

Over-expression and analysis of two *Vitis vinifera* carotenoid biosynthetic genes in transgenic *Arabidopsis*

by

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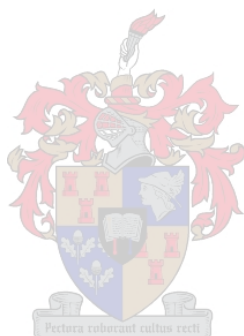
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Prof VR Smith

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis 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.

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Date



SUMMARY

Plants have evolved photosynthetic systems to efficiently harvest sunlight energy for the production of carbohydrates, but these systems also are extremely susceptible to an excess of light. To combat the potential damaging effects of light, plants have developed various mechanisms to control and cope with light stress. These mechanisms include the movement of either leaves, cells (negative phototaxis) or chloroplasts to adjust the light-capturing potential, the adjustment of the light-harvesting antenna size through gene expression or protein degradation, the removal of excess excitation energy either through an alternative electron transport pathway or as heat. However, the latter mechanism based on thermal dissipation, remains the most effective to rid the plant of damaging excess light energy. This process involves several carotenoid pathway pigments, specifically the de-epoxidised xanthophyll cycle pigments. The process and extent of thermal dissipation in plants can be measured and quantified as non-photochemical quenching (NPQ) of chlorophyll fluorescence by using well-established methodologies. Several *Arabidopsis* and *Chlamydomonas* mutants affected in the xanthophyll cycle have been isolated. These mutants have provided evidence for the correlation between the de-epoxidised xanthophyll cycle pigments and NPQ as well as better understanding of the operation of the xanthophyll cycle and the related carotenoid biosynthetic enzymes. This key photoprotective role of the xanthophyll cycle is therefore a promising target for genetic engineering to enhance environmental stress tolerance in plants. Several genes from the carotenoid biosynthetic pathway of grapevine (*Vitis vinifera* L.) were isolated previously in our laboratory. The main aim of this study was to over-express two xanthophyll cycle genes from grapevine in *Arabidopsis* and to analyse the transgenic population with regards to pigment content and levels as well as certain photosynthetic parameters. The transgenic lines were compared with wild type *Arabidopsis* (untransformed) plants and two xanthophyll cycle mutants under non-limiting conditions as well as a stress condition, specifically a high light treatment to induce possible photodamage and photoinhibition.

Transgenic *Arabidopsis* lines over-expressing the two *V. vinifera* xanthophyll cycle genes, β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*), were established following *Agrobacterium* transformation. In addition to the untransformed wild type, two NPQ mutants, *npq1* (lacking violaxanthin de-epoxidase) and *npq2* (lacking zeaxanthin epoxidase), were used as controls throughout this study. The transgenic lines were propagated to a homozygous T3-generation, where stable integration and expression of the transgenes were confirmed in only 16% and 12% for *VvBCH* and *VvZEP* lines, respectively. No phenotypical differences could be observed for the transgenic lines compared to the wild type, but the *npq2* mutant showed a stunted and 'wilty' phenotype, as was previously described.

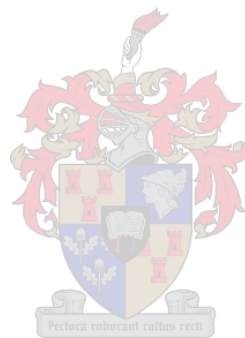
To evaluate the pigment composition of the transgenic lines a reliable and reproducible method was needed to analyse carotenoids from leafy material. To this end a new high-performance liquid chromatography (HPLC) method was developed for the quantitative profiling of eight major carotenoids and chlorophyll *a* and *b*. Emphasis was placed on baseline separation of the xanthophyll pigments, lutein and zeaxanthin as well as the *cis*- and *trans*-forms of violaxanthin and neoxanthin. The method effectively distinguished *Arabidopsis* wild type plantlets from the two *NPQ* mutant lines (*npq1* and 2) and could possibly find application for green leafy tissue samples in general.

The carotenoid content of the *NPQ* mutants were in accordance with previous reports. The lack of zeaxanthin epoxidase activity in the *npq2* mutant resulted in the accumulation of zeaxanthin under both low and high light conditions. This high level of zeaxanthin was found to cause an initial rapid induction of *NPQ* at low to moderate light intensities, but this difference disappeared at high light, where zeaxanthin formation induced considerable *NPQ* in the wild type. Similarly, the *npq1* mutant was unable to de-epoxidise violaxanthin to zeaxanthin under high light conditions, which resulted in severe inhibition of *NPQ* induction. Furthermore, these mutant plantlets were shown to be more susceptible to photoinhibition compared to that of the wild type.

The over-expression of *VvBCH* resulted in a marked increase in the xanthophyll cycle pool pigments (violaxanthin, antheraxanthin and zeaxanthin) and reduced β -carotene levels under both low and high light conditions compared to that of the wild type, indicating elevated β -carotene hydroxylase activity possibly due to over-expression of the *VvBCH* gene. Similar to the induction of *NPQ* in the *npq2* mutant, the increased levels of zeaxanthin in the *VvBCH* lines did not offer any additional photoprotection. This would suggest that the heightened zeaxanthin levels observed for the *VvBCH* lines do not necessarily enhance photoprotection, however it may protect the thylakoid membrane against lipid peroxidation as has been shown previously. The *VvZEP* lines however, showed reduced levels of zeaxanthin in high light conditions to that of the wild type, probably due to the competing epoxidation and de-epoxidation reactions of the xanthophyll cycle. This reduction in zeaxanthin synthesis in the *VvZEP* lines resulted in significant reduced *NPQ* induction compared to that of the wild type, a phenomenon also observed for the *npq1* mutant. Similar to the *npq1* mutant, these lines displayed significantly increased photoinhibition, which may be due to photodamage of the reaction centers if one considers the lowered photosystem II photochemistry efficiency and reaction center openness of these lines compared to the wild type. This may suggest that even small reductions in zeaxanthin amounts can result in an increase in photoinhibition, under high light conditions.

This study and its results provide fundamental information regarding two grapevine-derived carotenoid pathway genes and their possible physiological roles. Moreover, studies like these provide information that is essential when possible

biotechnological approaches are planned with this central plant metabolic pathway in mind. The results highlighted the complex regulation of this pathway, necessitating attention to flux control, simultaneous manipulation of several pathway genes, and the measurement of other compounds derived from this pathway when evaluating the possible applications of the carotenoid pathway of plants.



OPSOMMING

Plante het, deur middel van evolusie, sisteme ontwikkel om effektief die son se stralingsenergie te absorbeer en vir koolhidraatproduksie te gebruik. Alhoewel hierdie sisteme die ligenergie vasvang, is hul sensitief vir 'n oormaat lig. Om hierdie sisteme te beskerm, het plante meganismes ontwikkel om die oormaat ligenergie veilig te verwyder en enige skade te herstel. Hierdie meganismes sluit die volgende in: die beweging van blare, selle en chloroplaste; die verandering van die antennagrootte vir ligvaslegging deur geenregulering en proteïendegradering; die verwydering van oormatige ligenergie deur 'n alternatiewe elektrontransportweg en deur die omskakeling van ligenergie na hitte. Van al hierdie meganismes is die hitte-omskakeling die doeltreffendste. Dit betrek die pigmente van die xantofilsiklus en kan verder ook gemeet word as nie-fotochemiese blussing van chlorofil-fluoresensie ("*non-photochemical quenching*", *NPQ*)

Die xantofilsiklus is vir die eerste keer in 1962 opgemerk toe vasgestel is dat hierdie siklus deur lig beheer word. Sedertdien is verskeie *Arabidopsis*- en *Chlamydomonas*-mutante geïsoleer wat in hul xantofilsiklus beïnvloed is. Hierdie mutante het die belangrike fisiologiese funksies van die xantofilsiklus in die beskerming van die fotosintesesisteme en die ensieme wat hierby betrokke is, uitgelig. Die belangrikheid van die xantofilsiklus in fotobeskerming is dus 'n belowende teken vir genetiese manipulerings om plante meer weerstandbiedend teen ligstreskondisies te maak. Verskeie gene van die karotenoïedbiosintese-pad in wingerd (*Vitis vinifera* L.) is in ons laboratorium geïsoleer. Die hoofdoel van hierdie studie was om twee xantofilsiklusgene van wingerd in *Arabidopsis* oor te druk en om die karotenoïedinhoud en -vlakke, asook sekere fotosintese parameters van die transgeniese lyne, te bestudeer. Hierdie transgeniese lyne is vergelyk met die wildetipe (ongetransformeerde *Arabidopsis*) en twee *NPQ*-mutante tydens ongestresde en gestresde toestande, spesifiek onder hoë ligstreskondisie om moontlike fotobeskerming en fotobeskadiging te induseer.

Transgeniese *Arabidopsis*-lyne wat die β -karoteenhidroksilase- (*VvBCH*) en seaxantin-epoksidase- (*VvZEP*) gene oorruidruk, is na *Agrobacterium*-transformasie gegenereer. Die ongetransformeerde wildetipe en twee *NPQ*-mutante, *npq1* (defektief in violxantin-deëpoksidase) en *npq2* (seaxantin-epoksidase), is as kontroles gedurende hierdie studie gebruik. Die transgeniese lyne is gepropageer deur die T3-generasie en die stabiele integrasie, en transkripsie van die transgene bevestig in net 16 en 12% vir *VvBCH* en *VvZEP* onderskeidelik. Geen fenotipe verskille is vir hierdie lyne opgemerk nie, behalwe die *npq2*-mutant wat 'n dwergagtige en verlepte voorkoms gehad het, wat ook ooreenkom met vorige bevindinge.

Om die karotenoïedpigment-inhoud in die transgeniese lyne te evalueer was 'n akkurate en herhaalbare metode nodig om karotenoïedpigmente in blaarmateriaal te ontleed. 'n Nuwe hoëdoeltreffendheid-vloeistofchromatografie (HDVC) -metode is ontwikkel om kwantifiseerbare profiele vir die hoofkarotenoïede en chlorofil *a* en *b* te

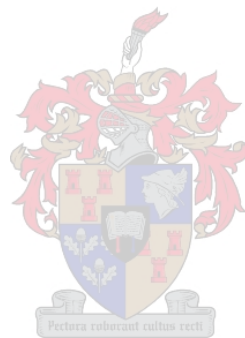
lewer. Die ontwikkeling van die nuwe metode was veral van belang vir die sorgvuldige skeiding van die xantofilpigmente, seaxantin en luteïen, sowel as die *sis-* en *trans-*vorme van violaxantin en neoxantin. Hierdie metode het dit moontlik gemaak om die wildetipe en die twee *NPQ*-mutante (*npq1* en *2*) van mekaar te kan onderskei en sal moontlik vir enige blaarmateriaal aangewend kan word.

Die karotenoïedinhoud wat in die *NPQ*-mutante waargeneem is, was in lyn met gepubliseerde data. Die gebrekkige seaxantin-epoksidase in die *npq2*-mutant het tot die opgaring van seaxantin gelei en verhoogde indusering van *NPQ* onder lae tot matige hoë lig tot gevolg gehad. Hierdie effek het verdwyn onder hoë lig, waar die vorming van seaxantin in die wildetipe tot hoë *NPQ*-induksie gelei het. Aan die ander kant was die *npq1*-mutant nie in staat om violaxantin na seaxantin onder hoë lig te deëpoksiedeer nie. Dit het tot gevolg gehad dat die *NPQ*-induksie van hierdie mutant betekenisvol geïnhibeer is. Verder is gewys dat hierdie mutant meer vatbaar is vir foto-inhibisie as die wildetipe.

