kimura, 2004), and with that of the authentic standards. The chl (*a* and *b*) concentration of the extracts was determined spectrophotometrically using the extinction coefficients in 100% methanol as described by Lichtenthaler and Wellburn (1983) and was expressed as nmol chl.mg^{-1} FD tissue. The absorbance measured at 666 nm and 653 nm was used in the following equations: chl *a* $=15.65 \times A_{666}$ - $7.34 \times A_{653}$; chl *b* $=27.05 \times A_{653}$ - $11.21 \times A_{666}$. The carotenoid concentration was determined from the standard curves and expressed as mmol.mol^{-1} chl *a*.

4.2.10 Physiological analysis of WT and *Vvβ-LCY* transgenic populations
4.2.10.1 Chlorophyll fluorescence
Modulated chlorophyll fluorescence measurements (FMS2 fluorimeter, Hansatech, King's Lynn, UK) were made on eight five-week-old T_4 generation plantlets per independent *A. thaliana Vvβ-LCY* line and *A. thaliana* WT control plants.

The PAR (Photosynthetically Active Radiation)/temperature leaf clip was placed on a fully-expanded rosette leaf. Plants were covered with a black bag and dark-adapted for 30 min. The fluorimeter's modulating light was turned on and F_o was measured. A saturating light pulse (PAR=~9000 µmol photons m^{-2}s^{-1} for 0.8 s) was then applied and F_m was determined. After allowing the fluorescence intensity to return to F_o, the actinic light was switched on at 21 µmol photons m^{-2}.s^{-1}. After 2 min the saturating light pulse was again applied to measure F_m'. The intensity of the actinic light was then increased and after 2 min F_m' again measured. This procedure was repeated to give measurements at a series of PAR values from 21 to 1856 µmol photons m^{-2}.s^{-1} After the measurement at 1856 µmol photons m^{-2}.s^{-1}, the leaf was darkened and recovery of F_m' measured for 25 min. After the pulse, the minimum fluorescence in the light-adapted state, F_o', was determined in the presence of the far red light by removal of the actinic light. Standard fluorescence nomenclature was used (Van Kooten and Snel, 1990) for the calculation of F_v/F_m, F_v'/F_m', NPQ, *qP*, *Φ*PSII, relative ETR, Q_r/Q_t, qE and qI.

4.2.10.2 Lipid peroxidation
The number of malondialdehyde (MDA) equivalents was determined in 24 plantlets five week-old T_4 generation plantlets per independent *A. thaliana Vvβ-LCY* line under LL and immediately following the application of a HL stress and *A. thaliana* WT control plants.

Lipid peroxidation was measured using the thiobarbituric acid-reactive-substances (TBARS) assay (Hodges *et al.*, 1999). Plant leaf tissue treated (LL and HL exposure) and harvested was FD overnight, ground to a fine powder and aliquoted (2 mg).

Ethanol (80% (v/v); 100 µL) was added, followed by centrifugation (3 000 g for 10 min at RT). The samples were diluted to 250 µL and a 100 µL aliquot was added to an equal volume of (i) (–) TBA solution [20% (w/v) trichloroacetic acid and 0.01% (w/v) BHT] (ii) (+) TBA solution [0.65% (w/v) TBA (Sigma-Aldrich), 20% (w/v) trichloroacetic acid and 0.01% (w/v) BHT]. The samples were vortexed vigorously, heated to 95°C in a heating block for 25 min, coole d at RT and centrifuged as before. Absorbances (Ultrospec III, Amersham-Pharmacia Biotech) were recorded at 440, 532 and 600 nm. The amount of MDA equivalents formed was calculated as follows and expressed per gram FD tissue: MDA equivalents (nmol.mL^{-1}) = $\left[\dfrac{(A-B)}{157000}\right]\times10^{6}$;

where A = $\left[\left(A_{532+TBA}-A_{600+TBA}\right)-\left(A_{532-TBA}-A_{600-TBA}\right)\right]$ and B = $\left(A_{440+TBA}-A_{600+TBA}\right)\times0.0571$

4.3 RESULTS

A functional β-LCY was isolated from *V. vinfera* cv Pinotage leaf tissue. The isolation and molecular characterisation of this gene has been comprehensively detailed by Young (2004).

4.3.1 Generation and molecular characterisation of *Vvβ-LCY* transgenic populations

In order to investigate the *in planta* role of grapevine β-LCY, the model plant *A. thaliana* was selected as the heterologous host. *A. tumefaciens*-mediated transformation of WT *A. thaliana* proceeded using the plasmid pART27.

Twenty-four putative primary transformants (T$_1$) were selected based on their ability to germinate and grow under antibiotic selective pressure (25 µg.mL^{-1} kanamycin). Integration of the *Vvβ-LCY* encoding gene into the model plant's genome was verified in all 24 primary transformants by Southern blot analysis, displaying between one and five integrations of the transgene (results not shown). Northern blot analysis revealed that all 24 primary transformants expressed the heterologous *Vvβ-LCY* gene (results not shown). The T$_2$-progeny seeds from the self-pollinated T$_1$-plants were harvested for all 24 transformant lines.

4.3.2 Segregation of the *Vvβ-LCY* transgenic populations

A stable homozygous transgenic T$_4$ population was obtained and four independent *A. thaliana Vvβ-LCY* transgenic lines were randomly chosen for subsequent investigations, namely L4.7 (5), L7.8 (1), L15.5 (4) and L18.10 (2). The number of integration events in each independent line is indicated in brackets and illustrated in (Fig. 4.1A), along with the corresponding transgene expression levels (Fig. 4.1B). There was no apparent correlation observed between the number of integration events and the level of transgene expression.
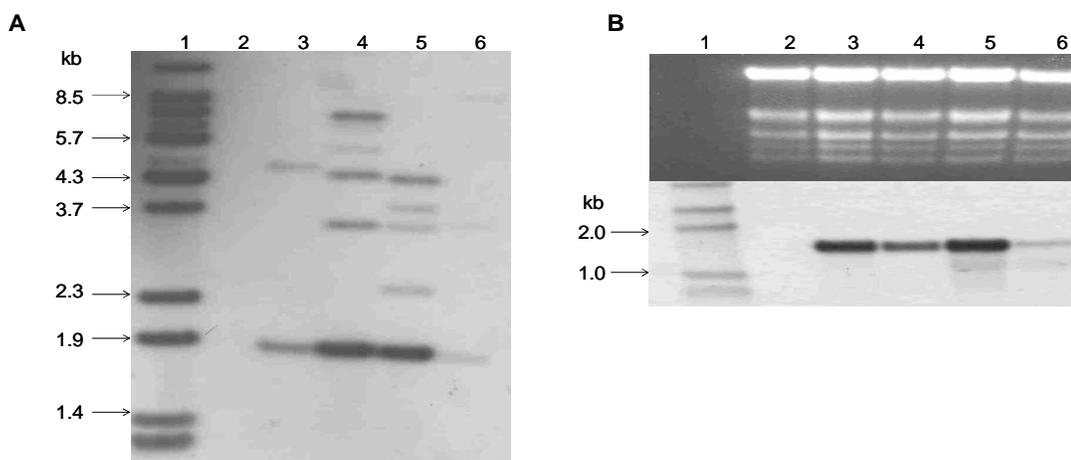
Figure 4.1. Genetic analysis of T_4 generation independent *A. thaliana* transgenic lines overexpressing *Vvβ-LCY*.

A. Southern blot analysis. Lanes **(1)**, λ DNA *Bst*EII-digested marker, with fragment sizes in kb; **(2)**, untransformed *A. thaliana* WT gDNA control; **(3-6)**, independent *A. thaliana Vvβ-LCY* transgenic lines L7.8, L4.7, L15.5 and L18.10, respectively.

B. Northern blot analysis. Lanes **(1)** High range RNA ladder (Fermentas Inc., Hanover, MD, USA), with fragment sizes in kb; **(2)**, untransformed *A. thaliana* WT total RNA control; **(3-6)** independent *A. thaliana Vvβ-LCY* transgenic lines L7.8, L4.7, L15.5 and L18.10, respectively.

4.3.3 Phenotypical analysis of the WT and *Vvβ-LCY* transgenic populations

Overall growth rate; rosette formation; leaf size, number, texture and colour; hypocotyl elongation; bolt formation and length of the *Vvβ-LCY* transgenic lines were not visibly different from the WT. Total number of seeds produced, germination rates and final germination percentage were also not significantly different from WT.

In addition, no appreciable differences in the morphology of the four T_4 generation independent *Vvβ-LCY* transgenic lines were observed relative to the WT following the application of a HL treatment (1400 μmol photons m^{-2}.sec^{-1}) or the withholding of water for a period of 18 days (results not shown).

4.3.4 Pigment concentrations, hormone levels and MDA equivalents in the WT and *Vvβ-LCY* transgenic populations before (LL) and after HL stress

Under LL conditions chl *a* and *b* concentrations of the *Vvβ-LCY* lines were not significantly different to that of the WT (Table 4.2), however chl *a* of line L7.8 was significantly higher than that of line L18.10. Chl *a/b* ratio of the transgenic lines was similar to that of the WT. The total carotenoid pool size of the *Vvβ-LCY* transgenic lines was smaller than that of the WT, however, this difference was only significant in lines L4.7 and L15.5. These differences can largely be attributed to decreases in two components, namely lutein and neoxanthin. The decrease in lutein was, however, not met by an increase in *β*-carotene, the product of lycopene cyclisation. In fact, the *β*-carotene concentration in line L4.7 was significantly lower than that of WT. Of the xanthophyll pigments the only significant difference between the *Vvβ-LCY* lines and the WT was that line L18.10 had an appreciably lower antheraxanthin concentration.

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The reduced levels of neoxanthin observed in the transgenics are largely due to *cis*-neoxanthin which comprises about 90% of total neoxanthin. The *cis*-isomer in the *Vvβ-LCY* lines was 25% to 46% lower than WT (results not shown). The decreased neoxanthin amounts in the *Vvβ-LCY* lines were not accompanied by increased levels of the phytohormone ABA (Table 4.3). SA levels in the *Vvβ-LCY* transgenics were similar to those of the WT with the exception of line L4.7 which had a 62% higher SA concentration than the WT. Similarly, the concentration of IAA in the *Vvβ-LCY* lines was not different to that in the WT. The number of MDA equivalents under LL conditions was similar in all lines (Table 4.4).

Exposure to HL (full winter sunlight, ~1400 µmol photons $m^{-2}.s^{-1}$ for 30 min) resulted in a significant decrease in chl *a,* chl *b* and the chl *a/b* ratio in line L7.8 but not in any of the other *Vvβ-LCY* lines or in the WT (Table 4.2; superscripts to left of means). After HL treatment the total carotenoid pool in all the *Vvβ-LCY* transgenic lines was similar to that of the WT (Table 4.2; superscripts to right of means). This is due to a significant decrease (5%) in the WT total carotenoid pool size but a significant increase (15%) of total carotenoids in line L4.7. HL stress did not significantly change the total carotenoid pool in lines L7.8, L15.5 and L18.10. As observed under LL, the neoxanthin concentration of the *Vvβ-LCY* lines was lower than the WT following HL. HL did not significantly alter the *cis*-neoxanthin levels in all the *Vvβ-LCY* lines and in the WT, however, the *trans*-neoxanthin concentrations were affected (results not shown). In contrast to LL, no significant differences were found in lutein concentration between the *Vvβ-LCY* lines and the WT. In line 4.7 HL caused a 22% and 13% increase in lutein and total *β*-carotene levels, respectively. As expected, HL induced xanthophyll cycling, converting violaxanthin to zeaxanthin and antheraxanthin. Consequently, the deepoxidation status of the total xanthophyll cycle pool - (Z + A) / (Z + A + V) - was significantly greater in all lines after light treatment (Table 4.2, superscripts to left of means). The total xanthophyll cycle pool (Z + A + V) of all light stressed *Vvβ-LCY* lines was larger than that of the light stressed WT, however, this was only significant in lines L7.8 and L18.10. Once again, the decreased neoxanthin concentrations in all *Vvβ-LCY* lines were not met by increased ABA levels (Table 4.3; superscripts to right of means). Furthermore, no changes in ABA concentrations were induced following HL exposure (Table 3.3; superscripts to left of means). The transgenic lines had similar SA concentrations to that of the WT (Table 4.3) with the exception of line L4.7 with an unexplainably, significantly higher level. Although no significant differences were recorded between the levels of IAA in the *Vvβ-LCY* lines and the WT post HL application, HL stress caused the concentration of IAA in all the transgenic lines and the WT to increase. However, this increase was only significant in the WT and *Vvβ-LCY* lines L4.7 and L18.10. Malondialdehyde equivalents following HL were similar in the WT and lines L4.7 and L7.8 (Table 4.4). However, HL treatment caused a higher degree of lipid peroxidation (as indicated by an increase in MDA equivalents) in lines L15.5 and L18.10.

Table 3.2: Pigment concentrations (mean ± standard error) in leaves of *A. thaliana* WT and *Vvβ-LCY* transgenic plants before and after HL stress. Chl *a* and chl *b* are expressed as nmol.mg^{-1} FD leaf tissue and carotenoid pigments as mmol.mol^{-1} chl *a*. Significant differences were recorded at $P \leq 0.05$ from ANOVA and the Unequal N HSD test. Where superscripts to the right are different it indicates that the difference between the row means is significant. Where superscripts to the left are different it indicates that the difference between before and after HL stress is significant.

Compound	WT	L4.7	L7.8	L15.5	L18.10
Before light stress:					
Total Carotenoids	[a]461.6±5.92[a]	[a]387.0±5.78[b]	[a]448.6±6.17[a]	[a]391.6±15.93[b]	[a]408.1±19.60[ab]
Neoxanthin	[a]34.2±0.73[a]	[a]25.4±0.53[b]	[a]26.0±0.47[b]	[a]19.9±0.95[c]	[a]18.8±2.03[c]
Lutein	[a]152.8±3.48[a]	[a]120.6±5.67[b]	[a]123.2±4.74[b]	[a]116.8±7.09[b]	[a]106.6±8.32[b]
Violaxanthin	[a]24.8±0.36[a]	[a]24.8±0.84[a]	[a]27.6±3.85[a]	[a]30.3±1.30[a]	[a]31.2±2.06[a]
Antheraxanthin	[a]2.6±0.12[a]	[a]2.3±0.21[a]	[a]3.1±0.16[ab]	[a]2.9±0.18[ab]	[a]3.8±0.33[b]
Zeaxanthin	[a]3.8±0.28[a]	[a]3.5±0.45[a]	[a]3.5±0.27[a]	[a]3.0±0.67[a]	[a]2.5±0.95[a]
β-Carotene	[a]243.4±4.30[ab]	[a]210.3±2.23[c]	[a]265.2±3.08[a]	[a]218.7±9.03[bc]	[a]245.2±8.30[ab]
V + A + Z[1]	[a]31.2±0.19[a]	[a]30.6±1.20[a]	[a]34.2±3.75[a]	[a]36.2±1.65[a]	[a]37.4±3.22[a]
(A + Z) / (V + A + Z)	[a]0.21±0.01[a]	[a]0.19±0.02[a]	[a]0.20±0.02[a]	[a]0.16±0.02[a]	[a]0.16±0.02[a]
Lutein / (V + A + Z)	[a]4.90±0.13[a]	[a]3.99±0.30[ab]	[a]3.81±0.36[ab]	[a]3.27±0.25[b]	[a]2.90±0.26[b]
Chl *a*	[a]15.4±0.93[ab]	[a]13.8±0.86[ab]	[a]17.2±0.84[a]	[a]15.1±1.09[ab]	[a]13.4±0.81[b]
Chl *b*	[a]9.5±0.62[a]	[a]8.8±0.48[a]	[a]10.5±0.56[a]	[a]10.3±1.10[a]	[a]9.1±0.91[a]
Chl *a*/*b*	[a]1.63±0.01[a]	[a]1.57±0.02[a]	[a]1.64±0.01[a]	[a]1.50±0.05[a]	[a]1.49±0.06[a]
After light stress:					
Total Carotenoids	[b]438.9±4.33[a]	[b]443.6±10.14[a]	[a]460.5±13.47[a]	[a]442.6±7.59[a]	[a]433.9±17.75[a]
Neoxanthin	[a]32.6±2.49[a]	[a]25.3±0.82[b]	[a]24.2±1.88[b]	[a]21.1±1.31[b]	[a]19.8±0.51[b]
Lutein	[a]137.0±5.39[a]	[b]146.7±5.10[a]	[a]129.7±2.43[a]	[a]138.5±2.45[a]	[a]125.6±13.10[a]
Violaxanthin	[b]11.3±0.88[ab]	[b]10.1±0.33[a]	[b]13.8±0.76[bc]	[b]12.3±0.87[abc]	[b]14.0±0.45[c]
Antheraxanthin	[b]8.3±0.23[a]	[b]9.1±0.48[ab]	[b]11.1±0.34[c]	[b]9.3±0.11[abc]	[b]10.2±0.36[bc]
Zeaxanthin	[b]14.1±0.65[a]	[b]15.7±0.38[ab]	[b]17.4±1.09[b]	[b]14.9±1.10[ab]	[b]16.9±0.56[ab]
β-Carotene	[a]235.6±3.25[a]	[b]236.9±5.29[a]	[a]264.3±9.15[b]	[a]246.5±8.41[ab]	[a]247.3±6.64[ab]
V + A + Z	[b]33.8±0.84[a]	[b]34.9±0.39[a]	[a]42.3±1.57[c]	[a]36.4±1.50[ab]	[a]41.2±1.00[bc]
(A + Z) / (V + A + Z)	[b]0.67±0.02[ab]	[b]0.71±0.01[a]	[b]0.67±0.01[ab]	[b]0.66±0.02[ab]	[b]0.66±0.01[b]
Lutein / (V + A + Z)	[b]4.08±0.21[a]	[a]4.21±0.16[a]	[a]3.08±0.07[b]	[a]3.82±0.15[ab]	[a]3.05±0.32[b]
Chl *a*	[a]13.9±0.58[a]	[a]13.1±0.83[a]	[b]12.9±0.64[a]	[a]15.3±0.94[a]	[a]13.8±0.26[a]
Chl *b*	[a]8.9±0.38[a]	[a]8.8±0.48[a]	[b]8.4±0.37[a]	[a]9.9±0.64[a]	[a]9.0±0.16[a]
Chl *a*/*b*	[b]1.55±0.01[a]	[a]1.50±0.03[a]	[b]1.54±0.01[a]	[a]1.55±0.01[a]	[a]1.54±0.01[a]

[1] V, Violaxanthin, A, Antheraxanthin, Z, Zeaxanthin

Table 3.3: Phytohormone profiles (mean ± standard error) in leaves of *A. thaliana* WT and *Vvβ-LCY* transgenic plants before and after HL stress. Phytohormones are expressed as ng.g^{-1} FD leaf tissue. Significant differences were recorded at $P \leq 0.05$ from ANOVA and the Unequal N HSD test. Where superscripts to the right are different it indicates that the difference between the row means is significant. Where superscripts to the left are different it indicates that the difference between before and after HL stress is significant.