Die ooruitdrukking van die *VvBCH* het gelei tot 'n verhoogde xantofilsikluspoel (violaxanthin, anteraxantin en seaxantin) en verlaagde β -karoteenkonsentrasie onder beide lae en hoë ligkondisies. Hierdie veranderinge in die karotenoïedhoeveelhede kan die gevolg wees van die moontlike verhoogde aktiwiteit van die β -karoteenhidroksilasegen in hierdie transgeniese lyne. Soortgelyk aan die *npq2*-mutant, het die addisionele seaxantin in die *VvBCH*-lyne geen verhoogde fotobeskerming teen hoë lig verseker nie, maar kan moontlik die tilakoïemembraan teen lipied-oksidasie beskerm, soos vroeër beskryf. In teenstelling hiermee, het die ooruitdrukking van die *VvZEP* geen tot verlaagde seaxantinvlakke onder hoë ligkondisies gelei, wat die gevolg kan wees van die kompetisie tussen die epoksidase en de-epoksidase reaksies van die xantofilsiklus. Hierdie verlaagde seaxantin in die *VvZEP*-lyne het tot betekenisvolle inhibisie van *NPQ*-induksie gelei. Hierdie inhibisie van *NPQ*-induksie is ook in die *npq1*-mutant geabsorbeer. Die verlaagde *NPQ*-induksie in die *npq1*-mutant en die *VvZEP*-lyne was as gevolg van fotoskade aan die reaksiesenters, soos geïllustreer deur die groter aantal reaksiesenters wat gesluit was, die verlaagde fotosisteen II-fotochemiese-effektiwiteit, sowel as die verhoogde foto-inhibisie (stadige vrystellende *NPQ*-komponent, *NPQ_S*) in hierdie plante. Dit wil voorkom asof selfs 'n klein vermindering in seaxantinhoeveelhede verhoogde foto-inhibisie onder hoë ligkondisies tot gevolg kan hê.

Hierdie studie is van belang om fundamentele inligting rakende twee karotenoïedbiosintese gene en hul moontlike biotegnologiese waarde te bepaal. Studies soos hierdie verskaf voorts ook waardevolle inligting wat in ag geneem moet word wanneer in biotegnologiese benadering met hierdie metaboliese pad in gedagte, beplan word. Hierdie resultate lig ook die komplekse regulering van die karotenoïedbiosintese pad, die gesamentlike manipulerings van verskeie gene en die bepaling van die gevolge op ander moontlike komponente afkomstig van hierdie sentrale pad wanneer 'n biotegnologiese benadering in hierdie pad geëvalueer word, uit.

This thesis is dedicated to my parents.



BIOGRAPHICAL SKETCH

Anika Elma Brackenridge was born in Pretoria, South Africa, on 18 April 1980. She attended Wonderboom Primary and matriculated with distinction from Overkruin High school in 1998. She enrolled at Stellenbosch University in 1999 and obtained a BSc degree (*Cum laude*) in Molecular and Cellular Biology in 2001. In 2002 she received the degree BScHons in Genetics. In 2003 she enrolled at the Institute for Wine biotechnology for an MSc degree in Wine Biotechnology.



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My **family** and **friends**, for their love and belief in my abilities;

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PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of Plant Physiology, except Chapter 3 which is prepared according to the style of the Journal of Chromatography A to which it will be submitted for publication. Chapter 4 will form part of a manuscript that will be submitted to Plant Physiology.

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**

The xanthophyll cycle and related physiological processes in higher plants

Chapter 3 **TECHNICAL REPORT**

High-Performance liquid chromatography profiling of the major carotenoids in *Arabidopsis thaliana* leaf tissue
(Accepted in *Journal of chromatography A*)

Chapter 4 **RESEARCH RESULTS**

Over-expression and analysis of two *Vitis vinifera* carotenoid biosynthetic genes in transgenic *Arabidopsis*
(This chapter will form part of a manuscript targeted for publication in *Plant Physiology*)

Chapter 5 **GENERAL DISCUSSION AND CONCLUSIONS**

I hereby declare that I was a co-contributor to a joined article by Me KL Taylor, a PhD student at the Institute for Wine Biotechnology, with respect to the research planning and execution, the data analysis and the interpretation, as well as the write-up of the experimental section of the data represented in Chapter 3. I was the primary contributor with respect to the experiments conducted and the presentation and interpretation of the data in the multi-author article presented in Chapter 4. My supervisors, Prof MA Vivier and Prof VR Smith, were involved in the conceptual development and continuous critical evaluation of the study.

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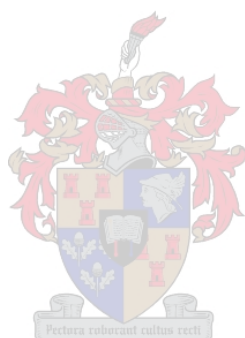
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**GENERAL INTRODUCTION
AND
PROJECT AIMS**



1.1 INTRODUCTION

Plants have evolved efficient systems to capture solar energy and to convert it into chemical energy. Large light-harvesting complexes (LHC) have evolved for efficient light absorption under limiting light conditions. However, in excess light the photosynthetic apparatus can be irreversibly damaged if plants absorb more light energy than they are able to use for photosynthesis. Moreover, environmental conditions such as drought, extreme temperature, or nutrient deprivation can further limit the ability of a plant to utilise light energy (Demmig-Adams and Adams, 1992).

Plants have devised various mechanisms to cope with a changing light environment (Demmig-Adams and Adams, 1992). Some algae and plants use a physical mechanism such as the movement of leaves, cells (negative phototaxis) or chloroplasts to regulate light uptake. Furthermore, chloroplasts are able to regulate the light harvested for photosynthesis by changing the physical size of the LHC; this occurs via regulation of gene expression of different LHC components, or by protein degradation (Anderson, 1986; Melis, 1991; Walters and Horton, 1994; Escoubas et al., 1995; Lindahl et al., 1995; Maxwell et al., 1995). Excessive light energy can also be removed by an alternative non-assimilatory electron transport pathway where oxygen can be reduced directly or through the oxygenase reaction catalysed by Rubisco (photorespiration) (Mehler, 1951; Biehler and Fock, 1996; Park et al., 1996). Despite all these mechanisms, photodamage is still a real consequence of photosynthesis. This is mainly due to the interaction of unstable intermediates with oxygen resulting in the formation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Krieger-Liszkay, 2004). Protection against ROS is affected by antioxidant systems involving specific molecules, including carotenoids and tocopherols, and antioxidant enzymes including superoxide dismutase and ascorbate peroxidase (Jespersen et al., 1997; Kliebenstein et al., 1998). Carotenoids and tocopherols are also able to quench or detoxify triplet chlorophylls (^3Chl), inhibit lipid peroxidation and stabilise the thylakoid membranes (Foyer et al., 1994; Demmig-Adams et al., 1996).

A well studied and effective photoprotection mechanism involves the dissipation of excessive light energy as heat. This mechanism plays an important role in the regulation of light-harvesting by photosystem II (PS II) and is controlled by a change in thylakoid pH (ΔpH). This process involves the de-excitation of ^1Chl (singlet chlorophyll) and can be measured as non-photochemical quenching (*NPQ*) of chlorophyll fluorescence (Demmig-Adams et al., 1996). Dissipation via *NPQ* involves a specific branch of the carotenoid pathway, the xanthophyll cycle and is triggered by an increase in ΔpH , resulting in protonation and binding of proteins related to the LHC (Björkman and Demmig-Adams, 1994; Demmig-Adams and Adams, 1996; Horton et al., 1996). As mentioned, the xanthophyll cycle is situated within the carotenoid biosynthetic pathway of plants (Hirschberg, 2001). It forms part of the

β -carotene branch, starting with the hydroxylation of β -carotene to zeaxanthin by β -carotene hydroxylase. The interconversion between zeaxanthin and violaxanthin is catalysed by two enzymes, zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), which are located on opposite sides of the thylakoid membrane (Yamamoto et al., 1962). VDE is situated on the stromal side of the thylakoid membrane and is activated by an increased acidity during high light conditions. Rapid conversion of violaxanthin to zeaxanthin proceeds, at a rate which exceeds epoxidation thereby resulting in zeaxanthin accumulation (Eskling et al., 1997). The elucidation of the distance between the xanthophyll and chlorophyll molecules in LHC II and their relative orientations has provided further evidence for the correlation between NPQ and xanthophyll cycling (Liu et al., 2004). The exact mechanism for NPQ is however, still under investigation. It has been proposed that the binding and protonation of PsbS (LHC associated protein) to zeaxanthin and to the LHC may cause a conformational change in the LHC (Gilmore, 1997), thus enabling NPQ. These results and others have proven unequivocally that xanthophyll cycle pigments play an important role in the dissipation of excitation energy.

Chlamydomonas and *Arabidopsis* mutants have been used to study the physiological importance of the xanthophyll cycle during plant growth and development under various conditions (Niyogi et al., 1997; Niyogi et al., 1998). These mutants have demonstrated the importance of xanthophyll cycling for the induction of NPQ, the role of xanthophyll pigments in lipid peroxidation of the thylakoid membranes, as well as the roles of the xanthophyll cycle enzymes. The *npq1* mutants (defective in the VDE gene) have provided evidence that NPQ induction and the extent of photoinhibition are associated with the de-epoxidation of violaxanthin to zeaxanthin. Furthermore, these mutants experienced higher levels of lipid peroxidation during prolonged exposure to high light in comparison to the wild type control. The *npq2* and *aba2* mutants (both defective in the ZEP gene) have shown that constitutive high levels of zeaxanthin results in rapid NPQ induction at low to moderate light intensities. This has however, not been correlated to increased photoprotection (Marin et al., 1996; Niyogi et al., 1998).

These mutants have enhanced our understanding of the operation of the xanthophyll cycle, the related carotenoid biosynthetic enzymes, and their physiological roles in adaptation to a changing environment. However, the *in planta* physiological roles of the individual gene products and their potential biotechnological application for enhanced plant stress tolerance needs to be investigated. To this end, transgenic lines over-expressing the genes encoding the products of the xanthophyll cycle either singly or in combination can be used to further our fundamental knowledge of photosynthesis or photoprotective mechanisms. This may be achieved by investigating the biochemical changes, specifically carotenoid levels under different stress conditions, and the physiological effects or roles of the heterologously expressed genes.

1.2 PROJECT AIMS

One of the current focuses of the Grapevine Biotechnology programme of the Institute for Wine Biotechnology is the improvement of *Vitis vinifera* L. towards environmental stress tolerance. The carotenoid biosynthetic pathway of plants not only forms a range of important pigments involved in various aspects of plant growth and metabolism, but several other compounds, including several growth hormones are derived from this pathway. Some of these have been linked to environmental stress protection and evidence already exist that biotechnological approaches involving carotenoid pathway members can lead to environmental stress protection (Borel et al., 2001; Davison et al., 2002). Several genes, directly or indirectly involved in this pathway were isolated from *Vitis vinifera* L. cv Pinotage in our laboratory (Young, 2004). These isolated genes are useful resources to investigate the physiological effect of these carotenoid biosynthetic gene products in an *in planta* environment and to establish their possible biotechnological application in grapevine improvement. This study forms part of the above initiative and used two carotenoid biosynthetic genes encoding a β -carotene hydroxylase and zeaxanthin epoxidase. These genes encode xanthophyll cycle enzymes and might be involved in protection against excessive light.