Phytohormone	WT	L4.7	L7.8	L15.5	L18.10
Before light stress					
SA	[a]0.67±0.14[a]	[a]1.42±0.16[b]	[a]0.50±0.8[a]	[a]0.40±0.001[a]	[a]0.56±0.04[a]
IAA	[a]0.75±0.09[a]	[a]0.70±0.08[a]	[a]0.73±0.60[a]	[a]0.73±0.13[a]	[a]0.83±0.11[a]
ABA	[a]0.33±0.001[a]	[a]0.35±0.003[a]	[a]0.31±0.01[a]	[a]0.31±0.01[a]	[a]0.32±0.003[a]
After light stress					
SA	[a]0.83±0.12[a]	0.81±0.11[a]	0.69±0.12[a]	0.54±0.10[a]	0.48±0.03[a]
IAA	[b]1.19±0.03[a]	[b]1.24±0.02[a]	[a]1.19±0.16[a]	[a]1.13±0.07[a]	[b]1.36±0.17[a]
ABA	[a]0.32±0.01[a]	[a]0.36±0.02[a]	[a]0.29±002[a]	[a]0.31±0.01[a]	[a]0.33±0.02[a]

[1] IAA is presented as the sum of itself and its precursor IAAN (indole acetonitrile; Kawaguchi and Syōno, 1996).

Table 3.4: MDA equivalents (TBARS; mean ± standard error) in leaves of *A. thaliana* WT and *Vvβ-LCY* transgenic plants before and after HL stress. TBARS are expressed as nmol.g^{-1} FD leaf tissue. Significant differences were recorded at $P \leq 0.05$ from ANOVA and the Unequal N HSD test. Where superscripts to the right are different it indicates that the difference between the row means is significant. Where superscripts to the left are different it indicates that the difference between before and after HL stress is significant.

Treatment	WT	L4.7	L7.8	L15.5	L18.10
Before light stress	[a]15.20±1.12[a]	[a]15.86±0.89[a]	[a]14.65±0.75[a]	[a]13.56±0.73[a]	[a]13.82±0.68[a]
After light stress	[a]15.69±1.02[a]	[a]15.24±1.22[a]	[a]17.08±1.28[a]	[b]24.20±1.37[b]	[b]23.48±1.20[b]

4.3.5 Chlorophyll *a* fluorescence characterisation of the WT and *Vvβ-LCY* transgenic populations

The changes in chlorophyll *a* fluorescence parameters with increasing levels of PAR are shown (Fig. 3.2) and the parameter values at the highest PAR tested, as well as dark-adapted F_0, F_m and F_v/F_m values, compared between the WT and all four *Vvβ-LCY* lines are presented (Table 3.5). Dark-adapted F_o, F_m and hence F_v/F_m, the potential or maximum quantum yield of PSII, was similar in the *Vvβ-LCY* lines and the WT. Increasing actinic irradiation caused a decrease in ΦPSII and qP and an increase in NPQ and in the relative ETR (Fig. 3.2). No significant differences were recorded between the *Vvβ-LCY* transgenics and the WT control for all fluorescence

parameters investigated at 1856 µmol photons m^{-2}.sec^{-1} (Table 3.5). NPQ appeared to be induced more rapidly and to a greater final extent in the *Vvβ-LCY* lines compared to the WT (Fig 3.2A; Table 3.5) however these apparent differences were at no point statistically significant. Furthermore, the *Vvβ-LCY* transgenics relaxed to the same degree as the WT after 25 min with comparable fast (qE) and slow (qI) NPQ relaxing kinetics (Table 3.5).
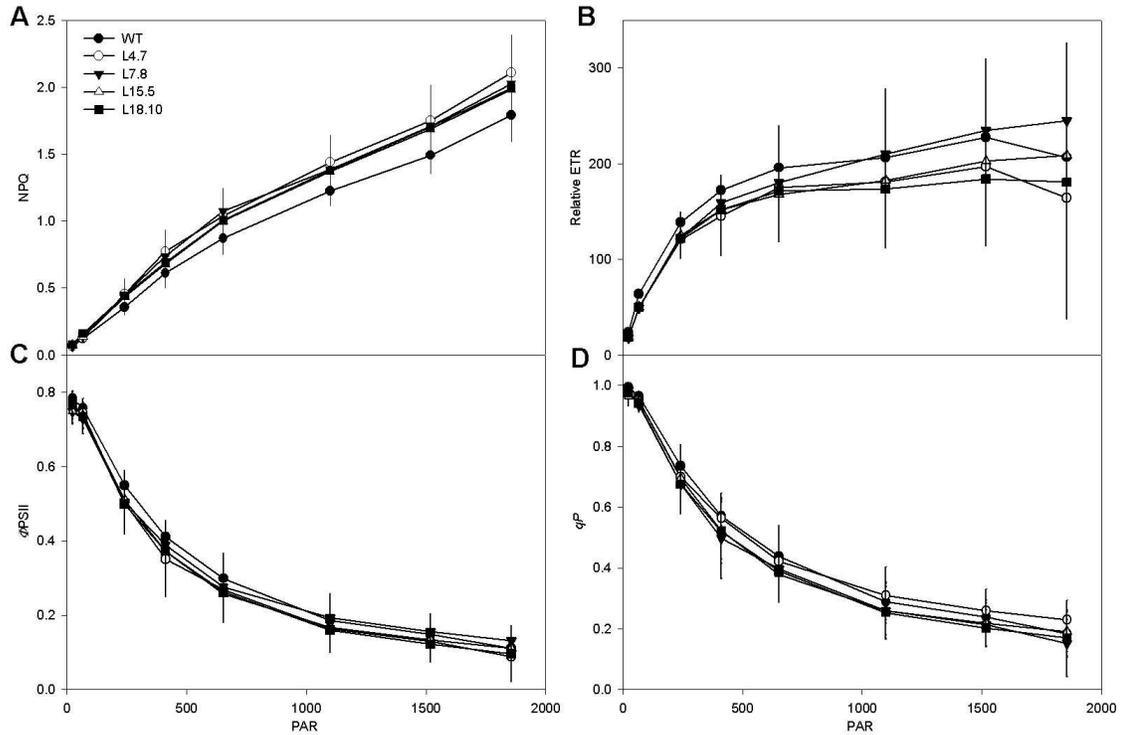
Figure 3.2. Light response curves of chlorophyll *a* fluorescence parameters in mature leaf tissue of five week-old *A. thaliana* WT and T$_4$ generation *Vvβ-LCY* transgenic lines L4.7, L7.8, L15.5 and L18.10.

(A) Non-photochemical quenching (NPQ), calculated as $(F_m'\text{-}F_m)/F_m'$. **(B)** The quantum efficiency of PSII photochemistry (ΦPSII), calculated as $(F_m\text{-}F_0)/F_m$ in the dark and $(F_m'\text{-}F_s)/F_m'$ in the presence of the actinic light. **(C)** The relative PSII electron transport rate, calculated as ΦPSII x PAR. **(D)** Photochemical quenching (qP), calculated as $(F_m'\text{-}F_s\text{-})/F_m'\text{-}F_0')$.

Table 3.5: Chlorophyll *a* fluorescence parameters (mean ± standard error) for leaves of five week old *A. thaliana* WT and *Vvβ-LCY* transgenic plants. Leaves were dark-adapted for 30 min, exposed to light at increasing PAR levels, up to 1856 µmol photons m^{-2}.s^{-1}, and then allowed to recover in the dark for 25 min. Different superscripts indicate that the differences between the WT and the *Vvβ-LCY* transgenic line means are significant at $P \leq 0.05$ using ANOVA and the Tukeys HSD test. Maximum (dark-adapted) quantum yields of PSII (F_v/F_m) and, for a PAR of 1856 µmol photons m^{-2}.s^{-1}, the effective quantum yields (ΦPSII), proportions of closed PSII reaction centers (Q_r/Q_t), quantum efficiencies of open PSII reaction centers (F_v'/F_m'), relative electron transport rates (Rel ETR), non-photochemical quenching (NPQ), and, after 25 min in the dark, the contributions of fast-relaxing quenching (qE) and slow-relaxing quenching (qI) to NPQ.

Light status	Parameter	WT	L4.7	L7.8	L15.5	L18.10
Light: PAR=1856 µmol photons m^{-2}.s^{-1}	F_0	575.0±17.52[a]	600.7±23.32[a]	608.3±38.24[a]	552.3±25.49[a]	593.5±11.72[a]
	F_m	3410.2±98.92[a]	3663.5±112.12[a]	3374.8±87.89[a]	3248.7±113.87[a]	3532.8±111.02[a]
	F_v/F_m	0.83±0.01[a]	0.84±0.002[a]	0.82±0.01[a]	0.83±0.004[a]	0.83±0.01[a]
	F_v'/F_m'	0.59±0.02[a]	0.57±0.02[a]	0.57±0.02[a]	0.57±0.02[a]	0.56±0.02[a]
	qP	0.18±0.03[a]	0.15±0.04[a]	0.23±0.03[a]	0.19±0.03[a]	0.17±0.02[a]
	ΦPSII	0.11±0.02[a]	0.09±0.03[a]	0.13±0.02[a]	0.11±0.02[a]	0.10±0.02[a]
	Rel ETR	206.7±39.62[a]	164.3±51.62[a]	244.9±33.48[a]	208.7±39.80[a]	180.7±32.95[a]
	Q_r/Q_t	0.82±0.03[a]	0.85±0.04[a]	0.77±0.03[a]	0.81±0.03[a]	0.83±0.02[a]
	NPQ	1.79±0.05[a]	2.11±0.119[a]	2.02±0.03[a]	1.98±0.16[a]	2.00±0.11[a]
Dark: Recovery time = 25 min	NPQ relax	0.26±0.02[a]	0.33±0.03[a]	0.29±0.04[a]	0.33±0.04[a]	0.36±0.03[a]
	qE	1.33±0.03[a]	1.55±0.10[a]	1.48±0.03[a]	1.44±0.12[a]	1.41±0.09[a]
	qI	0.47±0.02[a]	0.56±0.03[a]	0.55±0.05[a]	0.54±0.05[a]	0.59±0.04[a]
	qE/NPQ (%)	74±0.9[a]	73±0.7[a]	73±2.3[a]	73±1.3[a]	70±1.3[a]
	qI/NPQ (%)	26±0.9[a]	27±0.7[a]	27±2.3[a]	27±1.3[a]	30±1.3[a]

4.4   DISCUSSION

The β-carotene content in tomatoes (Dharmapuri *et al.*, 2002), rice (Ye *et al.*, 2000) and canola (Shewmaker *et al.*, 1999) has been significantly enriched following targeted expression of a β-*LCY*. During these experiments the role of β-*LCY* in the chromoplasts has been clarified. However, little knowledge is available regarding the role of a 'chloroplast-specific' β-*LCY*. To increase our fundamental knowledge of the regulation and functioning of chloroplastic β-*LCY*, a functional β-*LCY*, isolated from *V. vinifera* cv. Pinotage (Young, 2004) and named *Vvβ-LCY*, was overexpressed in *Arabidopsis*. *Vvβ-LCY* may be regarded to be a 'typical' β-cyclase as described by Cunningham *et al.* (1993, 1994 and 1996) and Pecker *et al.* (1996).

Vvβ-LCY transgenic populations resemble WT phenotypically and have similar phytohormone profiles

As the carotenoid biosynthetic pathway is integrally positioned in general plant metabolism, overexpression of the genes within this pathway has often altered the phenotype of the resulting transgenic plants (Niyogi *et al.*, 1998; Shewmaker *et al.*, 1999; Estévez *et al.*, 2001). In the current investigation, constitutive overexpression of *Vvβ-LCY* in *Arabidopsis* did not visually change the general plant morphology of the transgenics generated. Similar results have been reported by Rosati *et al.* (2000) and Dharmapuri *et al.* (2002) following fruit-specific expression of a β-*LCY* in a model plant. Furthermore, the independent constitutive overexpression of grapevine *ZEP* and *BCH* in *Arabidopsis* did not visually change the transgenic plant phenotype despite significant changes in some of the metabolite pools, particularly the xanthophylls (Brackenridge, 2006).

   The phytohormone profile covering ABA, SA and IAA was similar in the *Vvβ-LCY* transgenics and the WT. Furthermore, the application of a HL stress did not alter the *Vvβ-LCY* profile compared to that of WT. It is notable that the concentration of IAA in both the WT and transgenic lines was significantly higher after HL exposure. These increases are a result of normal plant response to an external trigger and are not specifically related to the introduced transgene as IAA is known to be upregulated in response to light (Slovin *et al.*, 1999; Reed, 2001). This study is the first to present the hormone profile in response to β-*LCY* overexpression and in combination with a light stress.

Constitutive levels of Vvβ-LCY lowers neoxanthin and lutein concentrations in the chloroplasts but does not appear to control carbon flux through the pathway

Constitutive overexpression of a β-*LCY* has been shown to overcome the normal regulatory mechanisms operating in the chromoplasts resulting in the constitutive accumulation of β-carotene (D'Ambrosio *et al.*, 2004). However, to date, the effects of constitutive overexpression of a β-*LCY* within the chloroplasts have not been reported.   The results obtained by Rissler and Pogson (2001) following *BCH*

antisense suppression in WT *Arabidopsis* were compared to the *Vvβ-LCY* overexpression work reported here as both experiments were attempting to accumulate *β*-carotene and to investigate the ensuing effects on carotenoid biosynthesis and general plant viability. In agreement with the current investigation, Rissler and Pogson (2001) found that *BCH* suppression did not alter the levels of chl *a* and *b* in the mutants. Furthermore, they observed no change in chlorophyll levels when plants were exposed to HL. Thus overexpression of *Vvβ-LCY* or suppression of *BCH* had not altered the rate of chlorophyll synthesis nor was the length of exposure to HL sufficient for photodamage of the chlorophyll-containing reaction centres to take place.

Rissler and Pogson (2001) found that reduced levels of the *BCH* transcript resulted in a decrease in the total carotenoid pool in five week-old plants largely due to decreases in violaxanthin and neoxanthin with 41% and 38% maximal reduction reported, respectively. Similarly, *Vvβ-LCY* overexpression decreased the total carotenoid pool in all transgenic lines largely due to a decrease in neoxanthin and, most notably, its *cis*-isomer.

Lutein levels in the *Vvβ-LCY* transgenics were different to those of the *BCH* mutants. The carotenoid biosynthesis pathway splits at lycopene, with the *ε*-carotene branch ending with lutein. It is thus possible that *Vvβ-LCY* overexpression directed the carbon flux away from lutein in favour of the *β*-branch. However, with the exception of antheraxanthin which increased slightly in line L18.10 and *β*-carotene which decreased significantly in line L4.7, no significant differences were recorded in the levels of *β*-carotene and any of the xanthophyll pigments (zeaxanthin, violaxanthin or antheraxanthin) when their concentrations in the *Vvβ-LCY* transgenics were compared to those of the WT. Thus it does not appear as if *Vvβ-LCY* overexpression has changed (increased/decreased) the carbon flux in the *β*-branch.

We compared the pigment profiles of two well-characterised lutein-deficient *Arabidopsis* mutants (*lut1* and *lut2)* with the pigment complement of the *Vvβ-LCY* transgenics (Pogson *et al.*, 1996). The decreased levels of lutein in the leaf tissue of the *lut1* and *lut2* mutants were offset by increases in violaxanthin and antheraxanthin, with *β*-carotene levels unchanging (Pogson *et al.*, 1998). In contrast, the decrease in lutein (and neoxanthin) in the *Vvβ-LCY* lines was not compensated for by an increase in xanthophylls but is instead the main cause for the lower total carotenoid pool although this was only significant in lines L4.7 and L15.5.

It thus seems that the rate of lycopene cyclisation is regulated by the amount of substrate (lycopene) available and not the abundancy of the cycling enzyme *β*-LCY. Misawa *et al.* (1994) and Römer *et al.* (2002) have speculated that the key regulatory steps determining substrate availability are controlled by PSY and/or PDS. It thus seems that constitutive levels of *Vvβ-LCY* controlled the bifurcation point between the *β*- and *ε*-branches, supporting the findings of Cunningham *et al.* (1996). However this occurs at the expense of neoxanthin and does not result in an increase in the carbon flux into the *β*-branch. For these reasons *Vvβ-LCY* does not appear to be a

regulatory step in the synthesis of β-carotene and/or the xanthophylls in chloroplasts. Enzyme kinetic analysis of *Vvβ-LCY* within *Arabidopsis* and quantification of the transcript levels of a number of genes recognised as control points within the pathway will provide additional evidence into the regulation of carotenogenesis in chloroplasts and define the role of a β-LCY.

Decreased lutein did not impact on the Vvβ-LCY transgenics' capacity for NPQ over a light range up to a maximum of 1856 μmol photons m^{-2}.s^{-1}

Light stress induces xanthophyll cycling, however, the concomitant effects of constitutive levels of *Vvβ-LCY* transcripts and HL on xanthophyll cycling in photosynthetically active tissues are not known. With the exception of line L4.7, HL did not change the total carotenoid complement in the transgenics.   In fact, in comparison to unstressed WT HL stress lowered the WT total carotenoid pool size due to slight decreases in lutein and β-carotene.   The decrease in the WT lutein/(Z + A + V) ratio provides evidence of a shift in the flux following transfer from LL to HL.   However, the levels of β-carotene and lutein were similar in the transgenics and the WT following HL exposure implying that the combination of light stress and constitutive levels of β-LCY did not alter the flux in the pathway from the ε-branch towards the β-branch in the leaf tissue.

   As expected, xanthophyll cycling occurred in response to HL whereby violaxanthin levels decreased in favour of zeaxanthin and antheraxanthin. An increase in the total xanthophyll cycle pool (Z + A + V) was recorded in the transgenic lines in comparison to the WT after HL, however this was only significant in lines L7.8 and L18.10. However, this did not translate into a larger deepoxidation ratio (Z + A)/(Z + A + V), a raw indication of a plants capacity for excess light dissipation during NPQ.   The pigment data indicates that the *Vvβ-LCY* transgenics and the WT should have an equivalent capacity for NPQ due to their similar zeaxanthin levels and deepoxidation ratios. However, the question remains as to whether decreased lutein levels at LL intensities impact on NPQ.   Previously the *lut1* and *lut2* mutants were shown to induce NPQ more rapidly due to a higher total xanthophyll pool (V + A + Z), but to a lower final extent than the WT, indicating that lutein plays a role in NPQ induction either directly of indirectly (Pogson *et al.*, 1998; Lokstein *et al.*, 2002). Although the average NPQ values in all four *Vvβ-LCY* transgenic lines investigated were higher than those of the WT they were not statistically significantly different at any of the PAR values tested.   Furthermore, the contribution of fast and slow relaxing components, qE and qI respectively, were similar in the transgenics and the WT. A number of studies have revealed that drastic alterations to plant pigments may take place (Niyogi *et al.*, 1998; Lokstein *et al.*, 2002) without deleterious consequences on NPQ as any shortfall in one specific pigment is counter-balanced by heightened levels of another.   It has also become clear that the xanthophyll cycle kinetics regulate NPQ rather than the absolute values of a specific pigment (Lokstein *et al.*, 2002). This can be attributed to the integral positioning of the xanthophylls bound to

the LHCs thereby maintaining the structural integrity of the photosystems and as such only a percentage of the detected compound can participate in NPQ of excess light energy. Consequently, the transgenics did not have an altered photosynthetic capacity nor was their NPQ ability improved upon or worsened.

Lower neoxanthin levels are potentially associated with increased lipid peroxidation in the Vvβ-LCY transgenics following HL exposure

Under LL conditions no significant differences were recorded in the number of MDA equivalents formed indicating that these growth conditions did not pose a threat of photodamage to the plant. However, on exposure to HL lines L15.5 and L18.10 had a significantly higher number of TBARS, 79% and 70% respectively, indicative of damage to the thylakoid membranes. β-carotene, lutein, violaxanthin and neoxanthin all function as antioxidants, with β-carotene closely associated with the chlorophyll molecules within the light harvesting complexes (LHCs). Of all the transgenic lines, line L15.5 and L18.10 showed the lowest levels of neoxanthin in comparison to the WT and the other two transgenic lines. Hence it is possible that the decrease in neoxanthin may account for the increase in lipid peroxidation. This degree of damage did not affect photosynthetic performance nor was it reflected as an increase in qI, the photoinhibition component of NPQ. These findings are partly supported by work conducted on ascorbate-deficient *Arabidopsis* mutants (Müller-Moulé *et al.*, 2004). No difference in the degree of lipid peroxidation was reported in mutants grown under LL or HL conditions. Furthermore, the lutein complement was the same under both growth conditions, although a decrease in β-carotene and, to varying degrees, neoxanthin was reported. An active role for neoxanthin and potentially lutein in lipid peroxidation is alluded to.

The role of Vvβ-LCY as a NSY cyclase paralog?

Phenotypically, the *Vvβ-LCY* lines responded to a brief exposure to HL or water deprivation in a manner identical to the WT. Contrastingly, D'Ambrosio *et al.* (2004) observed a drought tolerant phenotype in transgenic tomato plantlets accumulating high levels of β-carotene following constitutive overexpression of the tomato *β-LCY*. The authors speculated that the higher levels of β-carotene and β-carotene by-products (namely the photoprotective xanthophyll pigments and the phytohormone abscisic acid) may have conferred the drought tolerant phenotype observed in the transgenics. However, to date xanthophylls or ABA have not been measured to confirm this hypothesis. In the current investigation all the major carotenoids (carotenes and xanthophylls) present in the mature leaf tissue were quantified and observed significantly lower levels of lutein and neoxanthin under LL conditions. Furthermore, no changes in ABA concentrations in the *Vvβ-LCY* transgenics were determined in comparison to WT under LL.

The *Arabidopsis* genome does not contain a neoxanthin synthase (*NSY*) and thus it is believed that neoxanthin is synthesised by a bifunctional β-LCY (Fraser and

Bramley, 2004). It is thus feasible that overexpression of the *Vvβ-LCY* could have silenced the endogenous *Atβ-LCY* in the transgenic plants. In contrast, a NSY has been identified in tomato. Quantification of the transcript levels of the native *Arabidopsis β-LCY* could indicate whether this gene has indeed been downregulated by the heterologously expressed *β-LCY*.

4.5   CONCLUSION

Our results confirm that *β*-LCY controls the portioning of substrate between the *ε*-and *β*–branches of the carotenoid biosynthetic pathway. However, the conversion of lycopene to *β*-carotene is not regulated by *β*-LCY in photosynthetically active material, but instead this reaction is probably controlled by the amount of lycopene substrate present which, in turn, is determined by upstream rate-limiting steps. In this study it was shown that overexpression was not able to overcome these regulatory mechanisms to alter the carbon flux through the *β*-branch towards increased xanthophylls or increased levels of ABA. Instead the transgenic plants had decreased levels of lutein and neoxanthin. We postulate that the decreased neoxanthin results following silencing of the *Arabidopsis β-LCY* by *Vvβ-LCY*; as it may function as a NSY paralog. Transcriptional analysis is required to confirm this hypothesis. The reduced neoxanthin was also observed in the transgenics following exposure to HL stress and appears to be the main component contributing to the increase in lipid peroxidation taking place. Lutein has been shown to play a direct or indirect contributory role in NPQ yet the decreased lutein levels in the transgenics under normal growth conditions did not predispose the plants to a reduced capacity for NPQ following HL exposure. Detailed kinetic studies of *Vvβ-LCY in planta* and transcriptional analyses of genes regulating key control points will validate these findings. An overexpression strategy, whereby an upstream regulatory gene (eg. *PSY* or *PDS*) is overexpressed in combination with *β-LCY*, could also be investigated.

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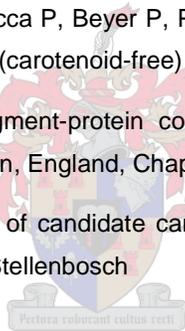
CHAPTER 5

RESEARCH RESULTS

In planta functional analysis of *Vv*NCED1 from grapevine in *Arabidopsis*

*In planta f*unctional analysis of *Vv*NCED1 from grapevine in *Arabidopsis*

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ABSTRACT

Water shortage is a major factor limiting plant growth and productivity and is predicted to worsen due to changes in the global climate. Abscisic acid (ABA) plays an important role in mediating a plant's response to a range of environmental stress factors, including drought. ABA formation is regulated by a 9-*cis* epoxycarotenoid dioxygenase (NCED), which has been shown to be upregulated during water stress and, when constitutively overexpressed, confers a drought-tolerant phenotype to the resulting transgenics. A full-length functional *NCED*, isolated from *Vitis vinifera* cv. Pinotage, was used for overexpression in *Arabidopsis*. A transgenic *A. thaliana* plant line collection was established and genetically characterised to confirm that the transgene was overexpressed. An *in planta* functional analysis of *VvNCED1* was conducted to assess the physiological effects of the transgene under normal growth conditions and following high light (HL) exposure and drought stress. In general, *VvNCED1* overexpression resulted in increased ABA levels, delayed seed germination and slightly stunted growth relative to the wild type (WT) control. Hormone profiling showed that salicylic acid levels were unaffected but indole acetic acid (IAA) levels were positively correlated to ABA concentrations. The transgenic lines had a lower chlorophyll complement than that of the untransformed control but, with the exception of violaxanthin, similar concentrations of carotenoids were found when expressed per mole chlorophyll *a*. The lower violaxanthin did not affect xanthophyll cycling in the transgenics following exposure to HL (2100 µmol photons $m^{-2}.s^{-1}$ for 30 min). Although the transgenic lines had a similar overall photosynthetic capacity to WT, non-photochemical quenching (NPQ) was, unexpectedly, markedly higher in the transgenics relative to WT. The fast-relaxing component of NPQ (qE) comprised the majority of this zeaxanthin-independent NPQ indicative of photoprotection. When the *VvNCED1* transgenics were water-stressed for 18 days, a marked increase in their tolerance to water stress was observed compared to the response of WT. Comparisons were drawn according to their pigment and hormone profiles, the maximum quantum yield of PSII, stomatal conductance, relative water content and soil moisture content and the degree of lipid peroxidation in the thylakoid membranes. Taken together these results provide a comprehensive summary of the physiological effects of *VvNCED1* on carotenoid biosynthesis and suggest a degree of crosstalk between ABA and IAA regulation. Furthermore, this study is the first reported case whereby *NCED* overexpression was found to increase the photoprotective capacity of the resulting transgenics and has extended our current knowledge of the factors affected during water stress and those parameters conferring drought tolerance.

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5.1 INTRODUCTION

The sesquiterpenoid hormone abscisic acid (ABA) was identified in the early 1960's (Addicott *et al.*, 1968) and was later established to be one of the important regulators co-ordinating plant responses to an applied external stress condition (Finkelstein and Rock, 2002; Seo and Koshiba, 2002; Xiong and Zhu, 2003). Furthermore, this ubiquitous hormone was found to play a central role in a number of plant physiological processes including the regulation of seed maturation and primary dormancy and influencing fruit development (Zeevaart and Creelman, 1988).

In higher plants the biosynthetic pathway of ABA was elucidated following $^{18}O_2$ feeding experiments, as well as a range of biochemical studies and molecular genetic analysis of a number of ABA-deficient mutants (Duckham *et al.*, 1991; Rock and Zeevaart, 1991; Marin *et al.*, 1996; Tan *et al.*, 1997; Burbridge *et al.*, 1999). These studies illustrated that ABA is primarily a downstream product of the carotenoid biosynthetic pathway beginning with the xanthophyll zeaxanthin. Under low light (LL) conditions, zeaxanthin epoxidase (ZEP) converts zeaxanthin to all-*trans*-violaxanthin (Demmig-Adams and Adams, 1996). The subsequent synthesis of 9-*cis*-neoxanthin and 9-*cis*-violaxanthin from all-*trans*-violaxanthin has not been completely unravelled, but is believed to involve a neoxanthin synthase (NSY; Nambara and Marion-Poll, 2005) and/or a *trans*-*cis*-isomerase (Strand *et al.*, 2000). The identification and comprehensive characterisation of the *viviparous 14* (*vp14*) mutant from maize was pivotal in establishing that the oxidative cleavage of these C_{40} 9-*cis* epoxycarotenoids is the primary dedicated step in ABA formation (Schwartz *et al.*, 1997; Tan *et al.*, 1997). This mutant was found to lack a *9-cis epoxycarotenoid dioxygenase* (*NCED*) which regulates the formation of xanthoxin, the direct precursor of ABA (Liotenberg *et al.*, 1999; Taylor *et al.*, 2000; Nambara and Marion-Poll, 2005). Subsequently, *NCED* homologues have been identified in a number of different dicotyledonous species, including tomato (Burbridge *et al.*, 1997, 1999), bean (Qin and Zeevaart, 1999), avocado (Chernys and Zeevaart, 2000), cowpea (Iuchi *et al.*, 2000), *Arabidopsis* (Iuchi *et al.*, 2001; Tan *et al.*, 2003), grapevine (Soar *et al.*, 2004), oranges (Rodrigo *et al.*, 2006), peanut (Wan and Li, 2006) and the flowers of *Gentiana lutea* (Zhu *et al.*, 2006).

The differential developmental, tissue-specific and stress-induced regulation of ABA biosynthesis has, to date, largely been studied on a transcriptional level. Under water limited conditions, transcript levels of a number of ABA biosynthetic genes were found to be upregulated, with *NCED* induction (Qin and Zeevaart, 1999) in both roots and shoots being prominent (Schwartz *et al.*, 2003; Xiong and Zhu, 2003; Nambara and Marion-Poll, 2005). Consequently high levels of ABA accumulated which resulted in decreased stomatal conductance. Improved drought tolerance has been reported following the inducible and/or constitutive overexpression of tomato *NCED1* in both tomato and tobacco (Thompson *et al.*, 2000a), bean *NCED1* in tobacco (Qin and Zeevaart, 2002) and *Arabidopsis NCED3* in *Arabidopsis* (Iuchi

et al., 2001). These findings have recognised *NCED* as a potential target for genetic manipulation in order to confer a drought-tolerant phenotype.

Previous work has established a strong correlation between *VvNCED1* expression, xylem sap and bulk leaf ABA, and stomatal conductance in grapevine canes following girdling experiments (Soar *et al.*, 2004). A relationship between increased ABA and reduced stomatal conductance following partial rootzone drying (PRD) of irrigated grapevine has also been shown (Stoll *et al.,* 2000). Thus *VvNCED1* expression, ABA levels, reduced stomatal conductance and increased tolerance to water stress are interdependence. The current study uses this well-established relationship to functionally analyse *VvNCED1* from grapevine (*V. vinifera* L. cv. Pinotage) in *Arabidopsis*. The native expression pattern of the isolated gene during berry development and in the shoot tips, leaves and flowers was also determined. The *VvNCED1* gene was constitutively overexpressed in *A. thaliana* and a homozygous population was established. The comprehensive analysis of this population and the comparisons with the wild type (WT) and relevant *Arabidopsis* mutants provided information on the whole plant phenotype, the leaf pigment and hormone profiles, the photosynthetic rate and non-photochemical quenching (NPQ) capacities. These analyses were also performed after a high light (HL) and drought stress treatment.

5.1   METHODS AND MATERIALS

5.2.1 Plant material, growth conditions and induction of abiotic stress conditions

V. vinifera L. (cv. Pinotage) fully-expanded photosynthetically active leaf tissue was harvested during the summer months from the Welgevallen experimental farm (Stellenbosch, RSA). Tissue was immediately flash frozen in liquid nitrogen and stored at -80°C prior to use.

A. thaliana ecotype Columbia, obtained from the *Arabidopsis* Biological Resource Centre (Ohio State University, CO, USA) was used as WT and as the host for *Agrobacterium tumefaciens*-mediated transformations. The growth conditions as well as the drought and light stress were as described in Chapter 4, section 4.2.1 of this dissertation, with the exception that the plants in this experiment were exposed to ~2100 μmol photons m^{-2}.s^{-1} at temperature of approximately 30°C for the light stress.

5.2.2   Plasmids, bacterial strains and growth conditions

All PCR products obtained were cloned into the pGEM(T) Easy vector system according to the manufacturer's specifications (Promega, Madison, WI, USA). Standard methodology for handling plasmids and bacterial strains were used and is the same to what is outlined in section 4.2.2 of Chapter 4.

5.2.3   DNA manipulations and visualisation

Genomic DNA was isolated from *V. vinifera* L. (cv. Pinotage) fully expanded leaves using a cetyltrimethylammonium bromide (CTAB)-based protocol as described by Steenkamp *et al.* (1994).   Plasmid DNA was isolated from cells of *E. coli* transformants by a modification of the rapid alkali method of Birnboim and Doly (1979) as described by Sambrook *et al.* (1989).   For PCR amplification conditions, DNA fragment purification and DNA and RNA quantification see section 4.2.3 of Chapter 4.

5.2.4   Isolation of total RNA and quantitative RT-PCR to determine the expression profile of *VvNCED1* in grapevine tissues

V. vinifera L. Pinot noir tissues were collected from plants in the field collections of the Istituto Agrario di San Michele all'Adige (Trento, Italy).   The berry samples were harvested in 2003 on 2nd July, 16th July and 6th August, and correspond to two weeks pre-véraison, véraison and two weeks post-véraison, respectively.   From a pool of fifty randomly selected berries, three pools of eight berries each were selected for RNA extraction.   The leaf, shoot tip and flower tissues were randomly selected and harvested in the 2005 growth season.

RNA was isolated from grapevine tissue according to the method described by Moser *et al.* (2004).   cDNA was synthesised from 1 μg of DNase I-treated (Promega) total RNA using the Superscript III Platinum first strand synthesis system (Invitrogen, Carlsbad, CA, USA) in a 20 μL reaction volume.   A duplicate reaction was performed without the reverse transcriptase to verify the absence of genomic DNA in the RNA extractions.   The forward primer was designed within the coding sequence, and the reverse primer within the 3'-untranslated region (UTR; See addendum to Chapter 5 Table 5.8).   Actin was employed as the house-keeping gene for relative quantification of expression.   The specificity of the primer pairs was verified by examination of the amplified PCR products in a 2% (w/v) agarose gel and melting curve analysis (data not shown).   Quantitative RT-PCR was performed using an Applied Biosystems (Foster City, CA, USA) ABI Sequence Detection System 7000 and the Platinum SYBR Green qPCR SuperMix UDG reagents (Invitrogen) according to the manufacturer's instructions.   Reaction mixtures contained 2 μL of cDNA (of a 1:25 dilution of the first-strand reaction) as template, 0.2 μM of each primer, 0.25 μL ROX and 6.25 μL of SYBR green reagent in a final volume of 12.5 μL.   The program for the PCR reactions was: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 15 s at 95°C and 30 s at 55°C.

Data was analysed using the Sequence Detection System (SDS) software (Applied Biosystems).   All PCR reactions consisted of at least three technical repeats.   The cycle threshold (Ct) value is directly related to the amount of PCR product and thus the original amount of target present (Leutenegger, 2001).   Hence transcript abundance of each gene was normalised independently to actin by subtracting of the Ct value of the house-keeping gene from the Ct value of

VvNCED1; where ΔCt = ΔCt$_{actin}$- ΔCt $_{VvNCED1}$. Transcript abundance of *VvNCED1* in both the control and sample tissues was obtained from the equation [(E$_{target}$) $^{ΔCt \ target \ (control-sample)}$/(E$_{reference}$) $^{ΔCtreference(control-sample)}$], where *E* is the PCR efficiency, as described by Ramakers *et al.* (2003), and is derived from the log slope of the fluorescence versus PCR cycle number in the exponential phase of each individual amplification plot, using the equation (1 + E) = 10^{slope}.

5.2.5   Construction of plant transformation vector

The full length cDNA copy of *VvNCED1* (see addendum A to this chapter) was excised from the pGEM(T) Easy construct and a two-step cloning strategy was followed to subclone the gene into the multiple cloning site of the primary cloning vector pART7 (Gleave, 1992). Firstly, a 429 bp *Sal*I/*Kpn*I fragment was cloned into the *Xho*I/*Kpn*I sites, followed by the larger 1466 bp *Kpn*I/*Spe*I fragment into the *Kpn*I/*Xba*I sites.   The resulting cDNA cassette was introduced into the dephosphorylated *Not*I sites of the binary plant transformation vector pART27 (Gleave, 1992), yielding *VvNCED1*-pART27. The resulting clone was sequenced to confirm that cloning had not introduced a frame-shift.   The recombinant binary construct was transferred to *A. tumefaciens* strain GV3101 by electroporation (1100 V, 201 Ω, 25 μF, 5 ms; EasyJet, EquiBio, Kent UK).

5.2.6 Transformation of WT *A. thaliana* and selection of putative positive transformants

Six *A. thaliana* WT plantlets were selected as the target tissue for *A. tumefaciens*-mediated transformation of the *VvNCED1*-pART27 construct via floral dipping as described by Clough and Bent (1998) using kanamycin resistance as the selection marker. An untransformed WT and transformed empty pART27 vector control were included.

5.2.7   Genetic analysis of *VvNCED1* transgenic populations

For the transgene integration and expression analysis of the final T$_4$ population, 12 individuals from four independent *VvNCED1* transgenic lines N3.5, N6.2, N10.6 and N17.2 were randomly chosen. In each case an *A. thaliana* WT was included as a negative control.

   Isolation of DNA and RNA as well as the conditions for Southern and northern hybridisation were as for section 4.2.6 of Chapter 4.

5.2.8   Phenotypical analysis of the WT and *VvNCED1* transgenic populations

The morphology and growth of at least 20 T$_4$ generation plantlets per independent *A. thaliana VvNCED1* line were compared with that of the WT.

5.2.9 Hormone analysis of the WT and *VvNCED1* transgenic populations

Phytohormone levels of nine eight week-old T_4 generation plantlets per independent *A. thaliana VvNCED1* line N3.5, N6.2 and N17.2 were determined under ambient growth conditions. Concomitantly, the hormone profile of nine eight-week-old T_4 generation independent *A. thaliana VvNCED1* lines N3.5 and N17.2 was determined after water had been withheld for a period of 18 days. *A. thaliana* WT plantlets were included as control.

The vapour phase extraction protocol and the EI-GC/MS conditions were comprehensively described in section 4.2.8 of Chapter 4. External calibration curves were constructed for each analyte over the range from 8-800 pg. The regression equations and their correlation co-efficients in addition to the limit of quantification (LOQ) for each phytohormone investigated in this experiment is detailed in Table 5.1). The hormone concentrations were determined from their respective standard curve and expressed as $ng.g^{-1}$ FD tissue.