A classical biotechnological approach was planned to investigate the *in planta* physiological effect(s) of these genes by generating independent transgenic lines over-expressing the *β -carotene hydroxylase* or the *zeaxanthin epoxidase* gene in the model plant *A. thaliana*. Since these genes function in the xanthophyll cycle and their products might be involved in photoprotection, the transgenic lines would be subjected to light stress conditions, to determine their effect on individual and total carotenoid pools and their possible physiological effect(s) on photosynthesis and photoprotective mechanisms. The transgenic lines to be generated, the untransformed controls and two available xanthophyll cycle mutants, *npq1* and *npq2* would constitute the genetic material/resources used in this study. The following specific aims were formulated for this study:

- i) *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* with plant expression cassettes containing the *Vitis vinifera* *β -carotene hydroxylase* (*VvBCH*) and *zeaxanthin epoxidase* (*VvZEP*) genes and the establishment of confirmed homozygous populations of these transgenic lines;
- ii) Evaluation of individual and total carotenoid pigment levels in the transgenic lines over-expressing the *VvBCH* and *VvZEP* genes, in comparison with wild type plants and the *NPQ* mutant lines, *npq1* and *npq2*, under normal as well as light-stressed conditions; and
- iii) Evaluation of photosynthetic parameters and photoprotective mechanisms in the transgenic lines, the wild type plants and the *npq1* and *npq2* mutants to assess the possible physiological effects of the transgenes.

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LITERATURE REVIEW

The xanthophyll cycle and related physiological processes
in higher plants



LITERATURE REVIEW

2.1 INTRODUCTION

Higher plants and algae are oxygenic photoautotrophic organisms that utilise light energy to produce carbohydrates via photosynthesis, a complex process involving successive reduction-oxidation (redox) reactions. These organisms have evolved large light-harvesting complexes (LHC) to ensure sufficient light capture, specifically during limiting light conditions. However, excessive light irradiation can also occur during long summer days. Absorbed excess light energy can have damaging effects on the photosynthetic apparatus, ultimately leading to reduced photosynthetic capacity, and even complete photosynthetic inhibition in extreme circumstances.

To cope with light as a changing environmental factor, plants can function optimally over a relatively broad range of light intensities, and they also have developed several mechanisms to minimise the damaging effects of light (see Figure 1). Some algae and higher plants have developed physical mechanisms to avoid excessive light absorption, such as the movement of leaves, cells (negative phototaxis), or chloroplasts (Brugnoli and Björkman, 1992; Björkman and Demmig-Adams, 1994). Chloroplasts are able to balance the absorption and utilisation of light energy by regulating the light harvested for photosynthesis and electron transport. These mechanisms include the adjustment of the size of the light-harvesting antennae associated with photosystems I and II (PS I and PS II) (Anderson, 1986; Melis, 1991). This can be achieved by changes in the LHC gene expression and protein degradation patterns (Walters and Horton, 1994a; Escoubas et al., 1995; Lindahl et al., 1995; Maxwell et al., 1995).

Excessive light energy in the photosynthetic apparatus can also be removed by an alternative electron transport pathway. There is evidence that non-assimilatory electron transport to oxygen plays an important role in dissipating excess excitation energy (Biehler and Fock, 1996; Heber et al., 1996). Oxygen functions as an electron acceptor either through an oxygenase reaction catalysed by Rubisco (photorespiration) or through direct reduction by electrons on the acceptor side of PS II (Mehler, 1951; Park et al., 1996). Direct reduction of oxygen by PS I is the first step in an alternative electron transport pathway, termed “pseudocyclic electron transport”, the “Mehler-ascorbate peroxidase reaction”, or the “water-water cycle” (Asada, 1999). This pathway involves the reduction of singlet oxygen produced on the PS I acceptor side by the thylakoid-bound isozymes superoxide dismutase (SOD) and ascorbate peroxidase (APX). These reactions generate water and monodehydroascorbate, which can be reduced directly by PS I to regenerate ascorbate (Asada, 1994; Asada, 1999). The electrons generated by PS II due to the oxidation of water, thus are consumed by the reduction of oxygen to water by PS I. This “pseudocyclic pathway” generates a change in pH (ΔpH) for ATP synthesis, but neither NADPH nor oxygen is produced.

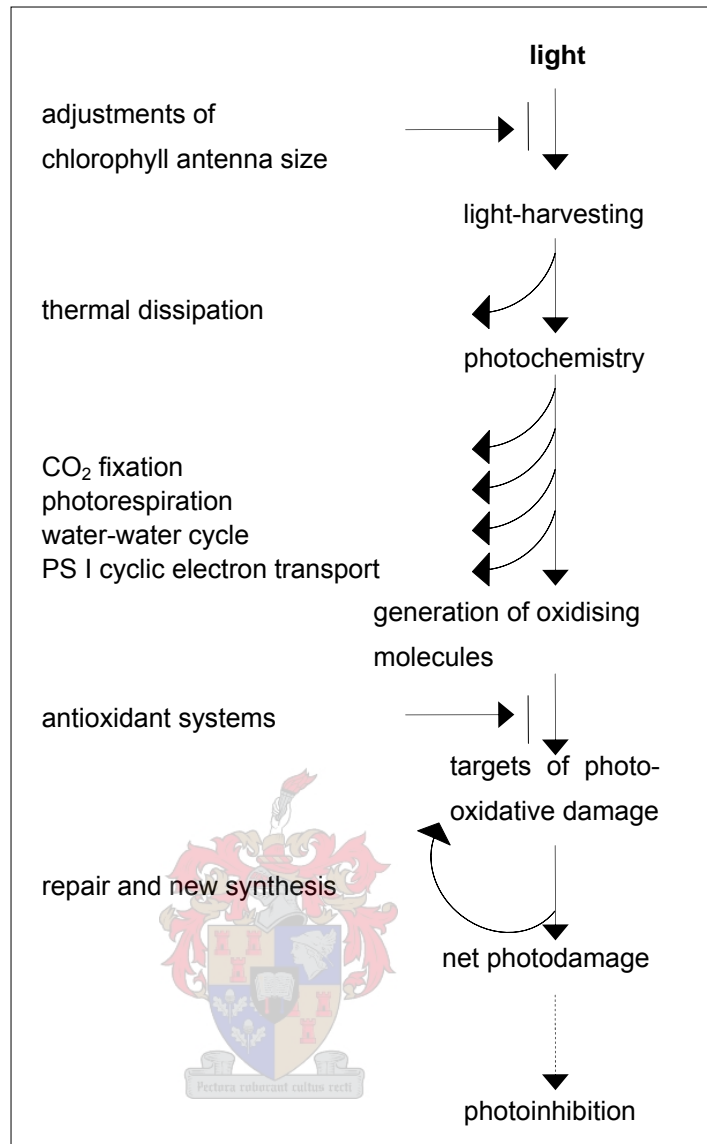


Figure 1: Schematic diagram of the photoprotective processes occurring within chloroplasts of plants (Niyogi, 1999).

One particular mechanism in plants involves the dissipation of the excess energy via carotenoid pigments, specifically the xanthophyll cycle pigments. This mechanism is very effective and can account for more than 75% of the photons absorbed. Several studies have focussed on the molecules and the mechanisms involved in thermal dissipation (Demmig-Adams and Adams, 1992; Björkman and Demmig-Adams, 1994; Horton and Ruban, 1994). In essence, a change in pH across the thylakoid membrane triggers a series of events that leads to the dissipation of excess light energy as heat (Niyogi, 1999). A PsbS protein, a member of the LHC superfamily of proteins (Kim et al., 1992; Morosinotto et al., 2003) as well as some carotenoids, especially the de-epoxidised xanthophyll pigments have been identified to play a key role in thermal dissipation in the PS II antenna pigment bed (Demmig-Adams, 1990; Gilmore et al., 1995; Demmig-Adams and Adams, 1996a;

Horton et al., 1996). Some of these xanthophyll pigments are closely associated with the LHC and form part of the xanthophyll cycle (Yamamoto et al., 1962).

Although these mechanisms protect the photosynthetic apparatus against excessive light conditions, photodamage could still occur as a consequence of photosynthesis. Photodamage to leaves exposed to excess light is partly attributed to the production of unstable intermediates by the photosynthetic electron transport system (Horton et al., 1996; Krieger-Liszky, 2004). The most important of these side reactions is the interaction of the unstable intermediates with oxygen to produce reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen. Plants have developed mechanisms to cope with the formation of ROS with the aid of several antioxidant molecules and scavenging enzymes. Accumulation of these antioxidant molecules and enzymes has been observed during excessive light treatments and their roles have been tested in mutant and transgenic organisms (Foyer et al., 1994; Allen et al., 1997; Alscher et al., 2002). Several antioxidant molecules have been identified, including several thylakoid membrane-bound molecules, such as carotenoids (especially xanthophylls) and tocopherols (especially α -tocopherol/vitamin E), ascorbate (vitamin C), and glutathione. These molecules have many functions as antioxidant agents in all biological systems, where they are able to quench or detoxify triplet chlorophyll (^3Chl) and ROS ($^1\text{O}_2$, O_2^- and OH), inhibit lipid peroxidation and stabilise the thylakoid membranes (Frank and Cogdell, 1993; Demmig-Adams et al., 1996b; Havaux, 1998).

This review will focus on the physiological and structural roles of the xanthophyll cycle pigments as part of the broader carotenoid pathway in plants. Their, integral role in protecting the photosynthetic apparatus of plants will form the central theme of the review. Aspects that will be covered include the biosynthesis of xanthophyll pigments, the location of the xanthophyll cycle pigments in the thylakoid membrane, the role of these pigments in photosynthesis and the mechanisms involved in thermal dissipation of excess energy. Finally, the transgenic and mutant approach to investigate the physiological roles and biotechnological importance/relevance of the xanthophyll enzymes will be discussed.

2.2 THE XANTHOPHYLL CYCLE AS PART OF CAROTENOID BIOSYNTHESIS IN PLANTS

The biosynthesis of carotenoids takes place in the plastids of plants by a specialised branch of the isoprenoid metabolic pathway that also produces a variety of other compounds, including tocopherols, quinines, chlorophylls, phytosterols and hormones such as gibberellins, cytokinins and abscisic acid (ABA) (Cunningham and Gantt, 1998; Fraser et al., 2002; Besumbes et al., 2004).

The carotenoid biosynthetic pathway begins with the five-carbon compound isopentenyl pyrophosphate (IPP), which is produced in the plastid via the mevalonate-independent pathway. The first step in carotenoid biosynthesis

(Figure 2) is the formation of the linear C₄₀ intermediate phytoene through a series of prenyl transferase reactions. Subsequently, phytoene undergoes four desaturation reactions, to generate lycopene. Lycopene is further cyclised and the resulting ring structures undergo varying degrees of hydroxylation and epoxidation to generate the range of carotenoid structures found in photosynthetic organisms.

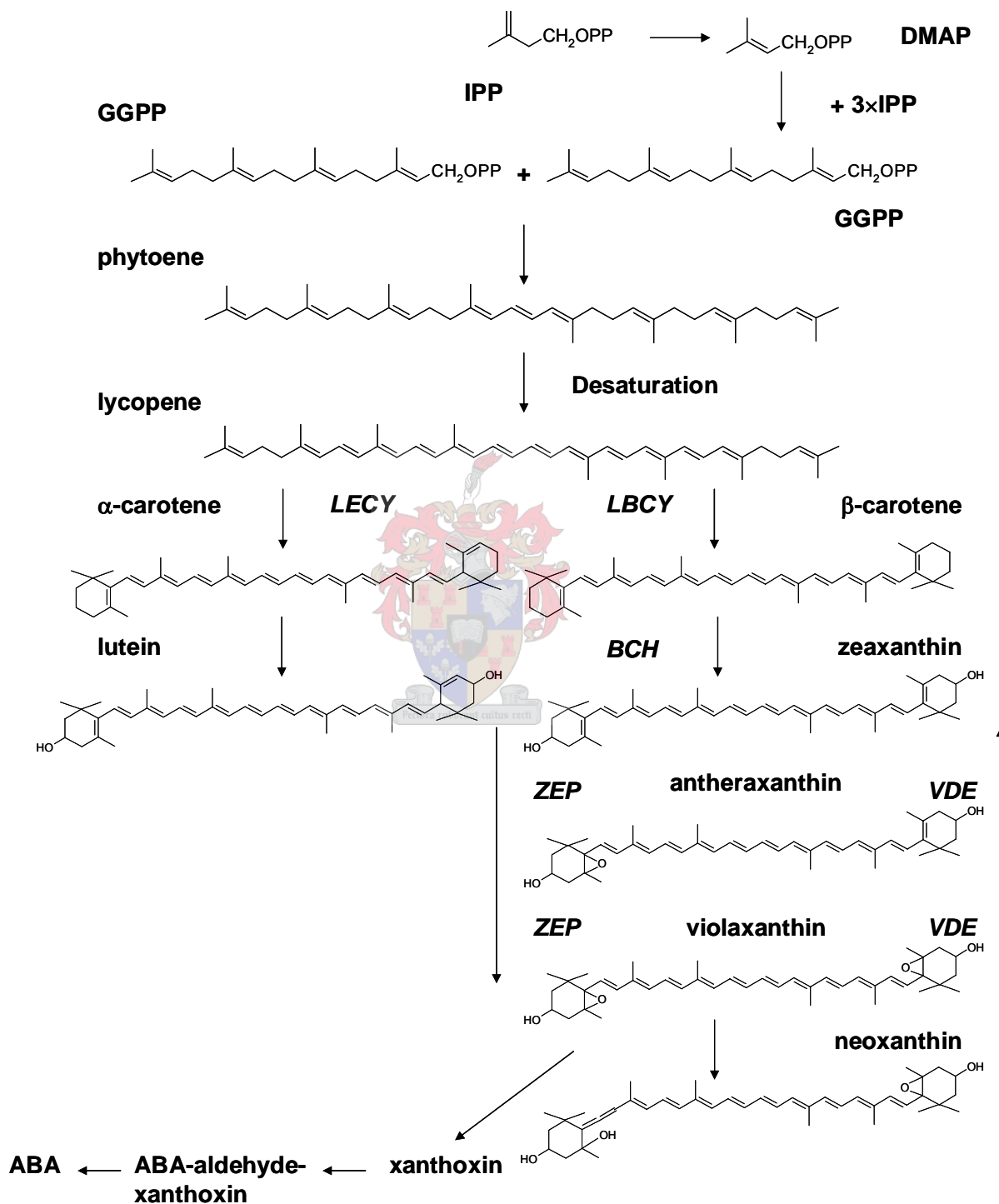


Figure 2: A simplified diagram of the carotenoid biosynthesis pathway in plants. LECY, lycopene ε-cyclase; LBCY, lycopene β-cyclase; BCH, β-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; ABA, abscisic acid (adapted from Hirschberg J (2001)).

Lycopene can undergo two different cyclisation reactions. Two enzymes have been identified which can either add a β -ring or an α -ring to lycopene. The lycopene β -cyclase enzyme adds β -rings to form β -carotene, whereas α -carotene is formed from the addition of one β -ring by lycopene β cyclase and the addition of one α -ring by lycopene ε -cyclase. The type of ring formed is dependent upon the enzymes involved (Cunningham and Gantt, 2001; Sandmann, 2001). The α -carotene branch is important for the production of lutein, the most abundant carotenoid found in photosynthetic tissue, whereas the β -carotene branch is important for the production of ABA and involves the xanthophyll cycle.

The first step in the β -carotene branch of carotenoid biosynthesis involves hydroxylation of β -carotene at C₃ of each ring of the hydrocarbons of the β -rings. Subsequently, zeaxanthin is produced via the step-wise addition of β -rings, catalysed by a β -carotene hydroxylase enzyme. Genetic evidence and functional analysis of an *Arabidopsis* β -carotene hydroxylase enzyme, supports the existence of separate hydroxylases specific for the β - and ε -rings (Pogson et al., 1996; Sun et al., 1996; Tian et al., 2003). The amino acid sequence of both the plant and bacterial β -hydroxylases predict the formation of transmembrane helices, suggesting a membrane-integral location *in vivo* and a series of conserved histidine motifs may be required for their activity (Bouvier et al., 1998). Over-expression and fruit specific expression of this gene resulted in the accumulation of the xanthophyll cycle carotenoids as well as β -cryptoxanthin. In tobacco, over-expression of the native β -carotene hydroxylase gene caused a two-fold increase in the xanthophyll cycle carotenoids (zeaxanthin, antheraxanthin and violaxanthin), resulting in reduced lipid peroxidation in these plants (Davison et al., 2002). From a nutritional perspective, fruit-specific expression of the β -carotene hydroxylase gene from *Capsicum annum* in tomatoes showed increased levels of β -cryptoxanthin and zeaxanthin (Bouvier et al., 1998; Dharmapuri et al., 2002).

The next step in carotenoid biosynthesis involves the xanthophyll cycle, which is the interconversion between three carotenoids; zeaxanthin, antheraxanthin and violaxanthin (Siefermann and Yamamoto, 1975a). The enzymes for this interconversion have been well documented and will be discussed in the sections to follow. An important end product of the β -carotene branch is the plant hormone, ABA, which can be produced from all-*trans*-violaxanthin, all-*trans*-neoxanthin and 9'-*cis*-neoxanthin molecules (Zeevaart and Creelman, 1988). ABA plays a key role in the developmental processes of plants, regulating seed maturation and maintenance of embryo dormancy (McCarty, 1995; Finkelstein et al., 2002) and mediating plant responses to abiotic environmental stresses such as low and high temperatures and water deficit (Zeevaart and Creelman, 1988; Hetherington and Quartano, 1991; Bohnert et al., 1995). An *Arabidopsis* mutant has shown that when ABA synthesis is inhibited, the plant's phenotype is severely affected. This mutant, *aba2* (defective in the epoxidation of zeaxanthin to violaxanthin in the xanthophyll cycle) accumulates zeaxanthin without production of ABA and shows a typical 'wilty' phenotype (Marin et

al., 1996). Furthermore, it has been shown that the epoxidation of zeaxanthin to violaxanthin appears to be the rate-limiting step in ABA biosynthesis (Liotenberg et al., 1999).

2.3 THE XANTHOPHYLL CYCLE AND THE ENZYMES INVOLVED

The xanthophyll or violaxanthin cycle was identified in higher plants and green algae and was first observed to respond to light-dark treatments by Sapozhnikov et al. (1957). Some algae groups, especially the *Bacillariophyceae*, *Chrysiophyceae*, *Xanthophyceae* and *Dinophyceae* have a similar diadinoxanthin cycle for photoprotection, which cycles between diadinoxanthin and diatoxanthin (Hager, 1980; Demmig-Adams and Adams III, 1993; Olaizola et al., 1994; Lohr and Wilhelm, 1999).

The xanthophyll cycle is localised in the chloroplasts of plants and involves the de-epoxidation and epoxidation interconversions of three xanthophylls (violaxanthin, antheraxanthin and zeaxanthin) (Yamamoto et al., 1962). This interconversion is catalysed by two enzymes that are localised on opposite sides of the thylakoid membrane. The zeaxanthin epoxidase enzyme on the stromal side of the membrane converts zeaxanthin to violaxanthin under limiting light conditions, whereas violaxanthin de-epoxidase on the thylakoid lumen side of the membrane converts violaxanthin back to zeaxanthin under high light conditions (Figure 3).

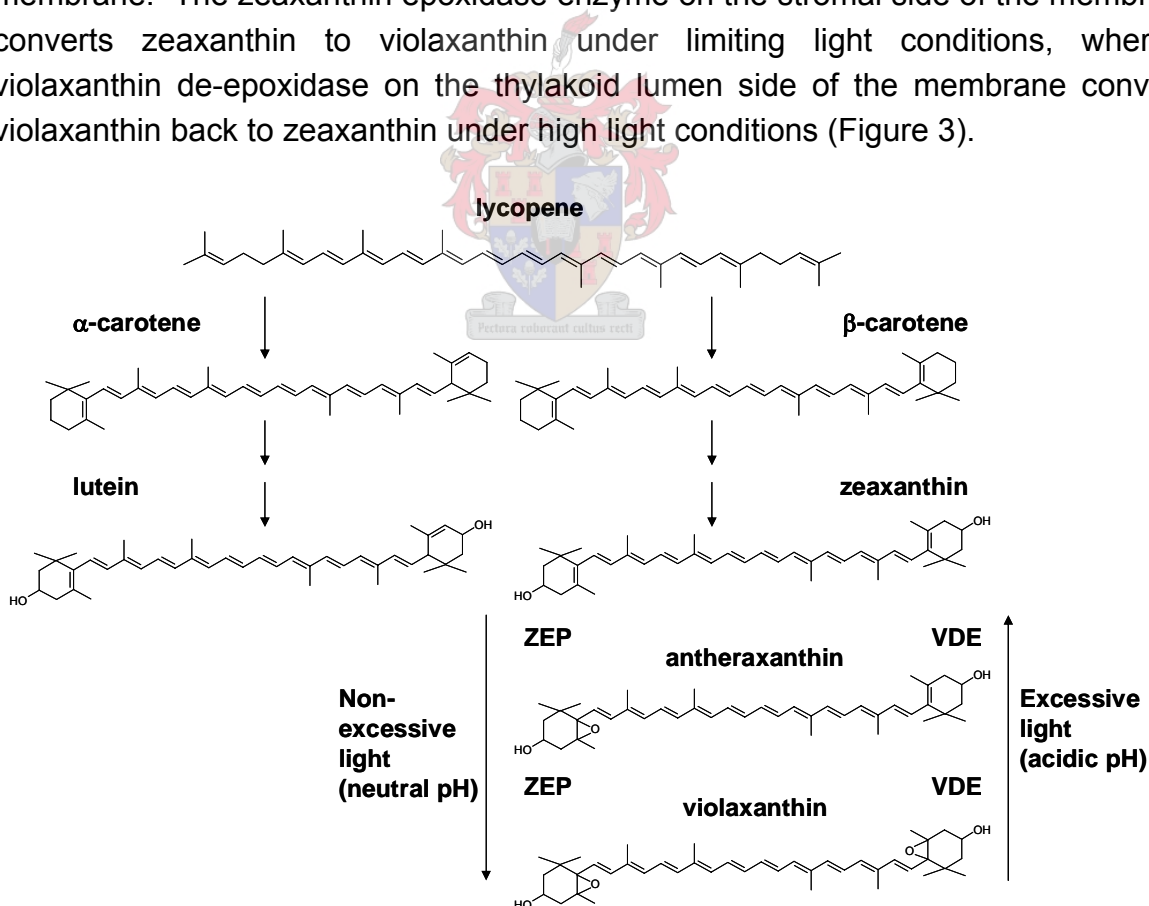


Figure 3. The biosynthetic pathway of the synthesis of cyclic carotenoids and the xanthophyll cycle in plants. The key enzymes of the xanthophyll cycle, zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), are both members of the lipocalin protein family. VDE is stimulated under excess light and ZEP under non-excessive light (Demmig-Adams and Adams, 2002).