Table 5.1: Calibration parameters determined for each phytohormone investigated

Phytohormone	Regression equation	r^2	LOQ (pg)
SA	y = 0.0093 x + 0.2511	0.9924	8
IAA	y = 0.0030 x + 0.0238	0.9964	8
ABA	y = 0.0014 x + 0.0009	0.9942	8

5.2.10 Pigment analysis of the WT and *VvNCED1* transgenic populations

Pigments were quantified for 30 five-week-old T_4 generation plantlets per independent *A. thaliana VvNCED1* line N3.5, N6.2 and N17.2 grown under LL and immediately following the application of a HL treatment. Carotenoid and chlorophyll (chl) contents of nine eight week-old T_4 generation plantlets per independent *A. thaliana VvNCED1* line N3.5 and N17.2 were determined following drought stress or a controlled watering routine. The latter experiment was conducted using an HPLC system that could not detect antheraxanthin. For the light treatment experiment, a new HPLC method was used (Taylor *et al.*, 2006) that could detect antheraxanthin. *A. thaliana* WT plantlets were included as control.

For sample extraction, HPLC running conditions and identification of carotenoids see section 4.2.9 of Chapter 4.

5.2.11 Physiological analysis of the WT and *VvNCED1* transgenic populations
5.2.11.1 Chlorophyll fluorescence

Standard modulated chlorophyll fluorescence measurements were performed using an FMS2 instrument (Hansatech, King's Lynn, UK) on six five-week-old T_4 generation plantlets per independent *A. thaliana VvNCED1* line N3.5, N6.2 and N17.2 grown under LL. F_o, F_m and were determined for 12 eight-week-old T_4 generation plantlets per independent *A. thaliana VvNCED1* line N3.5 and N17.2 which received a normal

watering regime or following water being withheld for 18 days. *A. thaliana* WT plantlets were included as control.

For the chlorophyll fluorescence protocol see section 4.2.10.1 of Chapter 4 with the amendment that measurements were taken at a series of PAR values from 21 to 1305 µmol photons $m^{-2}.s^{-1}$.

5.2.11.2   Lipid peroxidation

The number of malondialdehyde equivalents (MDA) was determined in nine eight-week-old T_4 generation plantlets per independent *A. thaliana VvNCED1* line N3.5 and N17.2 following drought stress or a controlled watering routine. *A. thaliana* WT plantlets were included as control.

Lipid peroxidation was measured using the thiobarbituric acid-reactive-substances (TBARS) assay (Hodges *et al.*, 1999). Leaf tissue was treated and harvested as described previously, with the exception that the samples were not frozen in liquid nitrogen but rather held on ice directly proceeding harvest. Tissue (~1 g) was homogenised in 80% (v/v) ethanol with glass beads (426-600 µm; Sigma-Aldrich) and centrifuged (3 000 g for 10 min at 4℃). Six 2 00 µL aliquots of the supernatant were placed in 1.5 mL self-lock eppendorf tubes and 200 µL of (i) (–) TBA solution [20% (w/v) trichloroacetic acid and 0.01% (w/v) BHT] was added to three tubes and (ii) (+) TBA solution [0.65% (w/v) TBA (Sigma-Aldrich), 20% (w/v) trichloroacetic acid and 0.01% (w/v) BHT] was added to the remaining three tubes. The samples were vortexed vigorously, heated to 95℃ in a boiling wa ter bath for 25 min and subsequently cooled on ice for 5 min. The samples were centrifuged as before and their absorbances (Ultrospec III, Amersham-Pharmacia Biotech) recorded at 440, 532 and 600 nm. The amount of MDA equivalents formed was expressed per gram fresh weight tissue and calculated as follows:

$$\text{MDA equivalents (nmol.mL}^{-1}) = \left[\frac{(A-B)}{157000} \right] \times 10^6 \, ;$$

where A $= \left[(A_{532+TBA} - A_{600+TBA}) - (A_{532-TBA} - A_{600-TBA}) \right]$ and B $= (A_{440+TBA} - A_{600+TBA}) \times 0.0571$.

5.2.11.3   Stomatal conductance

Twelve eight-week-old T_4 generation plantlets per independent *A. thaliana VvNCED1* line N3.5 and N17.2 following drought stress or a controlled watering routine were used for the determination of stomatal conductance. *A. thaliana* WT plantlets were included as control.

Stomatal conductance of the youngest fully-expanded rosette leaf between 6-8 h into the light period was measured using a steady state porometer (PMR-4; PP Systems, UK).

5.2.11.4   Water status: Relative water content and soil moisture content

Three eight-week-old T_4 generation plantlets per independent *A. thaliana VvNCED1* line N3.5 and N17.2 were used to determine the relative water content (RWC;

Munné-Bosch and Alegre, 2000) and the soil moisture content (SMC) following drought stress or a controlled watering routine. *A. thaliana* WT plants were included as control.

For determination of the RWC, the intact rosette was excised from the root system and weighed to determine the initial mass (P_i). The rosette was subsequently floated in distilled water at RT for 16 h to determine the turgid mass (P_t). The dried mass (P_d) was obtained by drying the sample at 70°C for 16 h. RWC was calculated using the equation: $\dfrac{(Pi - Pd)}{(Pt - Pd)}$. Similarly, the SMC was calculated using $\dfrac{(Si - Sd)}{Sd}$ following determination of the mass of the soil (and the roots) in the pots (initial mass, S_i) prior to drying out overnight (16 h) at 70°C (dried mass, S_d).

5.3 RESULTS

5.3.1 Expression profiles of the native *VvNCED1* in grapevine tissues

Relative to the expression of the housekeeping gene, actin, *VvNCED1* was found to be upregulated (2.2-fold) during véraison with basal levels recorded two weeks pre-véraison and two weeks post-véraison (Fig. 5.1A). When the vegetative tissues were analysed a ~5-fold increase in *VvNCED1* levels in leaf tissue and a ~13-fold increase in the flowers was found relative to shoot tips (Fig. 5.1B).
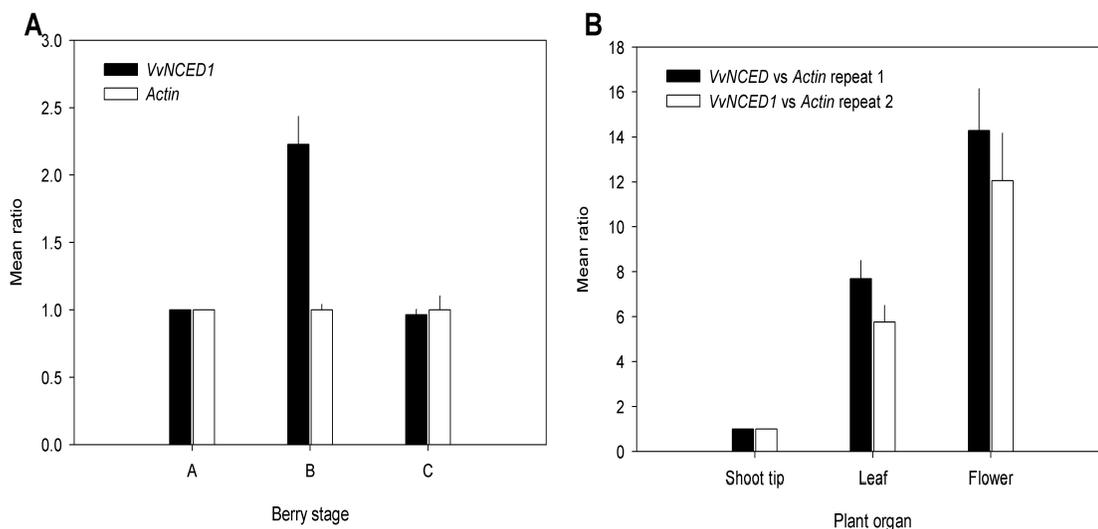
Figure 5.1. Quantitative RT-PCR analysis of *V. vinifera* L Pinot noir plant tissues showing developmental and tissue specific expression of *VvNCED1*.

(A) Berry stages A, B and C correspond to two weeks pre-véraison, véraison and two weeks post-véraison, respectively; **(B)** Vegetative plant organs normalised against actin and expressed relative to shoot tip.

5.3.2 Generation and molecular characterisation of *VvNCED1* transgenic populations

The functional analysis of the *VvNCED1* gene was performed in the model plant *A. thaliana*. *A. tumefaciens*-mediated transformation of WT *A. thaliana* yielded thirteen putative primary transformants (T_1), selected based on their ability to germinate and grow under antibiotic selective pressure (25 µg.mL^{-1} kanamycin). Integration of the *VvNCED1* encoding gene into the model plant's genome was verified in all 13 primary transformants by Southern blot analysis, displaying between one and ten integrations of the transgene (results not shown). Northern blot analysis revealed that 11 of the 13 primary transformants expressed the heterologous *VvNCED1* gene (results not shown). The T_1-progeny seeds from the self-pollinated T_0-plants were then harvested for all eleven northern positive lines.

5.3.3 Segregation of the *VvNCED1* transgenic populations

To obtain a stable homozygous transgenic T_4 population, each independently transformed T_1 plant line was self-propagated to the fourth generation. During this process, all putative transgenic lines were analysed according to their segregation under kanamycin selection, the presence of the transgene in the model plant's genome and the stable expression of the heterologous gene. Four independent *A. thaliana VvNCED1* transgenic lines were chosen for subsequent investigations, namely N3.5 (3), N6.2 (~9), N10.6 (3) and N17.2 (4). The number of integration events is indicated in brackets and illustrated in (Fig. 5.2), along with the corresponding transgene expression levels (normalised to RNA). There was no apparent correlation observed between the number of integration events and the level of transgene expression.
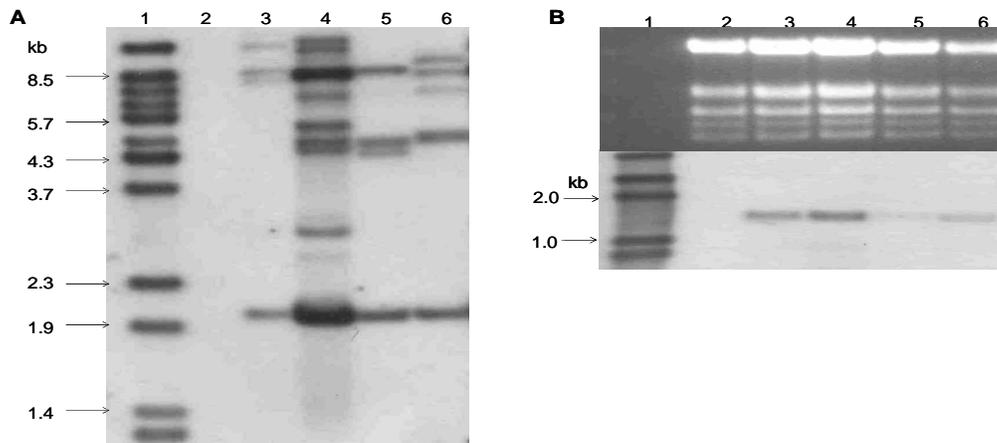
Figure 5.2. Genetic analysis of T$_4$ transgenic lines stably overexpressing *VvNCED1*.

A. Southern blot analysis. Genomic DNA (10 μg) was digested with *Sac*I, separated by gel electrophoresis and transferred to a nylon membrane. Lanes **(1)**, λ DNA *Bst*EII-digested marker, with fragment sizes in kb; **(2)**, untransformed *A. thaliana* WT gDNA control; **(3-6)**, independent *A. thaliana VvNCED1* transgenic lines N3.5, N6.2, N10.6 and N17.2 respectively

B. Northern blot analysis. Total RNA (10 μg) was isolated from leaf material, separated by gel electrophoresis (above) and transferred to a nylon membrane (below). Lanes **(1)** High range RNA ladder (Fermentas Inc., Hanover, MD, USA), with fragment sizes in kb; **(2)**, untransformed *A. thaliana* WT total RNA control; **(3-6)** independent *A. thaliana VvNCED1* transgenic lines N3.5, N6.2, N10.6 and N17.2 respectively.

5.3.4 Phytohormone profiling reveals increased ABA and associated phenotypes in *VvNCED1* transgenic populations

Significantly higher ABA concentrations than in WT were found in *VvNCED1* transgenic lines N3.5 and N17.2 (Table 5.2). These lines showed a significant delay in seed germination (Fig. 5.3). The general morphology of plantlets of line N3.5 was slightly smaller yet visually similar to that of the WT (Fig. 5.4). In contrast, the growth rate of line N17.2 was always slower than that of the WT and as such these plants were generally visually smaller in size (Fig. 5.4). There was no significant difference in SA levels between the transgenic lines and the WT. Line N3.5 had a significantly higher IAA concentration than WT.

ABA, SA and IAA levels in line N6.2 were not significantly different to the WT (Table 5.2). This line showed a more complete germination rate than the WT control, with no delay in seed germination (Fig. 5.3). Phenotypical analysis of plants of line N6.2 revealed a slight growth retardation at three weeks of age, but otherwise they were not visually different to WT (Fig. 5.4).

Seeds of transgenic line N10.6 germinated later than the WT control and to a lower final percentage after a period of 10 days (Fig. 5.3). Furthermore, the plantlets which developed had a considerably slower growth rate with an overall decreased leaf size and an altered leaf texture and colour (Fig. 5.4). Leaves appeared slightly purple due to the likely formation of anthocyanins and appeared to have a thicker waxy cuticle. The markedly reduced size of this mutant over its entire lifecycle rendered it impractical for physiological analyses and hence this line was excluded

from any further investigations. The remaining three lines were used for the full physiological analyses, but only lines N3.5 and N17.2, with the clear increases in ABA and associated phenotypes were used for drought and high light treatments.

Table 5.2: Phytohormone profiles in leaves of *A. thaliana* WT and *VvNCED1* transgenic plants grown under glasshouse conditions. Hormones are expressed as ng.g^{-1} FD leaf tissue. Different superscripts indicate that the difference between the row means is significant at *P*≤0.05 from ANOVA and the Tukey HSD test.

Hormone	WT	N3.5	N6.2	N17.2
SA	413.2±51.61[a]	311.0±29.99[a]	402.5±26.59[a]	408.3±26.08[a]
IAA[1]	2032.3±127.66[a]	6799.7±1096.95[b]	1094.3±306.41[a]	3055.8±415.27[ab]
ABA	41.2±2.4[a]	97.4±4.53[b]	68.9±10.86[a]	180.0±5.01[c]

[1] IAA is presented as the sum of itself and its precursor IAAN (indole acetonitrile; Kawaguchi and Syōno, 1996).
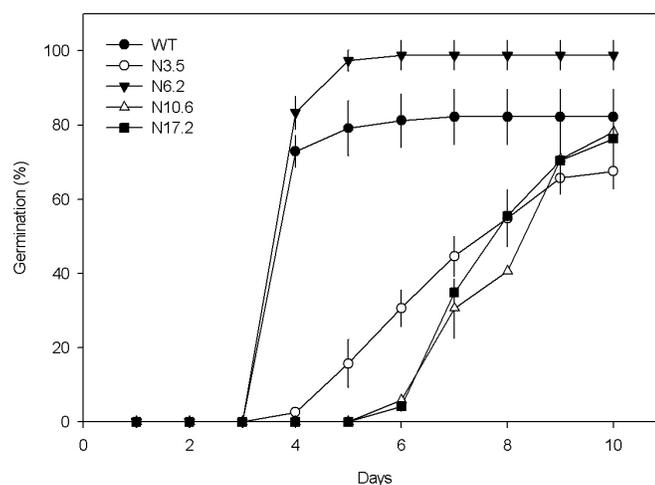
Figure 5.3. Percentage seed germination recorded in the WT control and T$_4$ generation *A. thaliana VvNCED1* transgenic lines N3.5, N6.2, N10.6 and N17.2 over a period of 10 days. Germination was scored when two fully-expanded green cotyledons had emerged.

WT **N3.5** **N6.2**

N10.6 **N17.2**
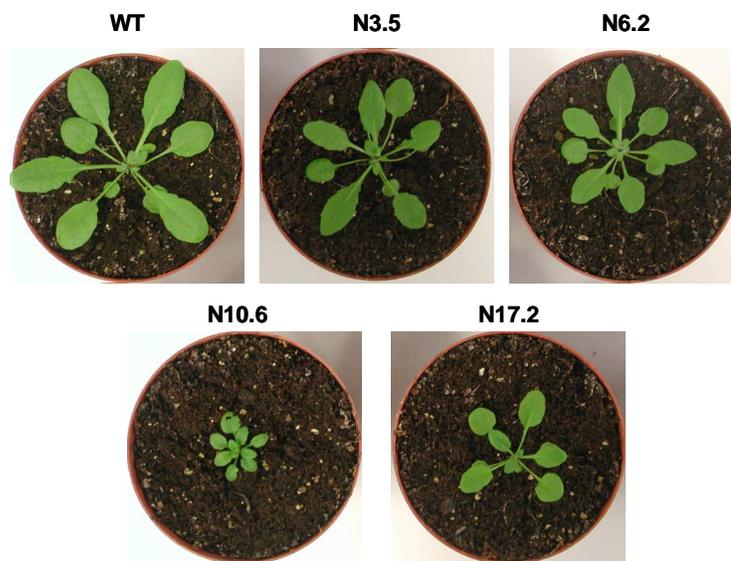
Figure 5.4. A typical example of three week-old T_4 generation *A. thaliana* plantlets heterologously overexpressing *VvNCED1.* Independent transgenic lines N3.5, N6.2, N10.6 and N17.2 are illustrated along with the WT control.

5.3.5 Pigment profiles of the WT and *VvNCED1* transgenic populations

The pigment concentrations of WT and three *VvNCED1* transgenic plant lines under ambient conditions are presented in Table 5.3. The *VvNCED1* lines with increased ABA levels had 21% to 37% smaller total carotenoid pools than WT. Line N6.2 which did not show a significant increase in ABA, also did not show any significant differences in total carotenoid pools at the 95% level. All the individual pigments except zeaxanthin occurred at lower concentrations in the *VvNCED1* lines than in WT, although for line N6.2 the difference was not significant for neoxanthin, lutein and β-carotene. The *VvNCED1* lines all had lower chl *a* (19-34% less) and chl *b* (18-32% less) concentrations than WT (Table 5.3). These differences were however only significant in line N3.5.

Table 5.3: Pigment concentrations (mean ± standard error) in leaves of *A. thaliana* WT and *VvNCED1* transgenic plants grown under growth room conditions (LL). Chl *a* and *b* are expressed as nmol.mg^{-1} FD leaf tissue and carotenoid pigments as mmol.mg^{-1} FD leaf tissue. Different superscripts indicate that the difference *b*etween the row means is significant at *P*≤0.05 from ANOVA and the Tukey HSD test.

Compound	WT	N3.5	N6.2	N17.2
Total Carotenoids	4557.0±232.80[a]	2866.6±231.96[b]	3606.1±322.53[ab]	3508.4±153.78[b]
Neoxanthin	479.4±32.45[a]	316.0±23.15[b]	372.6±32.00[ab]	369.1±14.0[b]
Lutein	2243.1±118.62[a]	1393.9±116.85[b]	1800.1±164.15[ab]	1757.8±75.38[ab]
β-Carotene	1271.3±56.43[a]	794.1±75.61[b]	1012.1±91.28[ab]	964.4±52.21[b]
Violaxanthin	330.2±22.42[a]	208.2±13.43[b]	237.1±21.81[b]	234.5±10.48[b]
Antheraxanthin	117.1±9.79[a]	69.0±3.72[b]	76.8±8.33[b]	81.8±2.75[b]
Zeaxanthin	115.9±5.78[a]	85.3±7.27[a]	107.3±10.45[a]	100.8±6.46[a]
V + A + Z[1]	563.2±31.61[a]	362.5±21.91[b]	421.3±36.53[b]	417.1±14.35[b]
(A + Z) / (V + A+ Z)	0.41±0.02[a]	0.43±0.01[a]	0.44±0.02[a]	0.44±0.01[a]
Lutein / (V + A + Z)	3.99±0.06[ab]	3.83±0.11[a]	4.27±0.08[b]	4.21±0.07[b]

[1] V, Violaxanthin; A, Antheraxanthin; Z, Zeaxanthin

Expressing the carotenoid concentrations on a chl *a* basis (see Niyogi *et al.*, 1997, 1998; Peterson and Havir, 2000; Rissler and Pogson, 2001 for similar presentation of carotenoids) (Table 5.4), lines N6.2 and N17.2 had significantly lower violaxanthin concentrations than WT, which resulted in a significantly lower pool of total xanthophyll cycle pigments (V + A + Z) and a higher lutein / (V + A + Z) ratio in these two lines. Similar results were obtained when the carotenoid concentrations were expressed per chl *b* (data not shown).

Table 5.4: Pigment concentrations (mean ± standard error) in leaves of *A. thaliana* WT and *VvNCED1* transgenic plants *b*efore and after HL treatment. Chl *a* and *b* are nmol.mg^{-1} FD leaf tissue and carotenoid pigments are mmol.mol^{-1} chl *a*. Significant differences were recorded at *P*≤0.05 from ANOVA and the Tukey HSD test. Superscripts to the right indicate that the difference *b*etween the row means is significant, while superscripts to the left indicate that the differences *b*etween *b*efore and after HL exposure are significant.

Pigment	WT	N3.5	N6.2	N17.2
Before light stress:				
Total carotenoids	^{a}320.6±5.63^a	^{a}303.0±5.34^a	^{a}311.5±3.29^a	^{a}309.0±4.40^a
Neoxanthin	^{a}33.6±0.57^a	^{a}33.5±1.01^a	^{a}32.2±0.39^a	^{a}32.5±0.59^a
Lutein	^{a}157.8±3.44^a	^{a}147.3±3.52^a	^{a}155.3±1.36^a	^{a}154.9±2.35^a
β-carotene	^{a}89.6±2.23^a	^{a}83.7±3.01^a	^{a}87.5±1.60^a	^{a}84.8±1.94^a
Violaxanthin	^{a}23.1±0.38^a	^{a}22.1±0.60^{ab}	^{a}20.4±0.27^b	^{a}20.7±0.53^b
Antheraxanthin	^{a}8.3±0.70^a	^{a}7.3±0.17^a	^{a}6.6±0.26^a	^{a}7.2±0.33^a
Zeaxanthin	^{a}8.2±0.31^a	^{a}9.0±0.47^a	^{a}9.4±0.83^a	^{a}8.9±0.33^a
V + A + Z	^{a}39.6±0.66^a	^{a}38.5±0.47^{ab}	^{a}36.5±0.71^b	^{a}36.8±0.27^b
(A + Z) / (V + A + Z)	^{a}0.41±0.02^a	^{a}0.43±0.01^a	^{a}0.44±0.02^a	^{a}0.44±0.01^a
Lutein / (V + A + Z)	^{a}3.99±0.06^{ab}	^{a}3.83±0.11^a	^{a}4.27±0.08^b	^{a}4.21±0.07^b
Chl *a*	^{a}14.3±0.95^a	^{a}9.4±0.61^b	^{a}11.6±1.02^{ab}	^{a}11.4±0.41^{ab}
Chl *b*	^{a}7.4±0.50^a	^{a}5.0±0.30^b	^{a}6.1±0.51^{ab}	^{a}5.9±0.18^{ab}
Chl *a/b*	^{a}1.93±0.02^a	^{a}1.89±0.03^a	^{a}1.89±0.02^a	^{a}1.93±0.02^a
After HL stress:				
Total carotenoids	^{a}312.4±7.49^a	^{a}303.7±5.79^{ab}	^{a}294.7±7.15^{ab}	^{b}284.9±5.42^b
Neoxanthin	^{b}29.9±0.34^a	^{b}30.4±0.42^a	^{a}32.9±1.75^a	^{b}29.2±0.93^a
Lutein	^{a}154.2±4.10^a	^{a}152.1±2.25^a	^{a}149.3±4.36^a	^{b}143.7±2.95^a
β-carotene	^{a}87.9±3.07^a	^{a}82.7±2.85^{ab}	^{b}74.9±1.00^b	^{b}74.5±3.06^b
Violaxanthin	^{b}9.4±0.06^a	^{b}9.2±0.14^a	^{b}10.2±0.66^a	^{b}7.9±0.11^b
Antheraxanthin	^{a}7.1±0.42^a	^{b}6.5±0.26^a	^{a}7.8±1.33^a	^{a}6.6±0.41^a
Zeaxanthin	^{b}23.9±0.69^a	^{b}22.8±0.58^a	^{b}20.8±1.06^a	^{b}23.1±0.85^a
V + A + Z	^{a}40.5±0.75^a	^{a}38.4±0.89^a	^{a}38.6±1.37^a	^{a}37.5±1.04^a
(A + Z) / (V + A + Z)	^{b}0.77±0.003^{ab}	^{b}0.76±0.004^{ab}	^{b}0.74±0.02^a	^{b}0.79±0.01^b
Lutein / (V + A + Z)	^{a}3.81±0.11^a	^{a}3.97±0.05^a	^{b}3.86±0.09^a	^{a}3.85±0.18^a
Chl *a*	^{a}13.5±0.43^a	^{a}11.1±0.70^b	^{a}10.3±0.30^b	^{a}12.1±0.30^{ab}
Chl *b*	^{a}7.0±0.21^a	^{b}6.0±0.29^b	^{a}5.6±0.19^b	^{a}6.5±0.24^{ab}
Chl *a/b*	^{a}1.93±0.02^a	^{a}1.86±0.03^a	^{a}1.84±0.02^a	^{a}1.88±0.04^a

Exposure to HL (full midday summer sunlight, about 2100 µmol photons m^{-2}.s^{-1} for 30 min) did not affect chl *a* or *b* concentrations, except for a slight increase in chl *b* in line N3.5. However, HL caused conversion of violaxanthin to zeaxanthin so that the deepoxidation status of the total xanthophyll cycle pool - (A + Z) / (V + A + Z) - was significantly greater in all lines after light treatment (Table 5.4, superscripts to left of means). For WT, N3.5 and N17.2, neoxanthin concentrations were also lower after the light treatment. Some other line-specific differences were found; HL exposure resulted in lower *β*-carotene concentrations in N6.2 and in lower *β*-carotene and lutein levels in N17.2. Hence, exposure to HL had resulted in significantly lower levels of all carotenoids (except antheraxanthin) in line N17.2, with the result that the total carotenoid pool in this line was significantly lower after HL than before the light treatment. HL treatment did not change the total carotenoid pool of the other two transgenic lines or WT.

As was observed under LL, the pigment concentrations on a chl *a* basis after HL exposure were largely similar in the *VvNCED1* lines and WT (Table 5.4, superscripts to right of means). The exceptions were the *β*-carotene concentration of line N6.2 and *β*-carotene and violaxanthin levels of N17.2, all of which were lower than in WT. Light stressed N17.2 had a significantly smaller total carotenoid pool than light stressed WT. The deepoxidation status of the xanthophyll cycle pigment pool in the light stressed *VvNCED1* lines was not significantly different to light stressed WT with the exception of line N17.2 which was higher.

5.3.6 Photosynthetic and quenching capacity of the WT and *VvNCED1* transgenic populations

The changes in chlorophyll *a* fluorescence parameters with increasing PAR levels are shown in Fig. 5.5 and the values at 1305 µmol photons m^{-2}.s^{-1}, as well as dark-adapted F_0, F_m and F_v/F_m values, compared between the WT and three *VvNCED1* transgenic lines are presented in Table 5.5. Dark-adapted F_v/F_m, the potential or maximum quantum yield of PSII, was slightly lower in the *VvNCED1* lines than in WT but the difference was only significant in the case of N17.2. The slightly lower F_v/F_m of the *VvNCED1* lines was due to slightly lower F_m, rather than higher F_0; in fact F_0 was also slightly lower for the *VvNCED1* lines. These lower minimal and maximal dark-adapted fluorescence yields probably reflect the lower concentration of chlorophyll in the *VvNCED1* lines compared with WT.

The response of ΦPSII (a measure of the proportion of the absorbed light used for photochemistry; Maxwell and Johnson, 2000), and hence the relative ETR (a measure of the overall photosynthetic capacity *in vivo*; Genty *et al.*, 1989) to increasing PAR was similar in all the lines. At none of the PAR levels were there significant differences in ΦPSII or ETR between the *VvNCED1* lines and WT (Fig 5.5). NPQ increased more sharply with increasing light at low PARs in the *VvNCED1* lines than in WT and attained a significantly higher NPQ at the highest PAR tested. However, this did not result in a greater proportion of open PSII reaction centers (*qP*)

in the transgenic lines relative to the WT. Neither was the quantum yield of open reaction centres significantly different between the *VvNCED1* lines and WT (F_v'/F_m', Table 5.5). Fast-relaxing quenching (qE) was appreciably (30-50%) higher in the *VvNCED1* lines than in WT; the transgenic lines also showed slightly higher slow-relaxing quenching (qI) than WT but the difference was only significant for N17.2. When expressed relative to the maximum NPQ obtained, qE formed a significantly higher percentage of NPQ and qI a significantly lower percentage of NPQ in the *VvNCED1* lines than in WT (Table 5.5).
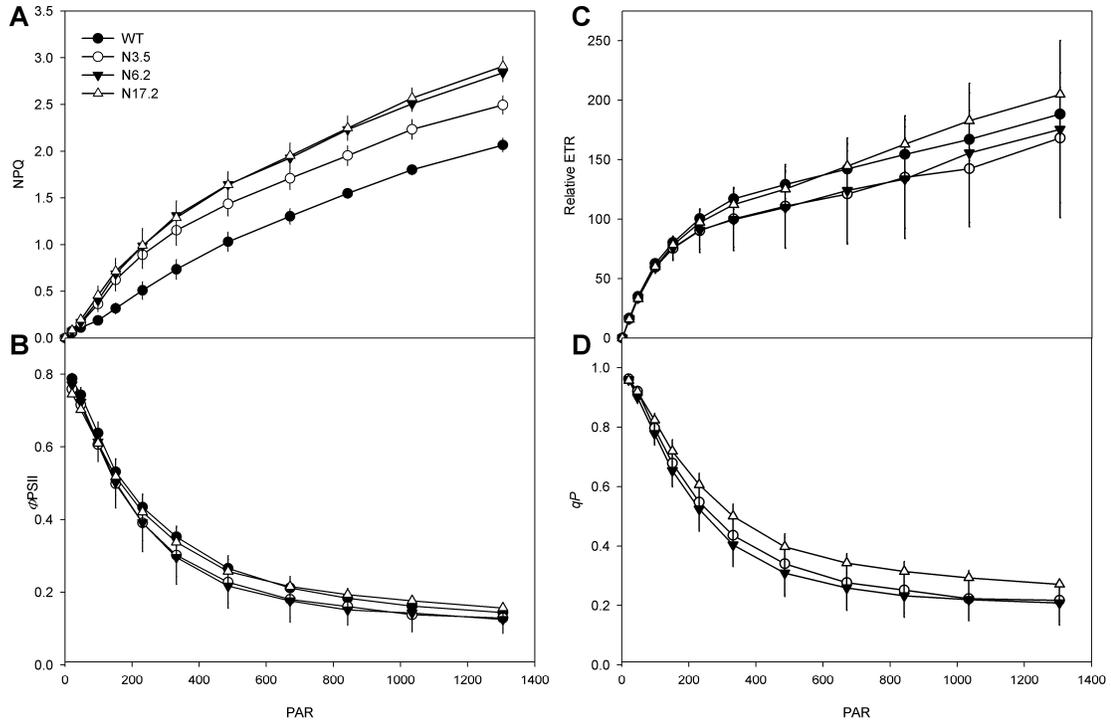
Figure 5.5. Light response curves of chlorophyll *a* fluorescence parameters in mature leaf tissue of five week-old *A. thaliana* WT and T$_4$ generation *A. thaliana VvNCED1* transgenic lines N3.5, N6.2 and N17.2.

(A) Non-photochemical quenching (NPQ), calculated as (F_m'-F_m)/F_m'. **(B)** The quantum efficiency of PSII photochemistry (ΦPSII), calculated as (F_m-F_0)/F_m in the dark and (F_m'-F_s)/F_m' in the presence of the actinic light. **(C)** The relative PSII electron transport rate, calculated as ΦPSII x PAR. **(D)** Photochemical quenching (*qP*), calculated as (F_m'-F_s-)/F_m'-F_0').

Table 5.5: Chlorophyll *a* fluorescence parameters (mean ± standard error) for leaves of five week-old *A. thaliana* WT and *VvNCED1* transgenic plants. Leaves were dark-adapted for 30 min, exposed to light at increasing PAR levels, up to 1305 µmol photons $m^{-2}.s^{-1}$, and then allowed to recover in the dark for 25 min. Different superscripts indicate that the differences *b*etween the WT and the *VvNCED1* transgenic line means are significant at $P{\leq}0.05$ using ANOVA and the Tukeys HSD test. Maximum (dark-adapted) quantum yields of PSII (F_v/F_m) and, for a PAR of 1305 µmol photons $m^{-2}.s^{-1}$, the effective quantum yields (*Φ*PSII), proportions of closed PSII reaction centers (Q_r/Q_t), quantum efficiencies of open PSII reaction centers (F_v'/F_m'), relative electron transport rates (Rel ETR), non-photochemical quenching (NPQ), and, after 25 min relaxation, the contri*b*utions of fast-relaxing quenching (qE) and slow-relaxing quenching (qI) to NPQ.

Light status	Parameter	WT	N3.5	N6.2	N17.2
Light: PAR=1305 µmol photons $m^{-2}.s^{-1}$	F_0	250.3±2.64[a]	237.5±1.84[a]	245.3±2.48[a]	250.3±1.97[a]
	F_m	1529.3±11.89[a]	1389.5±10.07[a]	1452.5±7.94[a]	1361.0±12.61[a]
	F_v/F_m	0.84±0.001[a]	0.83±0.001[ab]	0.83±0.001[a]	0.82±0.001[b]
	F_v'/F_m'	0.61±0.02[a]	0.59±0.004[a]	0.59±0.02[a]	0.58±0.01[a]
	qP	0.21±0.03[a]	0.22±0.04[a]	0.21±0.04[a]	0.27±0.01[a]
	*Φ*PSII	0.13±0.02[a]	0.13±0.02[a]	0.12±0.03[a]	0.16±0.01[a]
	Rel. ETR	167.6±23.77[a]	168.2±27.26[a]	162.9±32.97[a]	204.8±9.26[a]
	Q_r/Q_t	0.79±0.03[a]	0.78±0.04[a]	0.79±0.07[a]	0.73±0.01[a]
	NPQ	2.05±0.15[a]	2.49±0.03[b]	2.84±0.05[bc]	2.91±0.05[c]
Dark: Recovery time = 25 min	NPQ relax.	0.32±0.01[a]	0.27±0.01[a]	0.30±0.02[a]	0.29±0.03[a]
	qE	1.41±0.12[a]	1.80±0.02[b]	2.09±0.03[c]	2.13±0.02[c]
	qI	0.65±0.04[a]	0.70±0.02[a]	0.74±0.04[a]	0.78±0.03[a]
	qE/NPQ (%)	68±1.1[a]	72±0.4[b]	74±1.1[b]	73±0.7[b]
	qI/NPQ (%)	32±1.1[a]	28±0.4[b]	26±1.1[b]	27±0.7[b]

5.3.7 Significant parameters conferring drought tolerance to the *VvNCED1* transgenic populations

WT and *VvNCED1* line N3.5 and N17.2 plants from which water was withheld for 18 days and control plants which were watered over the 18 day period are illustrated (Fig. 5.6). The water-stressed *VvNCED1* plants looked similar to the watered plants, whereas in WT water stress caused conspicuous wilting and chlorosis of the leaves. This change in the appearance of the stressed WT plant occurred from day 14 onwards and the plants did not recover when subsequently rewatered (results not shown). Droughted WT plants had a significantly lower (aboveground) biomass than

watered WT plants (Table 5.6) whereas withholding water from the *VvNCED1* plants did not affect rosette biomass. Stressed and unstressed N17.2 plants developed less leaves than the WT or transgenic line N3.5 (Figure 5.6), reflected in the significantly lower biomass obtained for N17.2 compared with the other two lines.
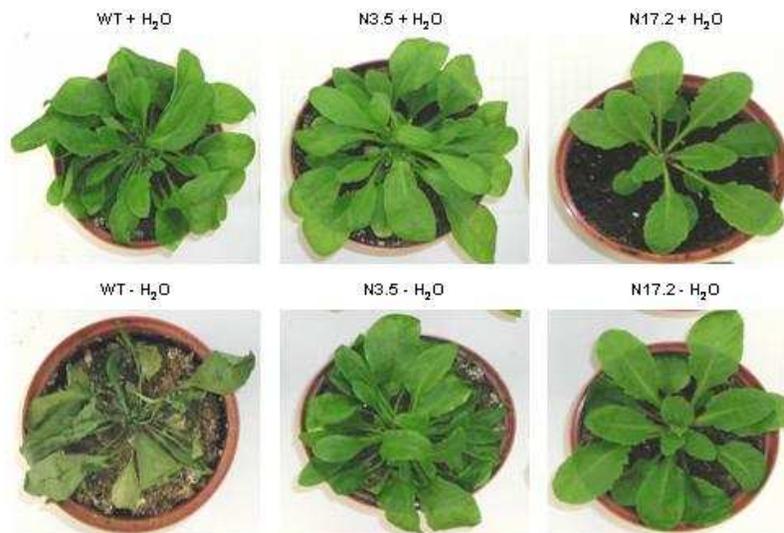
Figure 5.6. Eight week old T_4 generation *A. thaliana* plants WT and *VvNCED1* transgenic lines N3.5 and N17.2.

Plants were grown under a normal watering regime to five weeks of age and then either watered normally or deprived of water for 18 days. The photographs were taken on day 18.