2.3.1 VIOLAXANTHIN DE-EPOXIDASE

Violaxanthin de-epoxidase (VDE) is a 39.9 kDa nucleus-encoded protein with a pI of 5.4. VDE is localised in the lumen of the thylakoid and catalyses the stepwise removal of the 5-6 epoxide from violaxanthin with the aid of ascorbic acid to form zeaxanthin (Müller-Moulé et al., 2002). The enzyme has been shown to be induced by a change in lumen pH due to a photosynthetic pump in high light conditions. The increase in acidity enables the enzyme to associate with the thylakoid membrane where it catalyses the de-epoxidation of violaxanthin (Yamamoto et al., 1972, Hager and Holocher, 1994). This enzyme's properties and co-factors were reviewed by Rockholm and Yamamoto (1996).

Bratt et al. (1995) found that VDE was released from the membrane in a pH-dependent process, confirming the binding of this enzyme to the thylakoid membrane at a "docking site" for the conversion of violaxanthin to zeaxanthin (Liu et al., 2004). The de-epoxidation of violaxanthin is also dependent upon two other factors, namely the pigment pool size and the fraction of the violaxanthin pool that is free in the membrane (Siefermann and Yamamoto, 1974; Siefermann and Yamamoto, 1975b; Thayer and Björkman, 1990; Demmig-Adams and Adams, 1992; Yamamoto and Bassi, 1996). This was confirmed by de-epoxidation experiments performed with the *chlorina f2* mutant of barley. This mutant is depleted in chl *b* and lacks LHC proteins, so all the violaxanthin is free in the membrane. These experiments showed that the de-epoxidation of the *chlorina f2* mutant was faster and more complete, compared with wild type barley (Bassi et al., 1993; Peng and Gilmore, 2002). This suggests that the limiting step for the de-epoxidation of violaxanthin is the liberation of violaxanthin from the LHCs rather than the activation of VDE. VDE was isolated from romaine lettuce, tobacco and *Arabidopsis* and its protein sequence suggests that it forms part of the lipocalin protein family (Bugos and Yamamoto, 1996; Bugos et al., 1998).

2.3.2 ZEAXANTHIN EPOXIDASE

Zeaxanthin epoxidase (ZEP) is a 67 kDa protein localised on the stromal side of the thylakoid membrane and catalyses the stepwise addition of a 5-6 epoxide to zeaxanthin in the dark or under low light intensities (Siefermann and Yamamoto, 1975b). The optimal pH for this reaction is 7.5 and it requires the presence of oxygen, ferredoxin and ferredoxin-like reductives, as well as both NADPH and FAD as co-factors (Büch et al., 1995; Bouvier et al., 1996). Although the enzyme's optimal pH range is more neutral, this reaction occurs even in an acidified lumen (Gilmore et al., 1994). Furthermore, this epoxidation by ZEP is a slow process relative to the de-epoxidation by VDE.

ZEP was first isolated from *Nicotiana plumbaginifolia* mutants (*aba*) (Marin et al., 1996). Homologous sequences have been isolated from several other species including pepper, tomato, *Arabidopsis* and apricot (Bouvier et al., 1996; Burbidge et al., 1997; Hieber et al., 2000). Their protein sequences suggest they also form part

of the lipocalin protein family (Hieber et al., 2000). The regulation of the zeaxanthin epoxidase gene has been intensively studied in *N. plumbaginifolia* and to a lesser extent in *Vigna unguiculata* (Burbidge et al., 1997; Audran et al., 1998; Luchi et al., 2000; Thompson et al., 2000). It was found that both the *ZEP* mRNA and the protein were more abundant in the leaves than in the roots. Moreover during dehydration, levels of both increased in the leaves and roots, but the increase was more marked in the roots. *ZEP* mRNA levels were also shown to increase during seed development (Audran et al., 1998; Frey et al., 1999). Tobacco over-expressing the *ZEP* gene of *N. plumbaginifolia* resulted in increased levels of ABA, together with delayed seed germination (Frey et al., 1999). Thus, it is thought that zeaxanthin epoxidation constitutes a key regulatory step in ABA biosynthesis in non-chlorophyllous tissues.

2.4 THE LOCATION AND PROTECTIVE FUNCTIONS OF THE XANTHOPHYLL CYCLE PIGMENTS

2.4.1 LOCATION OF THE XANTHOPHYLL CYCLE CAROTENOIDS

The role of xanthophyll cycle carotenoids in photosynthesis is closely linked with their location in the chloroplast thylakoid membrane. They are associated with the LHCs of the photosynthetic apparatus, where their distribution may vary between the different components which constitutes the complexes (Yamamoto and Bassi, 1996).

Light harvesting is the primary step in photosynthesis and involves the capture of solar energy through a series of LHCs in the thylakoid membrane of chloroplasts. The solar energy captured through the LHCs is transferred to a photosynthetic reaction centre (RC) within the thylakoid membranes. The current models for higher plants and green algae depict a PS II core complex with an outer light-harvesting system (Barber and Kühlbrandt, 1999). The antennae are layered around the PS II centers, followed by the minor LHC components and the major LHCs, containing the major chlorophyll *a*- and *b*- (chl *a* and *b*) binding components. The LHC II is the most abundant integral membrane protein within the chloroplast; it exists as a trimer and forms the centre of the major complex. Furthermore, this complex binds the majority of the thylakoid chlorophylls. The minor antennae bind only a small fraction of the total chlorophyll, but are enriched with the xanthophyll cycle components (Yamamoto and Bassi, 1996). These minor complexes have been suggested to be the link between the PS II core and the LHC II. A typical LHC II system contains five LHC IIb and three or four minor complexes, which together forms a large oligomeric antenna in the thylakoid membrane (Bassi and Dianese, 1992; Jansson, 1994).

The structure of LHC II has been determined through electron crystallography and more recently by higher resolution X-ray crystallography (Figure 4) (Kühlbrandt et al., 1994; Liu et al., 2004). These models suggest that the monomeric structure of LHC II contains a polypeptide of about 232 amino acid residues, 13-15 chl *a* and *b* molecules, 3-4 carotenoids and one tightly bound phospholipid (Peter and Thornber, 1991; Nußberger et al., 1993; Ruban et al., 1999a). The carotenoids were identified

to be two molecules of lutein, one 9'-neoxanthin as well as a xanthophyll cycle carotenoid. The xanthophyll binding sites are of two types, two internal sites (L1 and L2), binding preferentially lutein and two peripheral sites binding neoxanthin (N1) and violaxanthin (V1) (Ruban et al., 1999a; Liu et al., 2004). The binding site for neoxanthin, N1 is highly selective for this xanthophyll, whereas the two lutein binding sites, L1 and L2 can also bind either violaxanthin or zeaxanthin, but with a lower affinity (Croce et al., 1999; Phillip et al., 2002). Furthermore, the orientation and distances between the two lutein and six chl *a* molecules are favourable for energy transfer from the carotenoid to the chlorophyll. Similarly, neoxanthin and two molecules of chl *b* are in a favourable orientation for the transfer of energy (Croce et al., 1999; Liu et al., 2004). The elucidation of these distances between the xanthophyll carotenoids and chlorophyll molecules, as well as their orientation in the LHC II supports their possible function as accessory light-harvesting pigments.

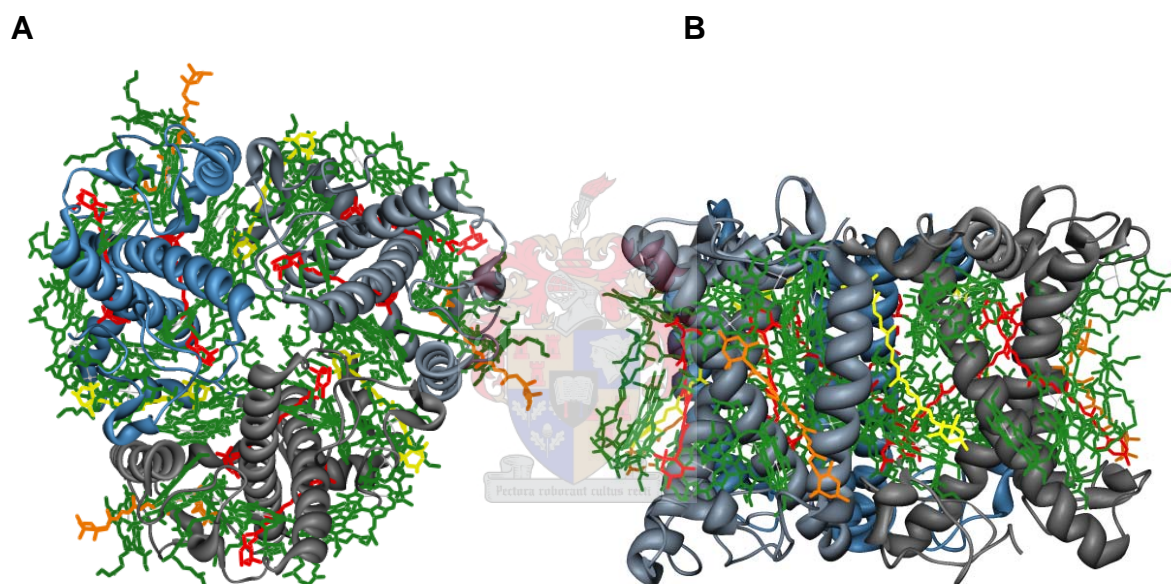


Figure 4. Pigments in the light-harvesting complex (LHC) II trimer (Liu et al., 2004). (A) A stereo view showing the pigment arrangement pattern in the LHC II trimer. This view is along the membrane from the stromal side. For clarity, the colours are assigned as follows: chlorophylls, in green; lutein in red; neoxanthin in yellow and xanthophyll cycle carotenoids in orange. (B) Pigment pattern in the trimer viewed from the side. Colour designation is the same as in A (reproduced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco).

It has been suggested that approximately 60% of the violaxanthin in the LHC is available for the de-epoxidation to zeaxanthin. Ruban and coworkers (Ruban et al., 1999a) have shown that violaxanthin is loosely bound to the LHC and can be removed by mild detergent treatment. Furthermore, its availability for de-epoxidation is increased if the isolated thylakoids are unstacked by removal of Mg^{2+} (Ruban et al., 1994a; Phillip and Young, 1995; Fäber and Jahns, 1996). It would seem that the availability of violaxanthin may be controlled by the organisation of the complexes in

the thylakoid membrane. Demmig-Adams and co-workers (Demmig-Adams and Adams, 1992; Demmig-Adams et al., 1995) have shown that violaxanthin availability may approach 100% when plants are grown in high light, which may be the result of light-dependent changes in the complex structure.

It is well established that the xanthophyll cycle pool size increases when plants are constantly grown in high light conditions, however it has not been determined whether more xanthophyll is bound to each LHC II or whether the additional xanthophyll is free in the membrane (Horton and Ruban, 2004). Experiments with LHC II-depleted thylakoids have suggested that xanthophyll pigments can additionally bind to other thylakoid proteins as well (Jahns, 1995; Krol et al., 1995).