The effects of drought stress (on day 18) on various physiological parameters are shown in Table 5.6. The maximum quantum yield of PSII, F_v/F_m, of water stressed WT plants was significantly lower than that of watered plants, due to both a lower F_m and a higher F_0. For the *VvNCED1* lines, however, withholding water did not result in a lower F_v/F_m than watered plants; in fact the N17.2 droughted plants had a slightly higher F_v/F_m than those which received water. Mean stomatal conductances (g_S) of the watered *VvNCED1* lines were considerably lower than that of the watered WT. Withholding water markedly lowered the g_S of the WT but not of the *VvNCED1* lines. In fact, droughted plants of transgenic line N3.5 showed a significantly higher mean g_S than the watered control plants. Over the 18 days without water, SMC declined markedly, so that at the end of the drought period SMC was significantly lower in the droughted pots than in the watered pots (Table 5.6). This difference was especially marked in the case of WT, where the mean SMC of WT droughted pots on day 18 was only 5% of that of their watered control pots, and less than 19 % of the mean SMCs of droughted pots containing either of the *VvNCED1* lines. The mean RWC of droughted WT plants decreased to 27% of that of the watered WT plants, whereas droughted *VvNCED1* plants had mean RWCs that were not significantly lower than those of the watered control plants (Table 5.6; superscripts to the left of means). ABA levels in water-stressed plants were significantly higher than in the watered

plants, but this difference was smaller for the *VvNCED1* lines than for the WT. Droughted WT had a mean ABA content 934% greater than the watered WT while ABA levels in water stressed N3.5 plants was 462% larger than the watered plants. In contrast, drought stress did not significantly alter the ABA concentration in transgenic line N17.2. Watered *VvNCED1* transgenic plants had similar SA levels resembling that of the WT. Drought stress did not change SA levels in either the WT or line N17.2 but N3.5 plants exhibited a 151% increase in SA concentration as a result of drought stress. The increased levels (235%) of IAA found in line N3.5 have been noted previously (section 5.3.4). However, of notable significance was the drastic increase in IAA levels in both the WT and line N3.5 following drought application with a remarkable 996% and 245% being recorded respectively. In contrast, N17.2 plants had similar levels regardless of the watering regime. Surprisingly, water-stressed WT plants had a lower TBARS value (the degree of lipid peroxidation, a measure of thylakoid membrane damage) than watered WT plants. Conversely, watered and droughted *VvNCED1* plants had similar mean TBARS values to each other and those of the watered WT.

The chlorophyll and carotenoid (per chl *a*) concentrations of the watered and droughted WT and *VvNCED1* transgenic lines N3.5 and N17.2 are presented (Table 5.7). No comparisons can be drawn between the results obtained here and those under LL conditions as the plants are in a different stage of their growth cycle (eight weeks as opposed to five weeks old) and a different HPLC method was used for quantification of the watered and drought treated plants (as discussed in the relevant methods section). Droughted *VvNCED1* line N17.2 had a 7% smaller total carotenoid pool than the water stressed WT and a 13% reduction in this pool compared to its watered control. Both watered and droughted plants belonging to line N3.5 had a total carotenoid complement closely resembling that of the WT. The watered WT and lines N3.5 and N17.2 had similar total carotenoid concentrations. However, drought treatment caused a decrease in both chl *a* and *b* in the WT and line N3.5 (Table 5.7; superscripts to left of the means) whereas the chlorophyll levels in line N17.2 were unaffected by drought treatment. All the individual pigments occurred at similar concentrations in the watered WT and watered *VvNCED1* lines with two exceptions. Line N3.5 had significantly lower β-carotene (11.2%) and zeaxanthin (57.7%) concentrations than the WT (Table 5.7; superscripts to right of the means). Droughted N3.5 plants had higher (7.9%) levels of β-carotene to that of the droughted WT plants while water stressed line N17.2 had lower concentrations of neoxanthin (10.3%), lutein (5.6%) and zeaxanthin (5.9%) in comparison to the levels of these pigments in the droughted WT. The sum of these slight differences accounts for the decrease observed in total carotenoids in the droughted N17.2 plants. All lines had a lower violaxanthin concentration with the WT and N3.5 showing increased zeaxanthin levels, indicative of xanthophyll cycling and resulting in a greater deepoxidation ratio – Z / (Z + V)- for the WT and N3.5 following drought treatment. In contrast, the decreased violaxanthin levels in line N17.2 were not met

by an increase in zeaxanthin, which was unchanged in the watered and droughted plants. Consequently, line N17.2 had a significantly lower deepoxidation ratio than that of the WT and line N3.5 following drought stress; although it was similar to that of the watered N17.2 plants. Drought treatment caused decreases in β-carotene levels of the WT and N17.2. In addition, neoxanthin and lutein levels fell in droughted N17.2 when compared to watered N17.2.

Table 5.6: Various parameters (mean ± standard error) measured following 18 days of drought application to the *A. thaliana* WT and *VvNCED1* transgenic lines N3.5 and N17.2. Control plants were watered normally over the 18 day period. Significant differences were recorded at $P{\leq}0.05$ from ANOVA and the Tukey HSD test. Superscripts to the right indicate that the difference *b*etween the line means within a treatment is significant, while superscripts to the left indicate that the differences *b*etween mean values recorded within a line and *b*etween watered and droughted treatments are significant

Variable (units)	WT		N3.5		N17.2	
	Watered	Droughted	Watered	Droughted	Watered	Droughted
F_0	[a]476.9±7.91[a]	[b]563.1±28.25[a]	[a]484.2±10.60[a]	[a]467.9±7.73[b]	[a]557.5±10.80[a]	[a]524.3 ±14.75[ab]
F_m	[a]2703.3±57.11[a]	[b]2203.8 ±79.73[a]	[a]2733.6±58.33[ab]	[a]2715.4±68.91[b]	[a]2897.4±42.80[b]	[a]2895.3±56.33[b]
F_v/F_m	[a]0.82±0.004[a]	[b]0.74±0.018[a]	[a]0.82±0.002[a]	[a]0.83±0.003[b]	[a]0.81±0.004[b]	[b]0.82±0.003[b]
g_s (mmol H_2O $m^{-2}.s^{-1}$)	[a]90.3±7.19[a]	[b]24.3±2.44[a]	[a]8.3±1.77[b]	[b]15.5±1.11[b]	[a]32.6±2.40[c]	[a]34.4±3.11[c]
Rosette *b*iomass (g)	[a]1.8±0.21[a]	[b]0.4±0.13[a]	[a]2.1±0.18[a]	[a]1.9±0.17[b]	[a]0.6±0.07[b]	[a]0.7±0.14[a]
RWC	[a]0.71±0.04[a]	[b]0.19±0.04[a]	[a]0.77±0.03[a]	[a]0.61±0.10[b]	[a]0.72±0.04[a]	[a]0.68±0.03[b]
SMC	[a]3.05±0.109[a]	[b]0.14±0.02[a]	[a]2.04±0.28[a]	[b]0.72±0.06[b]	[a]2.78±.0.37[a]	[b]1.03±0.09[c]
SA (μg.gr⁻¹ FD tissue)[1]	[a]413.2±51.61[a]	[a]403.8±65.90[a]	[a]311.3±29.99[a]	[b]781.0±56.64[b]	[a]408.3±26.08[a]	[a]384.7±24.60[a]
IAA (μg.gr⁻¹ FD tissue)[1,2]	[a]2032.3±127.66[a]	[b]22273.1±4191.87[a]	[a]6799.7±1096.95[b]	[b]23488.5±2478.01[a]	[a]3055.8±415.27[ab]	[a]4427.4±465.56[b]
A*B*A (μg.gr⁻¹ FD tissue)[1]	[a]41.2±2.49[a]	[b]425.9±54.16[a]	[a]97.4±4.53[b]	[b]547.6±60.64[a]	[a]180.0±5.01[c]	[a]225.9±14.96[b]
T*B*ARS (nmol.gr⁻¹ FW tissue)	[a]60.3±2.41[a]	[b]40.0±1.52[a]	[a]53.9±2.71[a]	[a]50.7±2.14[b]	[a]58.9±1.85[a]	[a]61.7±1.88[c]

[1] The values reported for the watered controls are the same as those given in Table 4.3.

[2] IAA is presented as the sum of itself and its precursor IAAN (indole acetonitrile; Kawaguchi and Syōno, 1996).

Table 5.7: Pigment concentrations (mean ± standard error) in leaves of *A. thaliana* WT and *VvNCED1* transgenic plants which followed a normal watering regime or following withholding of water for 18 days. Chl *a* and chl *b* are nmol.mg^{-1} FD leaf tissue and carotenoid pigments are mmol.mol^{-1}chl *a*. Superscripts to the right indicate that the difference *b*etween the line means within a treatment is significant, while superscripts to the left indicate that the differences *b*etween mean values recorded within a line and *b*etween watered and drought treatments are significant

Compound	WT		N3.5		N17.2	
	Watered	**Droughted**	**Watered**	**Droughted**	**Watered**	**Droughted**
Total Carotenoids	^{a}344.0±13.48^a	^{a}315.4±5.32^a	^{a}311.3±3.00^a	^{a}315.6±4.28^a	^{a}336.3±16.90^a	^{b}293.3±1.56^b
Neoxanthin	^{a}35.8±1.92^a	^{a}34.1±1.04^a	^{a}32.2±0.58^a	^{a}32.3±0.51^{ab}	^{a}35.2±1.71^a	^{b}30.6±0.25^b
Lutein	^{a}170.4±6.62^a	^{a}166.4±2.33^a	^{a}159.8±2.74^a	^{a}162.2±2.79^{ab}	^{a}179.4±9.41^a	^{b}157.1±1.25^b
β-Carotene	^{a}103.0±3.82^a	^{b}85.3±2.24^a	^{a}91.0±1.20^b	^{a}92.0±1.07^b	^{a}91.3±4.56^{ab}	^{b}80.3±1.54^a
Violaxanthin	^{a}29.6±1.41^a	^{b}22.6±0.39^a	^{a}26.2±0.36^a	^{b}22.2±1.07^a	^{a}26.5±1.11^a	^{b}21.9±0.44^a
Zeaxanthin	^{a}5.2±0.53^a	^{b}7.0±0.28^a	^{a}2.2±0.12^b	^{b}6.8±1.32^a	^{a}3.8±0.45^a	^{a}3.5±0.12^b
V + Z	^{a}34.7±1.53^a	^{b}29.6±0.26^a	^{a}28.4±0.37^b	^{a}29.0±.47^a	^{a}30.4±1.45^{ab}	^{b}25.3±0.53^b
(Z) / (V + Z)	^{a}0.15±0.01^a	^{b}0.24±0.01^a	^{a}0.08±0.004^b	^{b}0.23±0.04^a	^{a}0.13±0.01^a	^{a}0.14±0.003^b
Lutein / (V + Z)	^{a}4.93±0.12^a	^{b}5.62±0.004^a	^{a}5.63±0.05^b	^{a}5.60±0.008^a	^{a}5.91±0.10^b	^{a}6.22±0.14^b
Chl *a*	^{a}18.0±0.75^a	^{b}12.1±0.89^a	^{a}17.9±0.65^a	^{b}13.2±0.65^{ab}	^{a}14.4±0.99^b	^{a}14.8±0.23^b
Chl *b*	^{a}7.5±0.32^a	^{b}5.7±0.43^a	^{a}8.1±0.29^a	^{b}6.0±0.31^{ab}	^{a}7.0±0.52^a	^{a}6.9±0.12^b
Chl *a/b*	^{a}2.41±0.03^a	^{b}2.12±0.05^a	^{a}1.98±0.03^b	^{a}1.97±0.02^b	^{a}2.05±0.02^b	^{b}2.13±0.02^a

[1] No antheraxanthin could be detected.

5.4 DISCUSSION

Expression analysis of a NCED1 from Vitis vinifera L. cv Pinotage

Prior to the current investigation no spatial and/or temporal data was available on the expression profile of grapevine *NCED1* under unstressed conditions. Quantitative RT-PCR analysis in various plant tissues revealed that native *VvNCED1* expression is upregulated during véraison (and potentially leading up to véraison) and showed that incrementally larger amounts of transcript were present in the leaves and flowers when calculated relative to shoot tips.

Phytohormone profiling reveals increased ABA and associated phenotypes in VvNCED1 transgenic populations

Constitutive levels of *VvNCED1* did not confer a common phenotype or phytohormone profile to all of the resulting transgenic lines. Typically, however, *VvNCED1* overexpression resulted in constitutively high ABA levels, prolonged seed dormancy and a severe to slightly reduced plant growth rate. Although salicylic acid levels remained constant, IAA increased considerably in the transgenic lines. A degree of crosstalk has been proposed between the independent pathways synthesising ABA and IAA (Chinnusamy *et al.,* 2004). It is thus feasible that the increased IAA concentrations stimulate the cleavage reaction regulated by *VvNCED1* resulting in further increased ABA concentrations. Although conclusive evidence is still lacking, Wan and Li (2006) alluded towards auxin-regulation of ABA biosynthesis. However, the converse may be true: constitutive levels of *VvNCED1* regulating the constant cleavage of the epoxycarotenoids may induce IAA production. This aspect is of interest and requires considerable further investigation.

VvNCED1 overexpression lowers the total carotenoid and chlorophyll complement in the photosynthetically active tissue of the resulting transgenics

Previously, research has concentrated on establishing 9-*cis* neoxanthin and/or 9-*cis* violaxanthin as the substrates for NCED, however the effects following overexpression of this controlling enzyme on the concentration of the major pigments (carotenoids and chlorophylls) have not been established. All *VvNCED1* lines were found to have significantly lower concentrations of both chl *a* and *b*; however, the reduced chlorophyll levels recorded were not reflected as chlorosis of the photosynthetic tissue. Furthermore, despite the decline in chlorophyll content, no change was found in the chlorophyll *a*/*b* ratio, averaging 1.92±0.02 in all mature leaves. This suggests that there was no difference in the proportion of light harvesting complex (LHC) chlorophylls to reaction centre (RC) chlorophylls.

When carotenoids were expressed per gram FD tissue a decrease was found in the total carotenoid pool and in each of the individual pigments with the exception of zeaxanthin. It is proposed that the lower carotenoid levels are likely due to a lower

chloroplastidic pigment complement rather than a depletion of the carotenoid pool due to *VvNCED1* overexpression.

Furthermore, if pigments were expressed on a chl *a* basis, no difference was recorded in the total carotenoid pool or the levels of individual carotenoids in the WT and the transgenics with the exception of violaxanthin. This implies that the composition of carotenoids and chlorophylls in the LHC's is stochiometrically maintained through their co-regulation, likely via the common precursor geranylgeranyl pyrophosphate (Bartley and Scolnik, 1995). Furthermore, this observation supports the established data for the integral role these pigments play in maintaining the structural and functional integrity of the thylakoid membrane, and more specifically the photosynthetic apparatus.

It is possible that the decreased violaxanthin levels can be attributed to *VvNCED1* overexpression. Many questions still exist regarding the conversion or *trans*-violaxanthin to its *cis*-form as well as any potential inter-conversion between *trans*-violaxanthin and *tran*s-neoxanthin. As the current method was incapable of detecting *cis*-violaxanthin and as it is possible that the lower *trans*-violaxanthin levels observed was due to violaxanthin isomerisation; further research is required to validate this hypothesis.

Moreover, preferential *in vivo* reduction of *cis*-neoxanthin (neoxanthin is presented as the sum of its *cis*- and *trans*-forms) was not shown in the *VvNCED1* transgenics lines. The potential "pull" exerted by the constitutively expressed *VvNCED1* on a specific 9-*cis*-xanthophyll(s) is possibly being disguised due to the tight and complex regulation that exists within the carotenoid pathway. Depletion to varying degrees of a certain intermediate is probably quickly restored to steady-state levels. A similar finding was observed by Chernys and Zeevaart (2000).

Hence, although *VvNCED1* resulted in a smaller complement of carotenoids and chlorophylls, there was no apparent effect on xanthophyll cycling.

VvNCED1 transgenics have an enhanced capacity for NPQ over the entire actinic range investigated

All of the transgenic lines induced NPQ of chlorophyll *a* fluorescence more rapidly and to a greater final extent than the WT. Of particular significance was that qE of the observed NPQ in the transgenics exceeded that of the WT while qI was slightly lower, indicating reduced photodamage. A similar increase in NPQ has been reported previously (Sharma *et al.* 2002; Ivanov *et al.* 1995) following the application of ABA to both barley and sorghum. These authors have suggested that the increased capacity for xanthophyll-related NPQ is due to the reduced requirement exogenous ABA placed on the ABA precursor pool and in particular the xanthophylls. HPLC analysis under two different light conditions has shown that the light-induced deepoxidation state of the xanthophyll cycle pool in the *VvNCED1* transgenics is similar to that of the WT. Hence *VvNCED1* overexpression conferred a higher qE (and total NPQ) to the transgenic lines which is not associated with an increased

xanthophyll pool and specifically higher zeaxanthin concentrations. NPQ thus appears to occur independently of zeaxanthin. It is possible that the increased NPQ is instead related to the phenotype conferred by *VvNCED1* overexpression. Less light energy will be absorbed by the transgenic plants due to a decreased complement of carotenoids and chlorophylls. *VvNCED1* overexpression has resulted in increased ABA concentrations which are coupled to a reduction in stomatal conductance. Thus although the efficiency of photochemistry in the *VvNCED1* transgenic lines was the same as that of the WT, it is possible that the photosynthetic rate is reduced due to limited uptake of CO_2 as stringent stomatal control is exercised by the greater ABA abundance. This reduction may allow for a more rapid NPQ to be induced with a greater final magnitude. A zeaxanthin-independent mechanism of NPQ has been proposed which requires the presence of this pigment, but is not dependent on its formation for activation (Finazzi *et al.*, 2004; Demmig-Adams and Adams, 2006).

Drought tolerance: An interrelationship between water limiting conditions, NCED expression and ABA production

The WT and *VvNCED1* overexpressing lines displayed obvious phenotypical differences after water was withheld for 18 days. Drought stress symptoms were manifested as significant browning and drastic loss of turgidity in the WT whereas the *VvNCED1* transgenics remained comparatively green and healthy. Of significance was that line N17.2 had a lower biomass throughout its lifecycle which seemed, unexplainably, to be correlated to ABA levels.

Water deficit in the WT plants resulted in a decreased F_v/F_m, primarily due to an increasing F_0, confirming that the photosynthetic apparatus is susceptible to this stress and therefore the overall efficiency of PSII has decreased. This is further accounted for by a decrease in both chl *a* and *b* levels under limiting water conditions. A similar finding was reported by Jung (2004) and is most likely due to membrane disintegration following oxidative stress (Moran *et al.*, 1994; Alonso *et al.*, 2001). Nevertheless, droughted WT had apparent reduced lipid peroxidation in comparison to the watered WT plants and both *VvNCED1* transgenic lines. It is possible that this is due to the severe disintegration of the thylakoid membrane and hence this method is only able to measure the number of MDA equivalents in the intact membrane(s). Alternatively it is possible that the drought stress has induced a number of enzymatic defense responses. Jung (2004) observed that drought stressed mature leaves were found to have increased catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and glutathione reductase (GR) antioxidant activities specifically targeted to combat increased H_2O_2 levels. It is possible that the drought stress perceived by the WT plants upregulated each of these activities, accounting for the decrease observed in lipid peroxidation. Increases in ABA are observed in the WT and line N3.5 in response to drought stress. In agreement with Qin and Zeevaart (2002) it is likely that the native *NCED* promoter is capable of

driving stronger expression than the CaMV 35S promoter, however, it seems that constitutively high ABA levels are capable of regulating guard cell movement permanently affording a major advantage to the transgenic plants that strong induced expression of a native *NCED* is not able to match.