2.4.2. XANTHOPHYLL CYCLE COMPONENT'S FUNCTION IN LHC ASSEMBLY AND MEMBRANE STABILISATION

Carotenoids have important structural and physiological roles in the thylakoid membranes. They are necessary for the proper assembly and stability of the LHCs (Plumley and Schmidt, 1987; Paulsen et al., 1990; Paulsen et al., 1993; Croce et al., 1999; Hobe et al., 2000) as well as the stability of the membrane itself (Ourisson and Nakatani, 1994; Tardy and Havaux, 1997).

In vitro compositional analyses of isolated LHCs have shown that carotenoids are required for the accurate assembly of the LHC and that different carotenoids are able to assemble the LHC to varying degrees (Plumley and Schmidt, 1987; Paulsen et al., 1990; Paulsen et al., 1993). Phillip and co-workers (Phillip et al., 2002) have shown that carotenoids with a 3-hydroxy- β -end group is a specific requirement for accurate assembly. Furthermore, LHCs reconstituted with zeaxanthin and lutein were more resistant to proteolytic attack and maintained energy transfer at higher temperatures compared with LHCs containing violaxanthin and neoxanthin (Hobe et al., 2000).

Carotenoids, especially the xanthophylls, are also essential for thylakoid membrane stability (Havaux, 1998). The incorporation of carotenoids into artificial membranes (liposomes) can either have no specific orientation in the membrane and remain within the hydrocarbon inner part, for instance β -carotene, or can be located perpendicular to the bilayer with its polar regions anchoring it the head group regions on both sides of the membrane, such as zeaxanthin (Subczynski, 1991). This incorporation of carotenoids has led to the assumption that the thermodynamic and mechanical properties of the membrane are influenced by the orientation and structure of individual carotenoids. A similar orientation has also been found for zeaxanthin and lutein associated with lipid molecules in the human macula (Bone and Landrum, 1984). Havaux and co-workers (Havaux et al., 1996; Tardy and Havaux, 1997) have shown that thylakoid membranes prepared from illuminated leaves are substantially less fluid than membranes prepared from dark adapted leaves. Moreover, the enhanced zeaxanthin concentration was also accompanied by a light-induced increase in membrane viscosity. This zeaxanthin-induced decrease

in thylakoid-membrane fluidity protects the thylakoid membrane against disorganisation at high temperatures. Transgenic lines over-expressing an endogenous β -carotene hydroxylase gene in *Arabidopsis*, which led to elevated levels of zeaxanthin, also exhibited enhanced heat tolerance (Davison et al., 2002). It is postulated that the rigidifying effect due to the presence of zeaxanthin can further possibly lead to decreased oxygen penetration inside the lipid bilayer. Furthermore, a rigidifying effect via carotenoids, in combination with their antioxidant properties, lead to decreased lipid peroxidation of the thylakoid membrane (Subczynski et al., 1991; Tardy and Havaux, 1997; Havaux and Niyogi, 1999).

2.4.3 REGULATION OF PHOTOSYNTHETIC LIGHT-HARVESTING: A ROLE FOR THE XANTHOPHYLL CYCLE

When light is absorbed by chlorophyll it excites an electron of that chlorophyll. The excitation energy has three possible fates: (i) it can cause electron transport through the electron transport chain and subsequently be used to reduce CO_2 (photochemistry); (ii) the excited electron can return to its ground state and release heat (non-radiative, or thermal dissipation); (iii) it can be released as a photon of light, at a wavelength longer than that of the absorbed photon (fluorescence) (Campbell et al., 1998). Over 75% of the photons absorbed during excessive light can be eliminated by thermal dissipation. This process involves the de-excitation of singlet chlorophyll (^1Chl) and can be measured as non-photochemical quenching (*NPQ*) of chlorophyll fluorescence (Demmig-Adams et al., 1996a and 1996b). Thermal dissipation is thought to protect photosynthesis by decreasing the lifetime of ^1Chl , thus minimising the generation of singlet oxygen ($^1\text{O}_2$) in the PS II LHC and RC. It is also thought that thermal dissipation prevents over-acidification of the lumen, generating a long-lived excited state of P680 (P680^+) and decreased rate of oxygen reduction by PS II (Niyogi, 1999; Xu et al., 2000).

Three major components of *NPQ* have been described; (i) a pH gradient-dependent or energy-dependent mechanism (*qE* also referred to as the fast relaxing component of *NPQ*, *NPQ_F*) (Briantias et al., 1979; Krause and Behrend, 1986; Maxwell and Johnson, 2000), (ii) state transition, the redistribution of energy from PS II to PS I, which is usually more prominent in algae (*qT*) (Staehelein and Arntzen, 1983; Horton and Lee, 1985; Allen, 1992), and (iii) photoinhibition (*qI* also referred to as the slow relaxing component of *NPQ*, *NPQ_S*) of photosynthesis, which is a more sustained effect of excessive light (Demmig et al., 1987; Demmig-Adams et al., 1989; Maxwell and Johnson, 2000). The major component for *NPQ* is *qE*, with lesser contributions of *qI* and *qT*. The contribution of *qT* to *NPQ*, especially, has been observed to be very low in higher plants (Maxwell and Johnson, 2000).

Hence, the predominant mechanism for thermal dissipation is the rapidly reversible pH-dependent thermal dissipation and involves the xanthophyll cycle pigments, zeaxanthin and antheraxanthin in the PS II antenna pigment bed (Demmig-Adams and Adams, 1992; Björkman and Demmig-Adams, 1994; Horton et

al., 1996). The first evidence for the correlation between qE and zeaxanthin formation was observed by Demmig et al. (1987). Under light conditions the induction of qE is directly correlated to the amounts of zeaxanthin present. Since then numerous researchers have found that this correlation holds true for a wide range of environmental conditions including a wide range of photon flux densities (PFD) (Demmig-Adams, 1990; Demmig-Adams and Adams, 1993). Further evidence using isolated LHCs, have indicated that the addition of zeaxanthin, or a combination of zeaxanthin and antheraxanthin, results in significant reduction of chlorophyll fluorescence (Gilmore and Yamamoto, 1993). However the addition of other xanthophyll pigments, such as violaxanthin and lutein only induced a marginal reduction of chlorophyll fluorescence (Wentworth et al., 2000).

Thermal dissipation and the formation of zeaxanthin and antheraxanthin are associated with the pH gradient across the thylakoid membrane. Excess light is thought to cause a small increase in ΔpH , additional to the increase in ΔpH due to the photosynthetic proton pumping (Horton et al., 1996). It has been suggested that the ΔpH is regulated within narrow limits and that small changes in ΔpH affords control of NPQ. The small increase in ΔpH is an immediate signal of excess light, which triggers the feedback regulation of light-harvesting by thermal dissipation. The requirement for a low lumen pH has been shown by the addition of nigericin, which eliminates ΔpH and leads to the inhibition of qE . Shikanai et al., (1999) discovered several *Arabidopsis* mutants defective in the photosynthetic proton pump, which leads to a low ΔpH . These mutants had decreased qE levels. A low lumen pH does not have to be generated by light-dependent reactions to induce qE . qE can also be induced in thylakoid membranes *in vitro* by either lowering the pH of the buffer or by generating a ΔpH via ATP hydrolysis and reverse proton pumping by ATP synthase (Gilmore and Yamamoto, 1992; Krieger et al., 1992). All these phenomena illustrate the importance of ΔpH for the induction of qE .

The mechanistic role of ΔpH in driving xanthophyll cycling and thermal dissipation of excess energy has been intensively studied. A decrease in lumen pH leads to the protonation of the lumen exposed domains of specific polypeptides in the LHC associated with PS II (Crofts and Yerkes, 1994; Horton and Ruban, 1994; Walters et al., 1994b; Walters et al., 1996; Bergantino et al., 2003). The decreased lumen pH also results in activation of violaxanthin de-epoxidase, which is thought to migrate to the thylakoid membrane and bind to the LHC at a prescribed docking site (Hieber et al., 2000; Liu et al., 2004). There, violaxanthin associated with the LHC is converted to zeaxanthin (Pfundel and Bilger, 1994; Eskling et al., 1997). Macko et al. (2002) have suggested that the release of violaxanthin from the LHC and its diffusion within the thylakoid membrane may be necessary for de-epoxidation to proceed. The zeaxanthin in the thylakoid membrane is said to bind to the LHC, which decreases the fluorescence yield and increases thermal dissipation (Quenching 2, Figure 5) (Formaggio et al., 2001; Moya et al., 2001).

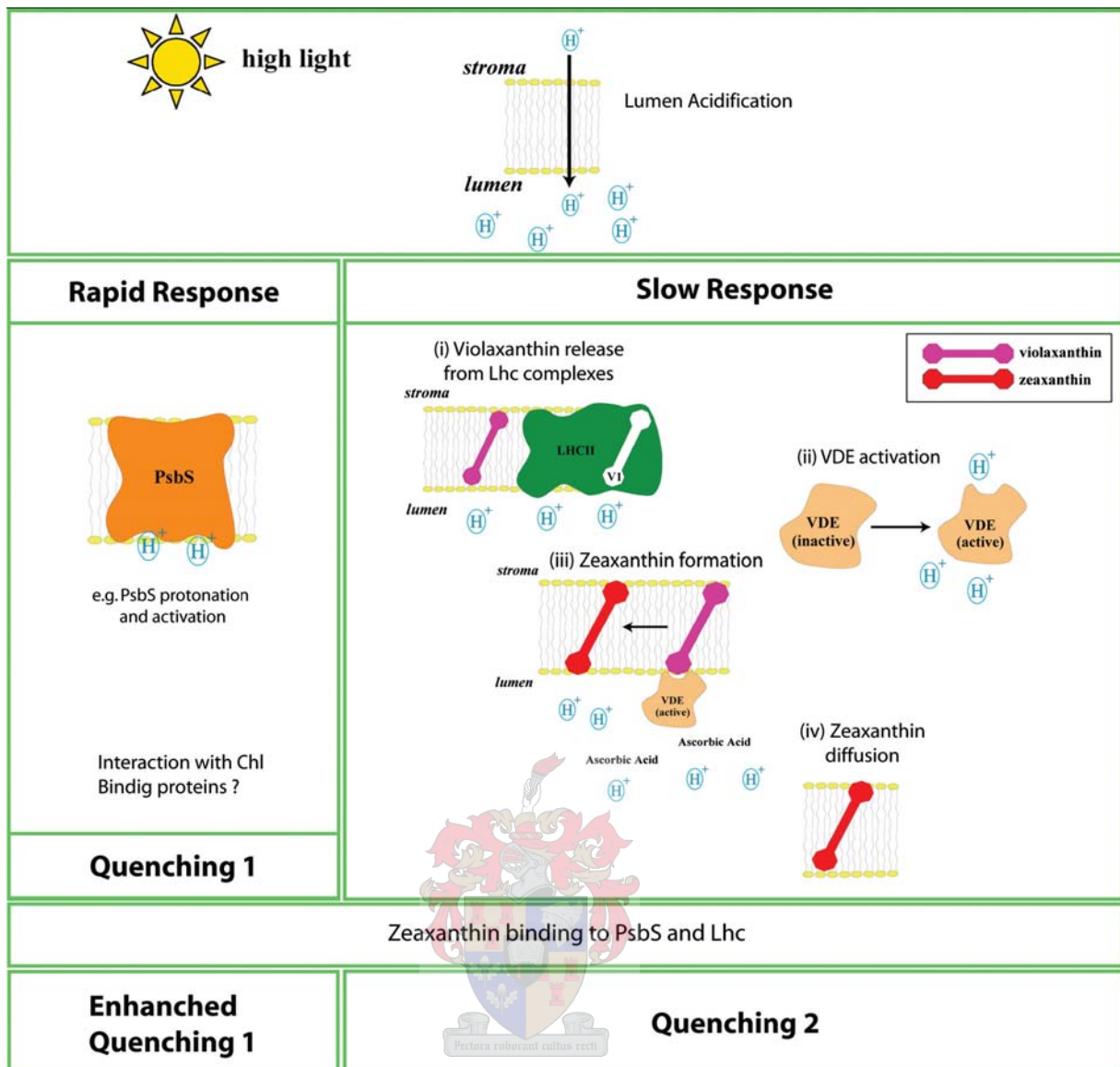


Figure 5. Representation of the xanthophyll cycle as stress signal transduction system in thylakoids. High light induces activation of rapid excitation energy quenching response triggered by low lumen pH (indicated as quenching 1). The rapid response is dependent on PsbS, whose mechanism of action is unknown. An alternative quenching is dependent on the xanthophyll cycle. Low pH triggers the activation of the xanthophyll cycle in several steps: (i) Release of violaxanthin from its site within the LHC II trimers; (ii) activation of violaxanthin de-epoxidase (VDE); (iii) Zeaxanthin production by VDE in the membrane lipid phase; and (iv) diffusion of zeaxanthin within the membrane. Zeaxanthin induces sustained quenching by binding to PsbS and to the allosteric site in the LHC antenna proteins (enhanced quenching 1 and 2) (Morosinotto et al., 2003).

Crimi et al., (2001) found that zeaxanthin is able to bind to a recombinant light-harvesting polypeptide, CP29 *in vitro* and that this induces a significant quenching effect compared to the native CP29. Zeaxanthin determines not only the extent of *qE*, but also the rate of quenching (Ruban and Horton, 1999b), while antheraxanthin-induced *qE* is manifested in the transient phase of *NPQ* (Havaux et al., 2000; D'Haese et al., 2004).

The binding of zeaxanthin and protonation of PS II proteins might cause a conformational change in the LHC, observed as a change in absorbance at 535 nm (Bilger et al., 1989; Bilger and Björkman, 1990; 1994; Ruban et al., 1993a). The binding of the PsbS protein, a member of the LHC superfamily, to zeaxanthin *in vitro* seems to be associated with the absorption change at 535 nm, and also with the development of *qE* (Aspinall-O’dea et al., 2002). In addition, the pH gradient increase may be the signal that is perceived by the PsbS protein, resulting in a structural change in the light-harvesting antenna and the development of *NPQ* (Li et al., 2000; Dominici et al., 2002; Morosinotto et al., 2003). This rapid response due to the Δ pH, involving the PsbS protein, is indicated as quenching 1 in Figure 5.

An *Arabidopsis* mutant, *npq4-1* is deficient in the PsbS protein and has provided evidence for the correlation between a conformational change observed at 595 nm and *qE*. This mutant has a reduced capacity for *qE*, but normal light-harvesting and photosynthetic efficiencies nevertheless are observed (Li et al., 2000; Peterson and Avir, 2000). Moreover, over-expression of PsbS resulted in a two-fold increase in *qE*, as well as resistance to photoinhibition. This indicates that this protein may be a limiting factor for the capacity of *qE*.

It seems that conformational changes caused by the binding of protons and zeaxanthin to PsbS and to other PS II proteins result in the formation of a quenching complex (Ruban et al., 2002). On the one hand, the binding of zeaxanthin to PsbS and the conformational change may be required for zeaxanthin to directly quench chlorophyll excited states (Ma et al., 2003). On the other hand, protonated PsbS may “deliver” zeaxanthin to its active site in the antenna to enable indirect quenching. Although the exact mechanisms are still unclear, proposed mechanisms are discussed in the following section.

2.4.4 PROPOSED MECHANISM(S) OF THERMAL DISSIPATION VIA THE XANTHOPHYLL CYCLE PIGMENTS

Horton et al. (2005) proposed that energy dissipation via the xanthophyll cycle pigments occurs in one or more of the proteins making up the LHC of PS II. Energy dissipation through these proteins is induced by a conformational change resulting from the synergistic effects of protein protonation and violaxanthin de-epoxidation to zeaxanthin. Although the operational model of the xanthophyll cycle has been well documented, the biophysical mechanism underlining its function is still under investigation. Two main mechanisms for thermal dissipation via the xanthophyll cycle have been suggested, (i) an indirect quenching process, which involves the formation of a quenching complex and changes in the organisation of the light-harvesting complexes and (ii) a direct chlorophyll-carotenoid interaction resulting in singlet-singlet energy transfer between them (Horton et al., 2005).

2.4.4.1 Indirect quenching involving conformational changes in the LHC

A number of studies have suggested that an indirect mechanism for quenching, involving the xanthophyll cycle pigments and a change in the organisation of the LHC, may be the primary mechanism for *NPQ*. It is thought that the xanthophyll pigments exert some control over the structure or organisation of the LHC II. Further evidence for the formation of a quenching complex has come from observations regarding the aggregation of isolated light-harvesting complexes in the presence of exogenous pigments (Ruban et al., 1993b; Ruban et al., 1996a). The addition of zeaxanthin to isolated LHCs led to stimulation of quenching, associated with the formation of LHC aggregates, and it appeared that zeaxanthin was not exerting a direct quenching effect (direct energy transfer from chlorophyll to zeaxanthin) (Ruban et al., 1994a; Phillip et al., 1996; Ruban et al., 1997; Wentworth et al., 2000; Wentworth et al., 2001; Polívka et al., 2002). In contrast, Ruban and coworkers (Ruban et al., 1994b, Ruban et al., 2002) reported high levels of quenching *in vitro* and *in vivo*, even in the absence of zeaxanthin. This suggests that the change in organisation of the LHC and binding of PsbS to the LHC may be the primary mechanism of *NPQ* and that zeaxanthin only adds to the quenching effect.

In this model, quenching is caused by a conformational change in one or more of the proteins of the LHC II system, enabling the shifting from a light-harvesting state to a quenching state. Two states of the PS II have been defined, the U state, which is capable of light-harvesting and the Q state, which is capable of allosteric quenching. The two states have different fluorescence lifetimes (Gilmore et al., 1995; Gilmore et al., 1998). In this model protonation of proteins within the PS II causes a change from the U state to the Q state (Horton et al., 2000). This change is induced by protonation of the proteins of PS II and by binding of both PsbS and zeaxanthin to the LHC. It would seem that the change in the conformation of PS II enables quenching via a binary reaction, possibly involving two chlorophyll molecules. This model also suggests that the role of xanthophyll cycle pigments in thermal dissipation is through allosteric control of the conformation of LHC (Wentworth et al., 2001).

2.4.4.2 Direct quenching involving the xanthophyll cycle pigment, zeaxanthin

Determination of the energy states of carotenoids have led to the suggestion that energy can be directly transferred from chlorophyll to carotenoids and from carotenoids to chlorophyll. The latter is important for a light-harvesting function of carotenoids, specifically involving violaxanthin (Ritz et al., 2000; Croce et al., 2001). The S_1 state (lowest singlet excited state) of violaxanthin is higher than that of chl *a*, hence violaxanthin can function as a light harvesting pigment and donate the excitation energy to chl *a*. The S_1 state of zeaxanthin is lower than that of chl *a* (Dreuw et al., 2003) so it is energetically possible for the S_1 state of zeaxanthin to accept excitation energy from chl *a*, and hence quench chlorophyll fluorescence. The term “molecular gear shift” has been used to describe the interconversions of the

xanthophyll cycle (Figure 6; Kwa et al., 1992; Andersson and Gillbro, 1995; Chynwat and Frank, 1995; Young and Frank, 1996).

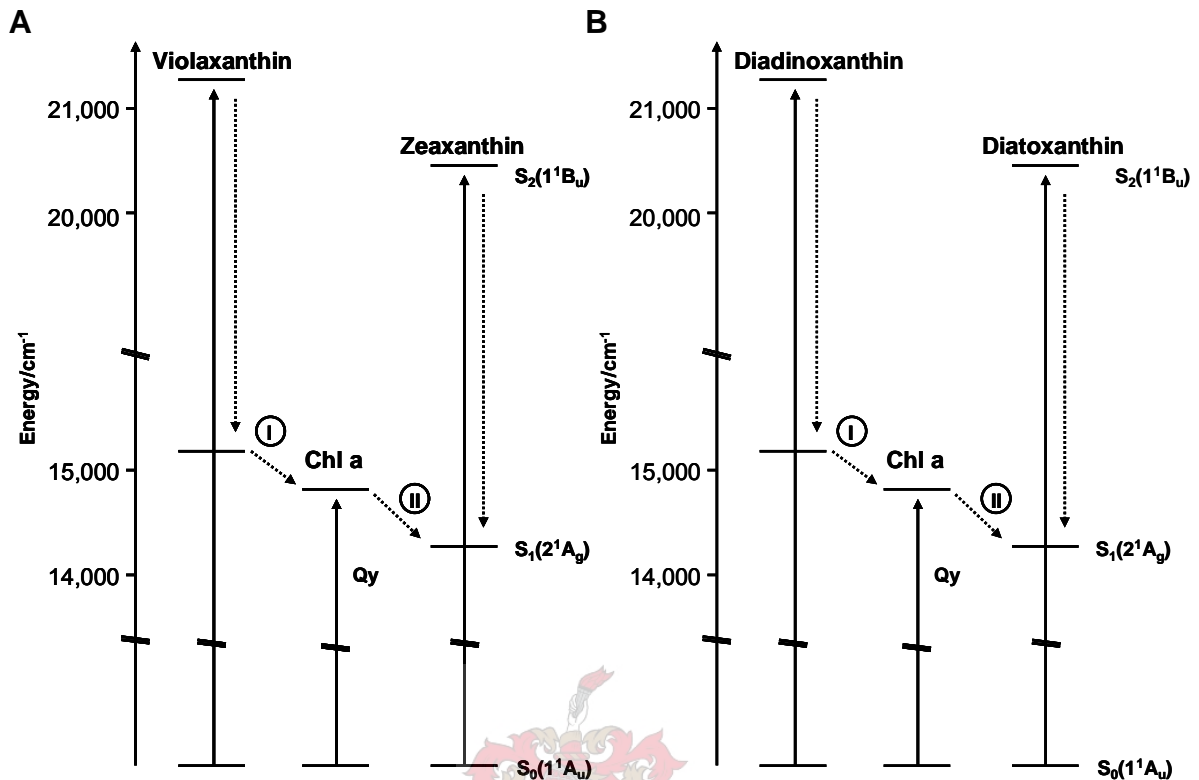


Figure 6. The “molecular gear shift” model showing the positions of the S_1 energies of the xanthophyll cycle carotenoids relative to chl a: (A) violaxanthin and zeaxanthin; (B) diadinoxanthin and diatoxanthin; I refers to light-harvesting and II to photoprotection. The ground state is indicated as $S_0(1^1A_u)$ and the low-lying singlet states are indicated as $S_1(2^1A_u)$ and $S_2(1^1B_u)$ (Young and Frank, 1996).