The RWC can be used as a measure of the water status, where greater water loss was correlated to greater membrane damage (Kocheva *et al.*, 2004). Indeed, the RWC of the droughted WT plants was significantly lower than that of the watered WT and droughted *VvNCED1* transgenic lines. The greater (at least intially) g_S of WT would have resulted in a greater cumulative water loss during the drought period, which is why RWC of droughted WT plants and SMC of the droughted pots containing the WT plants were considerably lower than for the *NCED* lines at the end of the drought period.

In summary, quantitative analysis of the induced *in vivo* effects of the applied stress within the leaf tissue of line N17.2 revealed no significant differences between the droughted and watered plants within this line. Drought treatment did, however, decrease the total carotenoid pool in this line due to decreases in all individual components (with the exception of zeaxanthin), however the chlorophyll complement was unaffected. In effect, overexpression of *VvNCED1* in this line had protected all the plants from a water deficit to the extent that no apparent stress was even perceived. In contrast, the WT suffered significantly under water limiting conditions with significant changes in almost every parameter investigated recorded.

An *Arabidopsis* mutant lacking *NCED3* (129B08/nced3) has recently been identified (Wan and Li, 2006) and shown to be hypersensitive to sorbitol-induced osmotic stress and more sensitive to drought stress than WT. This mutant supports the integral role of NCED in conferring tolerance to a water deficit. A more detailed investigation into its biochemistry and physiology would provide additional evidence of the key factors conferring drought tolerance.

A comprehensive "snapshot", taken on day 18 of drought tolerance, has revealed some of the parameters affected or directly responsible for the observed phenotype acquired. Of particular significance was the discovery that constitutively low stomatal conductance is key to drought tolerance. However, of concern was the high degree of variability which was shown to exist between the two *VvNCED1* transgenic lines investigated. Clearly, the current study has provided a baseline for subsequent in-depth investigations which should be conducted over the entire time course wherein plants are revealed to be drought tolerant or susceptible. In order to gain an understanding into the variability which exists between the transgenic lines and to confirm and expand upon the results obtained in the current study, a number of options should be investigated at various intervals over the 18 day drought tolerance period including:

- Transcriptional quantification of a number of known carotenogenesis control points to establish whether, and thus where, down-regulation had taken place;

thereby accounting for the decrease in the total pigment complement observed for the transgenics;

- Quantification of absolute ABA levels for determination of active and inactive ABA pools due to conjugation or catabolism and thereby providing a greater understanding of pathway flux; and
- A possible correlation between ABA levels and stomatal conductance should be ascertained to determine their degree of interdependence.

5.5 CONCLUSION

In conclusion, a full-length functional *NCED* from grapevine cv. Pinotage was analysed and characterised in terms of its occurrence in the *Vitis* genome as well as its spatial and temporal expression within a range of grapevine tissues. This is the first reporting of the effects of *NCED* overexpression on the pigment complement, interplay between carotenogenesis and hormone biosynthesis, and the photosynthetic and quenching capacity of the transgenics. Although constitutive levels of the *NCED* mRNA transcript decreased the total carotenoid and chlorophyll complement, after exposure to high light the transgenics displayed a larger capacity for NPQ, indicative of photoprotection. Furthermore, a wide range of parameters conferring drought tolerance and factors which are directly or indirectly affected during water stress were identified in this study providing a baseline for subsequent investigations. The increased ABA levels and decreased stomatal conductance of the transgenics conferred resistance under water-limiting conditions. This study presents a comprehensive functional analysis of the grapevine *NCED1* gene.

ACKNOWLEDGEMENTS

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ISOLATION AND SEQUENCE ANALYSIS OF *NCED1* FROM *VITIS VINIFERA* L. CV. PINOTAGE

INTRODUCTION

A number of 9-cis epoxycarotenoid dioxygenase (NCED) encoding genes have been isolated previously from a range of plant species including tomato (Burbridge *et al.*, 1997), bean (Qin and Zeevaart, 1999), avocado (Chernys and Zeevaart, 2000), and *Arabidopsis* (Iuchi *et al.*, 2001). This gene was found to cleave the xanthophylls 9-*cis* neoxanthin and/or violaxanthin and is the key regulatory step in abscisic acid (ABA) synthesis.

Isolation of the full length genomic DNA and cDNA copies of *NCED1* from grapevine proceeded employing degenerate primers and subsequently using a combination "PCR/subgenomics" approach.

MATERIALS AND METHODS

Isolation of full-length *VvNCED1* genomic DNA and cDNA copies

A genomics approach was used to isolate the full length genomic DNA (gDNA) and cDNA clones of *VvNCED1*. This involved the identification, retrieval and subsequent alignment of representative plant protein sequences for NCED using BLAST (Altschul *et al.*, 1990) on the National Centre for Biotechnology Information (NCBI) database (GenBank; http://www.ncbi.nlm.nih.gov). Alignment of the retrieved amino acid sequences enabled the recognition of conserved regions within these closely related plant species. Subsequent analysis of the aligned nucleotide sequences of these conserved protein sequences (ClustalW; http://www.ebi.ac.uk/clustlw/; Thompson *et al.*, 1994) facilitated PCR primer design in areas showing the least degree of degeneracy (Table 5.8).

A partial *VvNCED1* gene fragment (740 bp) was PCR-amplified from *V. vinifera* gDNA. This product was cloned and the sequences verified using the M13 forward- and reverse-sequencing primers on an ABI Prism 3100 Genetic Analyser (Central Analytical Facility, Stellenbosch University, Stellenbosch, RSA). The resulting partial gene fragment was PCR DIG-labelled as described by the manufacturer (Roche Diagnostics, Mannheim, Germany) and employed as a probe to screen *Hin*dIII-, *Kpn*I- and *Pst*I- pBlueskript II SK(+) (Stratagene, La Jolla, CA, USA) size-selected (6.0 kb, 2.3 kb and 1.7 kb, respectively) sub-genomic *V. vinifera* libraries. Following sequence analyses and alignment of these cloned fragments, the full length gDNA and cDNA copy was obtained. Alignment of the predicted amino acid sequence for the identification of the degree of homology to existing conserved protein domains was carried out using the ClustalW program while ChloroP (www.cbs.dtu.dk/services/ChloroP/; Emanuelsson *et al.,* 1999) was used for transit peptide prediction.

Table 5.8: PCR primers designed for amplification of the partial or full-length *VvNCED1* gene.

Primer name	Sequence (5'-3')
NCED5'	CCA MGC RTT CCA SAG MTG GAA MCA A[1]
NCED3'	CAC CAY YTC TTC GAY GGM GAC GG[1]
NCED5'_ATG	GTC GAC ATG GCT TCT CCT GCA GCT GC[2]
NCED3'_STOP	CAA TCT GAC ACC AAG CAG CCA TG[2]
RT-NCED5'	CCA CGG CAC CTT CAT AAG CT[3]
RT-NCED3'	TCT GCA ATC TGA CAC CAA GCA[3]

[1] Degenerate primers amplifying an internal *VvNCED1* fragment (740 bp).

[2] Gene specific primers amplifying the full-length genomic and cDNA copies (both 1833 bp in length) of *VvNCED1*.

[3] Gene specific primers designed in the coding sequence and 3' untranslated region (EST accession number TC48377) for amplification of a partial *VvNCED1* fragment (103 bp) during quantitative RT-PCR.

RESULTS

Isolation and sequence analysis of a grapevine *NCED* gene

A combination 'PCR/sub-genomic library' approach was adopted for the isolation of a *NCED* from *V. vinifera* cv. Pinotage since no sequences were available when the study started. Briefly, PCR with degenerate primers generated a partial *NCED* fragment (~740 bp) which was DIG-labelled and used to screen a 1.7 kb *Pst*I-*V. vinifera* sub-genomic library. Sequence analysis revealed that this genomic fragment contained a partial *NCED*. Two additional *V. vinifera* sub-genomic libraries were required to obtain the missing 5'-region (including the start codon); a 2.3 kb *Kpn*I- and a 6.0 kb *Hin*dIII-*V. vinifera* sub-genomic library. Screening of each sub-genomic library took place via PCR using gene-specific primers designed in the predicted region of overlap between the two libraries. In summary, a total of three *V. vinifera* L (cv Pinotage) sub-genomic libraries were generated in order to clone the full-length genomic DNA sequence of *NCED*. The gDNA sequence was found to be identical to the cDNA sequence, indicating a lack of introns. This gene has been designated *VvNCED1*.

VvNCED1 was found to contain an open reading frame of 1833 bp encoding a predicted protein product of 611 amino acids. The mature protein is predicted to contain a chloroplast transit peptide 70 amino acids in length which is characteristically particularly rich in serine residues (Von Heijne *et al.*, 1989). Sequence analysis revealed a predicted amphipathic α-helix involved in protein thylakoid membrane binding (LQSAAAMALDAVETALVA; Tan *et al.*, 2001) and four conserved histidine residues (Fig 5.7) proposed to be mandatory for iron sequestration and hence catalytic activity (Schwartz *et al.*, 1997; Tan *et al.*, 1997). A high degree of homology (99%) is exhibited to *V. vinifera* (cv. Shiraz) *NCED1* detailed by Soar *et al.* (2004) with potentially only a point mutation accounted for by the cultivar differences. Furthermore, *Vv*NCED1 was 73% homologous to *Vv*NCED2,

the other member in the grapevine NCED family (Soar *et al.*, 2004). The primary protein structure exhibits a marked degree of homology to other NCEDs in closely related plant species displaying, for example, 72% and 71% identity to bean and tomato, respectively. Phylogenetic tree analysis revealed *Vv*NCED1 to group more closely with NCED isoforms which play a known role in stress-induced ABA biosynthesis, namely *Arabidopsis* NCED3 (Tan *et al.*, 2003), tomato NCED1 (Thompson *et al.*, 2000) and bean NCED1 (Qin and Zeevaart, 1999; results not shown).

```
Phaseolus      --------MPSPASNTWINTTLPSSCSSPFKDLASTSSSPTTLLPFKKRSSS--NTNTIT  50
Lycopersicon   -----MATTTSHATNTWIKTKLSMPSS---KEFGFASNSISLLKNQHNRQSL--NINSSL  50
Vitis          MASPAAAAASTPNISACVKPKLCYSSS---SSSSSSSSALDLGSFSFHRPTIRPNINCSL  57
Arabidopsis    ---MASFTATAAVSGRWLGGNHTQPPL---SSSQSSDLSYCSSLPMASRVTRKLNVSSAL  54
Zea            ------MQGLAPPTSVSIHRHLPARSR------ARASNSVRFSPRAVSSVPPAECLQAPF  48
                           :   :           :  .    :           : .   .

Phaseolus      CSLQTLHYPKQYQPTSTSTTTTPTPIKPTTTTTTTTTPHRETKPLSDTKQPFPQKWN⌈FLQK⌉ 110
Lycopersicon   QAPPILHFPKQ-----SSNYQTPK------NNTISHPKQENNNSSSSST---SKWN⌊LVQK⌋ 96
Vitis          HSPSVLHFPKH-----TSSSTGVA-------TVKTTLPKEKDDFSSSVS---QQWN⌊FLQS⌋ 102
Arabidopsis    HTPPALHFPKQ-------SSNSPA-------IVVKPKAKESNT--------KQMN⌊LFQR⌋ 91
Zea            HKP-VADLPAP--------SRKPA-------AIAVPGHAAAPRKAEGGK---KQLN⌊LFQR⌋ 89
                     .  *                                       .:  *:.*

Phaseolus      ⌈AAATGLDMVETALVS⌉--HESKHPLPKTADPKVQIAGNFAPVPEHAADQALPVVGKIPKCI 168
Lycopersicon   ⌊AAAMALDAVESALTK⌋--HELEHPLPKTADPRVQISGNFAPVPENPVCQSLPVTGKIPKCV 154
Vitis          ⌊AAAMALDAVETALVA⌋--RETQHPLPKTADPRVQIAGNFAPVPEQPVQHSLPVDGRIPDCI 160
Arabidopsis    ⌊AAAAALDAAEGFLVS⌋--HEKLHPLPKTADPSVQIAGNFAPVNEQPVRRNLPVVGKLPDSI 149
Zea            ⌊AAAAALDAFEEGFVAN⌋VLERPHGLPSTADPAVQIAGNFAPVGERPPVHELPVSGRIPPFI 149
                *** .** *  :.       *   * **.**** ***:****** *..  : *** *::*  :

Phaseolus      DGVYVRNGANPLYEPVAGHHFFDGDGMVHAVKFTNGAAS-YACRFTETQRLAQEKSLGRP 227
Lycopersicon   QGVYVRNGANPLFEPTAGHHFFDGDGMVHAVQFKNGSAS-YACRFTETERLVQEKALGRP 213
Vitis          QGVYTRNGANPLHKPVAGHHLFDGDGMVHAVQFKDGDAS-YACRFTETQRLIQERDFGRP 219
Arabidopsis    KGVYVRNGANPLHEPVTGHHFFDGDGMVHAVKFEHGSAS-YACRFTQTNRFVQEROLGRP 208
Zea            DGVYARNGANPCFDPVAGHHLFDGDGMVHALRIRNGAAESYACRFTETARLRQERAIGRP 209
                .***.****** ..*.:***:***********::: .* *. ******:* *: **: :***

Phaseolus      VFPKAIGELHGHSGIARLLLFYARSLFQLVDGSHGMGVANAGLVYFNNHLLAMSEDDLPY 287
Lycopersicon   VFPKAIGELHGHSGIARLMLFYARGLFGLVDHSKGTGVANAGLVYFNNRLLAMSEDDLPY 273
Vitis          VFPKAIGELHGHSGIARLLLFYARGLFGLVDHSHGTGVANAGLVYFNRRLLAMSEDDLPY 279
Arabidopsis    VFPKAIGELHGHTGIARLMLFYARAAAGIVDPAHGTGVANAGLVYFNGRLLAMSEDDLPY 268
Zea            VFPKAIGELHGHSGIARLALFYARAACGLVDPSAGTGVANAGLVYFNGRLLAMSEDDLPY 269
                ************:***** *****.  :** : * ********** :*********

Phaseolus      HVRITSNGDLTTVGRYDFNGQLNSTMIA⌈H⌉PKLDPVNGDLHALSYDVVQKPYLKYFRFSAD 347
Lycopersicon   HVKVTPTGDLKTEGRFDFDGQLKSTMIA⌊H⌋PKLDPVSGELFALSYDVIQKPYLKYFRFSKN 333
Vitis          QIRVTPSGDLETVGRYDFEGQLRSTMIA⌊H⌋PKVDPVSGEMFALSYDVVQKPYLKYFRFSPK 339
Arabidopsis    QVQITPNGDLETVGRYDFDGQLESTMIA⌊H⌋PKVDPESGELFALSYDVVSKPYLKYFRFSPD 328
Zea            HVRVADDGDLETVGRYDFDGQLGCAMIA⌊H⌋PKLDPATGELHALSYDVIKRPYLKYFYFRPD 329
                ::::::   *** * **:**:***  .:****:**:** .*::. *******:.:****** *  .

Phaseolus      GVKSPDVEIPLKEPTMM⌈H⌉DFAITENFVVVPDQQVVFKLTEMITGGSPVVYDKNKTSRFGI 407
Lycopersicon   GEKSNDVEIPVEDPTMM⌊H⌋DFAITENFVVIPDQQVVFKMSEMIRGGSPVVYDKNKVSRFGI 393
Vitis          GDKSPDVEIPLAEPTMM⌊H⌋DFAITERFVVVPDQQXVFKLQEMISRGGSPVVYDKNKMSRFGV 399
Arabidopsis    GTKSPDVEIQLDQPTMM⌊H⌋DFAITENFVVVPDQQVVFKLPEMIRGGSPVVYDKNKVARFGI 388
Zea            GTKSDDVEIPLEQPTMI⌊H⌋DFAITENFVVVPDHQVVFKLQEMLRGGSPVVLDKEKTSRFGV 389
                * ** ****  :  :***:*:*  ****** .***.**:*  ****  **:  ** **:* :****:

Phaseolus      LDKNAKDANAMRWIDAPECFCF⌈H⌉LWNAWEEPETDEIVVIGSCMTPADSIFNECDESLKSV 467
Lycopersicon   LDKYAKDGSDLKWVEVPDCFCF⌊H⌋LWNAWEEAETDEIVVIGSCMTPPDSIFNECDEGLKSV 453
Vitis          LDKNATDASGMRWIEAPDCFCF⌊H⌋LWNAWEEPETDEVVVIGSCMTPPDSIFNECDEGLKSV 459
Arabidopsis    LDKYAEDSSNIKWIDAPDCFCF⌊H⌋LWNAWEEPETDEVVVIGSCMTPPDSIFNESDENLKSV 448
Zea            LPKHAADASEMAWVDVPDCFCF⌊H⌋LWNAWEDEATGEVVVIGSCMTPADSIFNESDERLESV 449
                * * * *..   :  * *:..*:****:******:    *.*:**********.****** ** *:**

Phaseolus      LSEIRLNLRTGKSTRRPIISD-AEQVNLEAGMVNRNKLGRKTQFAYLALALAEPWPKVSGFA 526
Lycopersicon   LSEIRLNLKTGKSTRKSIIENPDEQVNLEAGMVNRNKLGRKTEYAYLAIAEPWPKVSGFA 513
Vitis          LSEIRLNLKTGKSTRRPILPE-SEQVNLEAGMVNRNKLGRKTQFAYLAVAEPWPKVSGFA 518
Arabidopsis    LSEIRLNLKTGESTRRPIISNEDQQVNLEAGMVNRNMLGRKTKFAYLALAEPWPKVSGFA 508
Zea            LTEIRLDARTGRSTRRAVLPP-SQQENLEVGMVNRNLLGRESRYAYLAVAEPWPKESGFA 508
                *:*****:  :**.***:.::       :* ***.****** ***:..:****:****** ****

Phaseolus      KVDLFSGEVQKYMYGEEKFGGEPLFLP------NGEEEGDGYILAFVHDEKEWKSELQIV 580
Lycopersicon   KVNLFTGEVEKFIYGDNKYGGEPLFLPRD---PNSKEEDDGYILAFVHDEKEWKSELQIV 570
Vitis          KVDLSTGQVSKYIYGEQRYGGEPLFLPRD---PNSGREDDGYILAFVHDEKTWKSELQIV 575
Arabidopsis    KVDLTTGEVKKHLYGDNRYGGEPLFLPG-----EGGEEDEGYILCFVHDEKTWKSELQIV 563
Zea            KEDLSTGELTKFEYGEGRFGGEPCFVPMDPAAAHPRGEDDGYVLTFVHDERAGTSELLVV 568
                * :* :*:: *. **: ::**** *:*        .    *.:**:* ***** :   .*** :*

Phaseolus      NAQNLKLEASIKLPSRVPYGF⌈H⌉GTFIHSKDLRKQA- 615
Lycopersicon   NAMSLKLEATVKLPSRVPYGF⌊H⌋GTFINANDLANQA- 605
Vitis          NATNLQLEASVKLPSRVPYGF⌊H⌋GTFISSKELEKQA- 610
Arabidopsis    NAVSLEVEATVKLPSRVPYGF⌊H⌋GTFIGADDLAKQVV 599
Zea            NAADIRLEATVQLPSRVPYGF⌊H⌋GTFITGQELEAQAA 604
                ** .:.:**:::*****:**:****** ..:*  *.
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Figure 5.7. ClustalW alignment of the predicted amino acid sequence of the Pinotage *VvNCED1* gene to NCED protein sequences of related species, namely *P. vulgaris* (AAF26356), *L. esculentum* (CAD30202), *A. thaliana* (NCED3; NP_188062) and *Z. mays* (AAB62181). (*) and (:) denote identical and similar amino acids, respectively. The blocked text indicates the α-helix and the four conserved histidine residues.

A Southern blot, performed at high stringency, and probed with the full-length *VvNCED1* revealed two bands (Fig. 5.8) despite no internal *Eco*RV restriction site within the gene. A strongly detected additional hybridisation band approximately 3.0 kb in size was detected at low stringency (results not shown). Analysis of *VvNCED2* reported by Soar *et al.* (2004) revealed an internal *Eco*RV restriction site within this gene. In conjunction with their findings, we speculate that *VvNCED1* is present as a single copy in the grapevine genome but is a member of a two-gene family.
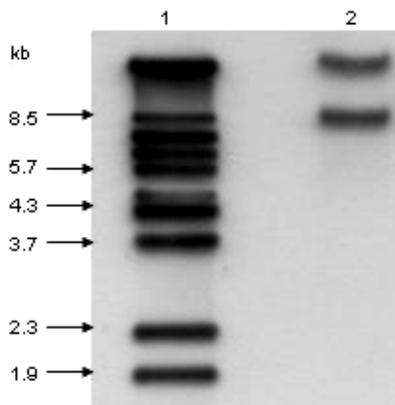

Figure 5.8. Southern blot analysis of *Eco*RV-digested *V. vinifera* L. (cv. Pinotage) genomic DNA. Genomic DNA (10 μg) was digested with *Eco*RV, separated by gel electrophoresis and transferred to a nylon membrane. Lane **(1)** λ DNA *BstE*II-digested marker with fragment sizes given in kb **(2)**, *Eco*RV-digested *V. vinifera* L. (cv Pinotage) genomic DNA.

DISCUSSION

The isolation of a *NCED1* from grapevine cv. Shiraz has previously been detailed by Soar *et al.* (2004). During the same time period, we isolated a *NCED1* from Pinotage, a local South African grapevine hybrid. *VvNCED1* was found to be a member of a two-gene family encoding a predicted protein of 611 amino acids and containing a number of conserved regions making it a typical 11, 12-double bond cleaving NCED. The putative amino acid sequences of the coding regions of both grapevine *NCED*1s were found to be identical; with a potential point mutation accounting for the cultivar differences.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 Carotenoids are central to plant metabolism, yet are not completely understood

Plants sustain most living organisms by converting solar energy into chemical energy via photosynthesis. Carotenoids play a central role in plant metabolism in general, and specifically in photosynthesis where they perform a myriad of functions. In addition to being integral structural and accessory light-harvesting components of the photosynthetic apparatus, their ability to quench reactive oxygen species formed under adverse conditions, thereby protecting the photosynthetic machinery against damage, is invaluable. Furthermore, carotenoids are precursors for the synthesis of the sesquiterpenoid abscisic acid (ABA).

Carotenoid biosynthesis has been intensively studied on the biochemical level, yielding comprehensive data on the pathway involved in biosynthesis as well as individual enzymes involved in the various steps to produce the end-products and secondary metabolites from this pathway. These studies and others have shown that the carotenoid pathway is central in plant metabolism and as such is tightly controlled.

Metabolism is influenced by genotype, but also by the environment of the organism. The ability to isolate individual genes from plant species and to analyse them with respect to their functions in their respective hosts or in model plant species, brings significant advantages to plant biology. Carotenoid metabolism is benefiting from these advances in various ways, but significant gaps exist in our understanding of the regulation and *in planta* functioning of each biosynthetic gene as well as the physiological functions of the metabolites formed within the pathway.

6.2 Functional analysis of two carotenoid biosynthetic pathway members from grapevine

As mentioned previously, a number of carotenoid biosynthetic genes, 31 in total, have been isolated from *Vitis vinifera* L. cv. Pinotage providing an invaluable resource for investigating the *in planta* role of the individual pathway members. Although not yet publicly available, the grapevine genome sequencing project has recently been completed (www.iasma.it), making available a vast amount of sequence information that will fast-track research activities in this non-model woody perennial plant. The present investigation was initiated prior to the availability of the complete sequence information of *V. vinifera*. Hence, employing a combination "PCR-subgenomic library" approach, five full-length carotenoid biosynthetic genes were isolated (Young, 2004), whereas the remaining 27 were identified and cloned from sequence information made available in the Expressed Sequence Tag (EST) database (unpublished data). Two of the originally isolated genes; namely *lycopene β-cyclase* (*β-LCY*; Chapter 4) and *9-cis epoxycarotenoid dioxygenase* (*NCED;* Chapter 5), were targeted for functional analyses as well as addressing pertinent

scientific questions regarding carotenogenesis as outlined in Chapter 1 of this dissertation.

The strategy involved the overexpression of the genes in the model plant *Arabidopsis* and subsequent phenotypic, genotypic, metabolic and physiological analysis of the homozygous transgenic populations.

6.2.1 Technologies developed and adopted for evaluating the *in vivo* role of grapevine carotenoid biosynthetic genes

Three technologies were either developed and/or adopted in our environment to facilitate functional analysis of the chosen genes. These technologies will be invaluable for future analyses of other genes in the carotenoid biosynthetic pathway. Moreover, with the grapevine genome now available, additional *omics* tools become available to study grapevine genes and processes. The three technologies that were crucial for the analysis of the genes in this study was a pigment profiling method developed for chloroplastic tissue (Chapter 3), a hormone profiling method (Chapters 4 and 5) as well as various physiological analyses (Chapters 4 and 5) and will be briefly discussed below.

Pigment profiling. As carotenoids are particularly light-sensitive and are susceptible to damage by a range of physico-chemical factors, we experienced current literature to be lacking in terms of handling and storage procedures for both the authentic standards and the samples. Furthermore, existing techniques were not able to show acceptable baseline separation of lutein from zeaxanthin as well as between the *cis*- and *trans*-isomers of violaxanthin and neoxanthin. Chapter 3 discusses all the precautions to be taken and details the pitfalls that may be encountered during preparation and storage of authentic standards as well as during the extraction of carotenoids from plant leaf tissue. Furthermore, a high performance liquid chromatography (HPLC) method using a combination of methanol, water and *tert*-butyl methylether (TBME) was set up for the reproducible step-by-step quantitative profiling of the principal carotenoids found in plant leaf tissue. Although this method has currently only found success for pigment quantification in *A. thaliana* leaf tissue and recently for *Nicotiana tabacum*, we believe that it could have broad-spectrum application.

Hormone profiling. Carotenoid biosynthesis is known to be a fluid cascade by means of multiple checks and balances, with considerable interplay within the pathway, due to multi-enzyme aggregates (Cunningham and Gantt, 1998), and between related pathways (Laule *et al.*, 2003). Due to the integral role phytohormones play during plant growth and development and in response to external stress factors, as well as their potential cross-talk between their biosynthetic pathways and those of carotenogenesis, we adopted an existing vapour phase extraction (VPE) protocol and electron impact–gas chromatography/mass spectrometry (EI-GC/MS; Schmelz *et al.*, 2003, 2004) method and modified and

validated it for application in our environment (see relevant materials and methods section in Chapter 4).

Physiological analysis. A number of chlorophyll *a* fluorescence methodologies were tested for optimal determination of a number of parameters allowing information to be gathered regarding the photosynthetic capacity, status of the photosynthetic machinery and the capabilities of the plants to quench excess light energy (non-photochemical quenching, NPQ) in a harmless manner.

6.2.2 Chloroplastic β-LCY apportions the flux at the bifurcation point, but is not a rate-limiting step and may function as a neoxanthin synthase (NSY) paralog

β-carotene and the active enzyme catalysing its synthesis play an undeniably key role in carotenoid metabolism. Using tomato as a model plant, the regulation and functioning of β-LCY within the chromoplasts has been elucidated. Although it is clear that β-LCY regulates the apportioning of substrate between the β- and α-branch (Cunningham *et al.*, 1996) in photosynthetically active tissues, the regulatory role of β-LCY specifically in β-carotene formation and within the general context of carotenogenesis is limited. Chapter 4 details the overexpression of grapevine β-*LCY* in *Arabidopsis* in order to further clarify the *in planta* role of this gene.

β-LCY controls carbon flow between the β-and α-branches of the carotenoid biosynthetic pathway. As expected, overexpression of β-*LCY* decreased lutein levels due to preferential portioning of lycopene into the β-branch. However, no concomitant increases in either β-carotene or the xanthophyll cycle pigments were reported indicating that β-*LCY* is not able to regulate the flow of carbon through the pathway and providing additional evidence to the fluidity of this pathway whereby pigment levels are continually balanced.

A number of role players that have been identified as control points within the pathway of which *PSY* and/or *PDS* are the likeliest upstream regulatory steps controlling the amount of substrate available for β-LCY (Misawa *et al.*, 1994; Romer *et al.*, 2002). A detailed transcriptional analysis as well as a comprehensive enzyme kinetic study of *PDS*, *PSY*, the native β-*LCY* and *Vvβ-LCY* in both the *Vvβ-LCY* lines and the WT may be beneficial in establishing exactly how regulation is achieved to balance out the carotenoid levels within the pathway.

Decreased neoxanthin levels may be associated with increased lipid peroxidation and supports a role for β-LCY as an NSY paralog. *Vvβ-LCY* overexpression resulted in significant decreases in neoxanthin (and specifically the *cis*-isomer) under both low light (LL) and following exposure to high light (HL). In parallel, the *Vvβ-LCY* transgenics exhibited higher malondialdehyde levels following exposure to excess light energy. Although not corroborated an essential role for neoxanthin, and

potentially lutein, has been suggested in preventing or at least reducing lipid peroxidation under HL stress.

It is possible that the decreased neoxanthin levels may be due to silencing of the *Arabidopsis β-LCY* by the *Vvβ-LCY*, as the former may function as a NSY paralog as NSY is not encoded for in the *Arabidopsis* genome. Although not conclusive, this finding provides evidence for the grapevine *β*-LCY to also substitute for a NSY, when required. Quantification of the transcript levels of the native *Arabidopsis β-LCY* could indicate whether this gene has indeed been downregulated by the heterologously expressed *β-LCY*.

Combined overexpression of β-LCY and an upstream regulatory gene may play a role in light stress management. A number of components may contribute to photoprotection when light energy is available in excess. Although zeaxanthin has been shown to be essential for photoprotection under saturating light conditions in *Arabidopsis* (Davison *et al.*, 2002), a xanthophyll cycle-independent mechanism of heat dissipation has recently been discovered (Finazzi *et al.*, 2004; Demmig-Adams and Adams, 2006). Furthermore, lutein has been shown to be involved directly or indirectly in NPQ of chlorophyll *a* fluorescence *in vivo* (Pogson *et al.*, 1998; Lokstein *et al.*, 2002). Although the combination of *Vvβ-LCY* overexpression and HL stress increased the size of the xanthophyll cycle pool slightly, this was not reflected in a larger de-epoxidation ratio nor in an altered NPQ capacity when the WT and the transgenics were compared. Furthermore, this provides evidence that the lower lutein levels had not compromised the plant's ability to induce and maintain NPQ.

It is possible that the advantages held by *β*-LCY overexpression will only become obvious following prolonged exposure to a HL stress. Experiments whereby transgenic *Vvβ-LCY* plants are grown continually under HL (>1000 μmol photons $m^{-2}.s^{-1}$) and exposed to extended periods (exceeding 30 min) of excess light might reveal a role for *β*-LCY in light stress management. Furthermore an overexpression strategy should be investigated whereby *β*-LCY and a gene regulating the amount of substrate available for *β*-LCY are co-expressed.

6.2.3 A comprehensive physiological analysis of *Arabidopsis VvNCED1* transgenics during both water availability and shortage

NCED has been unquestioningly established to be the key regulatory step in ABA production via cleavage of carotenoids, specifically the 9-*cis*-isomers of neoxanthin and violaxanthin (Xiong and Zhu, 2003; Nambara and Marion-Poll, 2005). The relationship between *NCED* overexpression, higher constitutive ABA levels, reduced stomatal conductance and drought tolerance has been repeatedly demonstrated (Thompson *et al.*, 2000; Iuchi *et al.*, 2001; Qin and Zeevaart, 2002). A similar interrelationship has been eluded towards by Stoll *et al.* (2000) and later by Soar *et al.* (2004) in grapevine. Chapter 5 discusses the constitutive overexpression of *VvNCED1* in *Arabidopsis* in order to corroborate the direct role of *Vv*NCED1 in

drought resistance and conduct functional *in planta* analyses for an increased understanding thereof. This is the first report where *NCED*-overexpressing transgenic lines were comprehensively analysed according to their physiology under conditions where water is abundant or limiting, and following exposure to HL. These analyses revealed a number of parameters involved or affected during drought tolerance.

Well-watered VvNCED1 transgenics have a lower pigment complement yet increased capacity for NPQ over a wide actinic light range when compared to WT. Although the 9-*cis* xanthophylls have been shown to be the substrate(s) for NCED, the concomitant effects of constitutive levels of this enzyme on the total and individual chlorophylls and carotenoids are lacking. Contrary to expectations, the lower pigment pool detected did not appear to be due to a specific depletion of carotenoids following *VvNCED1* overexpression but more likely due to a lower total chloroplastic pigment complement. This is evidence to the integral structural and functional role these pigments play in the thylakoid membranes and in various aspects of photochemistry and photoprotection. Furthermore, these findings corroborate the stringent control mechanisms that operate during carotenogenesis whereby the specific depletion of a single compound is rapidly distributed amongst all intermediates within the whole pathway.

Of particular significance was the novel finding of an increased capacity for NPQ in the well-watered *VvNCED1* transgenics and, in particular, a higher flexible thermal energy dissipation component (qE) when compared to that of WT. Previously the exogenous application of ABA was connected to an increase in xanthophyll-dependent NPQ (Ivanov *et al.*, 1995; Sharma *et al.*, 2002), most likely due to a reduced requirement exerted on the ABA precursor pool. *VvNCED1* overexpression was not associated with increased zeaxanthin concentrations, nor did it result in an increase in the deepoxidation ratio, traditionally used as a measure of the xanthophyll-dependent photoprotective capacity. Instead it is possible that the increased NPQ is coupled to the constitutively decreased stomatal conductance found in the transgenic plants. Although overexpression did not impact on the photochemical efficiency of the *VvNCED1* transgenics, we propose that the reduced chlorophyll and carotenoids complement absorb comparatively less light energy and that the rate of photosynthesis is slower due to decreased CO_2 uptake. It is possible that this combination necessitates a greater capacity for NPQ, however, this speculation requires a considerable amount of research for confirmation.

VvNCED1 overexpression impacts on the phenotype of well-watered plants and reveals potential interplay between the auxin and carotenoid biosynthetic pathways. In general and as expected, *VvNCED1* overexpression resulted in increased ABA levels which were associated with delayed seed germination and a slight to severe

retardation in the overall plant growth rate frequently to the extent of a lower above ground biomass.

Threshold levels of ABA have been proposed beyond which this phytohormone self-regulates its catabolism into phaseic acid (PA) and dihydrophaseic acid (Nambara and Marion-Poll, 2005). Previously, overexpression of *Phaseolus vulgaris NCED* in tobacco (Qin and Zeevaart, 2002) resulted in increased PA concentrations. Extension of the existing EI-GC/MS method is recommended for detection of these catabolites in order to quantify *NCED* overexpression more accurately and to further our existing knowledge of ABA regulation and subsequent breakdown.

The current study has provided the first experimental evidence of potential indole acetic acid (IAA) and ABA co-regulation in *NCED* transgenics. Many questions still exist regarding the exact mechanism of crosstalk between the two pathways however increased levels of the auxin IAA have previously been shown to be associated with decreased stomatal conductance (Assmann and Armstrong, 1999).

An array of parameters are involved and/or affected during drought tolerance. As mentioned previously, *VvNCED1* overexpression is met by a specific increase in ABA and hence a constitutively reduced stomatal conductance. Following withholding of water for a period of 18 days, stomatal conductance frequently decreased even further conferring drought tolerance and thereby maintaining plants with a green, healthy phenotype. Although it was clear that the native *Arabidopsis NCED* was upregulated during water stress, resulting in increased ABA and decreased stomatal conductance, it seems that the constitutively high ABA levels in the transgenics which maintain the guard cells in a closed state confers a greater advantage that native *NCED* upregulation in response to a perceived stress cannot match.

This study is the first one where drought tolerance was quantified relative to pigment levels and is profiled relative to SA and IAA concentrations. Furthermore changes in the maximum efficiency of photochemistry, F_v/F_m, the degree of lipid peroxidation, and the soil moisture content of the pots and relative water content of the plants were determined. The results of the two transgenic lines investigated did not mirror each other exactly; however, this study reveals some of the factors affected during drought acclimation and indicates those parameters that are integral during drought tolerance. Collectively, these results confirm and advance our existing knowledge of the role of NCED in drought tolerance and the specific mechanisms operating on a whole plant level. In conjunction with the *nced3/sto1 Arabidopsi*s mutant, the *Arabidopsis VvNCED1* transgenic lines generated in this study may provide further insights into the signal induction cascade initiated and the mechanisms operating under water limitation where it is critical to the wine, table grape and dried fruit industries.

6.3 CONCLUSION

This study has provided further evidence as to the strict regulatory control and fluidity that exists within the carotenoid biosynthetic pathway whereby individual pigment levels are constantly brought back into balance despite constitutive expression of one of the pathway gene members. Our current knowledge of the roles of the two target genes studied, namely *β-LCY* and *NCED*, has clearly been furthered. Gaps in our current understanding regarding the *in planta* role of *β-LCY* in the chloroplasts have been addressed as well as questions concerning the *in vivo* physiological effects of a functional NCED when water is abundant or scarce. Furthermore, a number of parameters have been identified for classification and conference of drought tolerance. The fundamental data acquired has set the scene for future investigations to further corroborate some of these recent findings and newly-established hypotheses.

Over the last couple of years some important advances have been made in generating crop species with an enhanced nutritional content (particularly *β*-carotene; Fraser *et al.*, 2002; D'Ambrosio *et al.*, 2004; Al-Babili and Beyer, 2005), decreased susceptibility to HL (Davidson *et al.*, 2002) and increased tolerance to drought (Iuchi *et al.*, 2001; Qin and Zeevaart, 2002; Wan and Li, 2006) following manipulation of specific carotenoid biosynthetic genes. The *in planta* analysis of all grapevine isoprenoids isolated within the Grapevine Biotechnology Programme is still in its incubator stage and comprehensive analyses of the remaining 27 genes in a model environment is ongoing.

As with most critical pathways in living organisms, carotenoid biosynthesis and regulation pose a considerable scientific challenge to unravel. Plants have mechanisms to ensure the functionality of this pathway, complicating overexpression (as used in this study) and silencing strategies that are typically applied. These analyses do provide valuable baseline information about individual genes. These baselines can be extended upon with other technologies such as transcriptomic, proteomic and metabolomic data in model plants to comprehend the full complexity involved in carotenogenesis. Grapevine is being developed as the first woody perennial model plant and this study makes a significant contribution towards understanding the role of two important carotenoid genes from grapevine, but also provides fundamental information that can be built on with the *omics* tools currently becoming available in grapevine research.

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