With the direct quenching mechanism, zeaxanthin may serve to de-activate the excited state of chl a by harmlessly dissipating the energy as heat, which can be observed as a reduction in chlorophyll fluorescence. Frank et al. (1996) have also suggested this possible route of chlorophyll de-activation by spectroscopic analysis of the algal carotenoids, diatoxanthin and diadinoxanthin.

Femtosecond transient absorption experiments on thylakoids with normal qE , suggest that excitation of the S_1 state of zeaxanthin is involved in qE (Ma et al., 2003). Recently Holt et al. (2005) showed that a carotenoid radical cation is formed upon excitation of chlorophyll under conditions of maximum, steady-state feedback de-excitation. The carotenoid radical was identified as zeaxanthin using femtosecond transient absorption measurements of various wild type, transgenic and mutant *Arabidopsis thaliana* plants. This suggests that the de-activation of chlorophyll during excessive light occurs by energy transfer to a zeaxanthin heterodimer, followed by ultrafast carotenoid radical cation formation.

The exact mechanism of NPQ is still under investigation and may involve a combination of both the indirect and direct mechanisms, but the results obtained thus

far clearly show that the xanthophyll cycle carotenoids play an important role in thermal dissipation.

2.5 MUTANT AND TRANSGENIC APPROACHES TO INVESTIGATE THE GENES INVOLVED IN THERMAL DISSIPATION BY THE XANTHOPHYLL CYCLE

Several mutants of the xanthophyll cycle of *Chlamydomonas* and *Arabidopsis* have been isolated by video imaging of chlorophyll fluorescence quenching (Niyogi et al., 1997a, Niyogi et al., 1998, Shikanai et al., 1998). These mutants have confirmed the physiological importance of the xanthophyll cycle pigments in thermal dissipation as well as the enzymes involved.

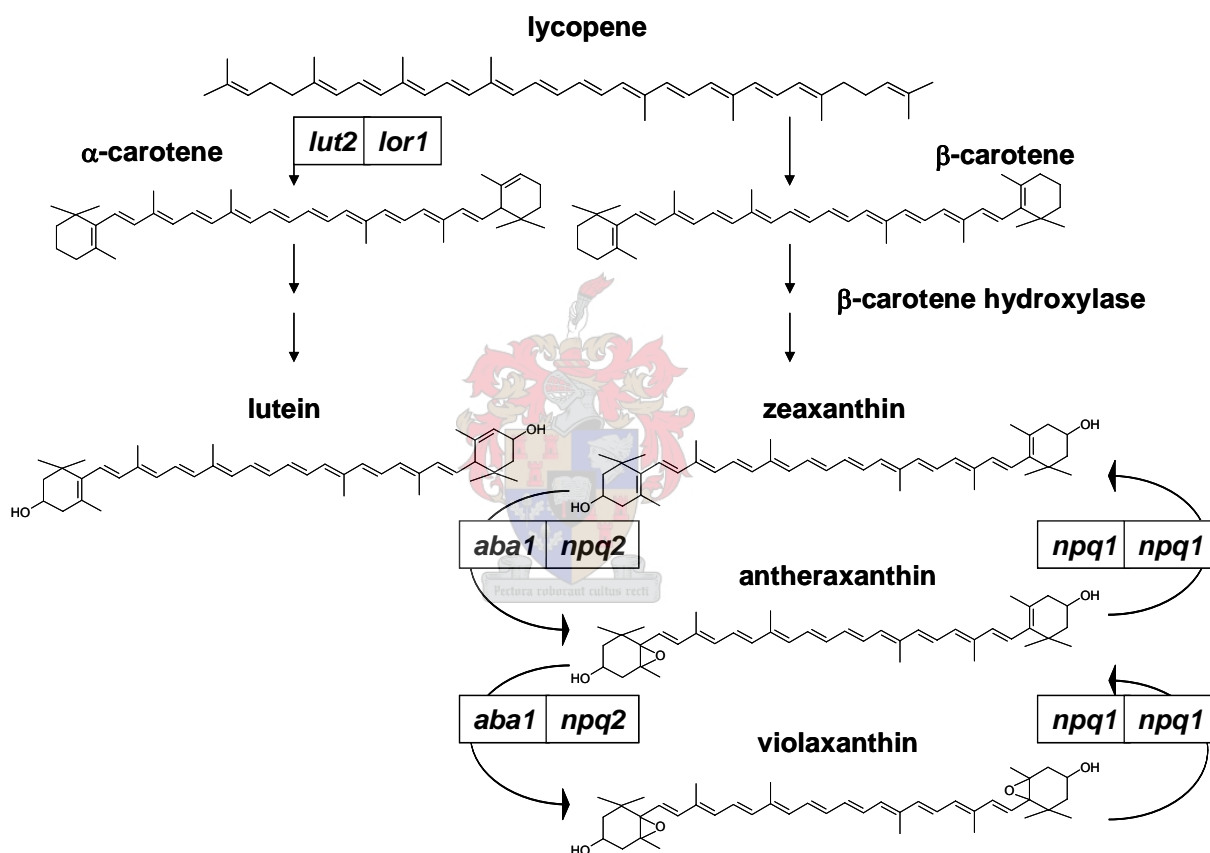


Figure 7. The xanthophyll biosynthetic pathway in green algae and plants. The steps that are blocked in mutants are indicated, with the *Arabidopsis* mutants occurring on the left and the *Chlamydomonas* mutants name on the right half of each box (Baroli and Niyogi, 2000).

The *Chlamydomonas* and *Arabidopsis* npq2 mutants, together with mutants aba1 (Koornneef et al., 1982) and aba2 (Marin et al., 1996) from *Arabidopsis* and tobacco, respectively, are defective in zeaxanthin epoxidase activity (Figure 7). These mutants accumulate zeaxanthin and contain only trace amounts of the epoxy-xanthophylls; antheraxanthin, violaxanthin and neoxanthin (Duckham et al., 1991; Niyogi et al., 1997, Niyogi et al., 1998). By comparing the kinetics of NPQ formation of the *Arabidopsis* npq2 mutant to the wild type, it is clear that NPQ is more rapidly induced when zeaxanthin is already present at high levels. However, over

time the wild type is also able to produce zeaxanthin to such extent, that there is no difference in the final induction of *NPQ* between the wild type and the *npq2* mutant. This is illustrated in Figure 8.

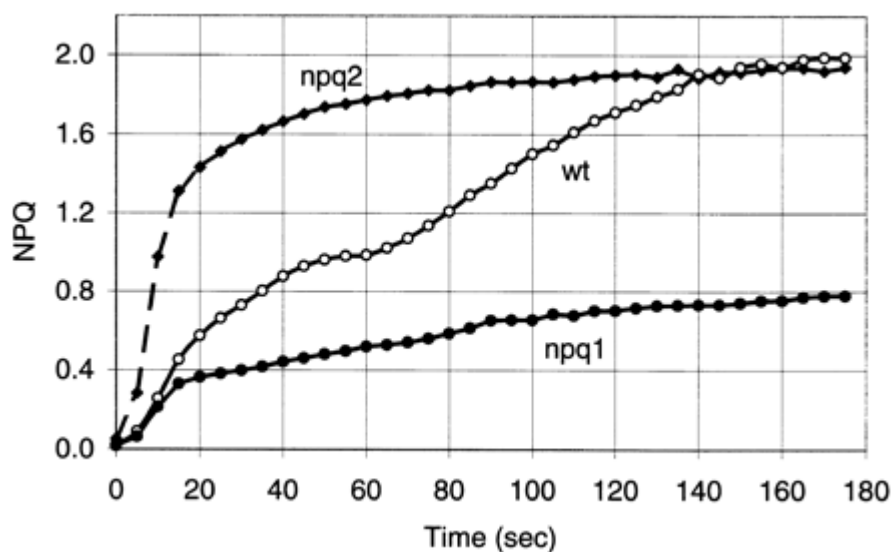


Figure 8. Rapid time course of *NPQ* induction in leaves of the wild type, *npq1* and *npq2* mutants of *Arabidopsis* (Niyogi et al., 1998).

It has further been demonstrated that the additional zeaxanthin in the *npq2* mutant did not enhance photoinhibition compared to that of the wild type, furthermore the *aba1* mutant was even more photoinhibited than the wild type (Tardy and Havaux, 1996; Hurry et al., 1997). However, transgenic lines over-expressing a native β -carotene hydroxylase in *Arabidopsis* accumulating zeaxanthin, showed enhanced protection against lipid peroxidation under high light conditions (Davison et al., 2002).

The *npq1* mutants of *Chlamydomonas* and *Arabidopsis* (Niyogi et al., 1997; Niyogi et al., 1998) further illustrated the importance of the xanthophyll cycle and the difference to which it contributed to photoprotective mechanisms in these organisms. These mutants are unable to produce zeaxanthin under high light due to their inability to de-epoxidase violaxanthin. This reduced formation of zeaxanthin severely inhibited the induction of *NPQ* (Niyogi et al., 1998), particularly in the *Arabidopsis npq1* mutant (Figure 8), whereas the *Chlamydomonas npq1* mutant was only partially inhibited (Niyogi et al., 1997a). This would suggest that *NPQ* induction in *Arabidopsis* is more dependent on the xanthophyll cycle for photoprotection. *NPQ* inhibition was also observed through antisense suppression of *VDE* in tobacco (Verhoeven et al., 2001). Moreover, during short term high light stress the *Arabidopsis npq1* mutant showed increased photoinhibition compared to the wild type. However, over a prolonged period of growth in high light, photoinhibition in the *npq1* mutant was similar to that of the wild type. This would suggest that in the long term, other photoprotective processes can compensate for the defect in *npq1*.

The non-photochemical mutants have greatly accelerated the knowledge regarding the importance of the xanthophyll cycle and the enzymes involved, in *NPQ*. However, their biotechnological application for the enhancement of stress tolerance in plants still needs to be investigated.

Conclusion:

The carotenoid pigments and specifically the xanthophyll cycle pigments have been conclusively linked to the ability of plants to not only “harvest” sunlight energy, but also protect the photosynthetic machinery, plant organs and organelles against the harmful effects of excess light. A large body of scientific data has been accumulated on the structural and functional aspects of these pigments. Moreover the possibility to individually as well as in combinations over-express (or silence) plant genes in various hosts and model plants, have started to provide information on the possible biotechnological roles these pigments could fulfil. The possibility to influence photoprotection and/or other environmental stresses through manipulation of this pathway is one of the possible biotechnological targets linked to the carotenoid pathway of plants.

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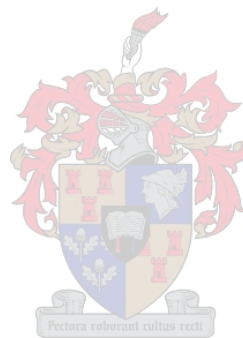
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TECHNICAL REPORT

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



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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 C₃₀ 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.

Key words: HPLC; C₃₀; authentic standards; carotenoids; quantification; plants; *Arabidopsis*

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3.1 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 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 C₁₈ reverse-phase column (monomeric or polymeric) has been used [29, 30]. However, more recently, a triacontyl (C₃₀) 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].

3.2 Experimental

3.2.1 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, 16h-light: 8h-dark cycle and a uniform photon flux density of 43 μmol photons.m⁻².sec⁻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.

3.2.2 Analytical materials

The following solvents were purchased from Sigma-Aldrich (Steinheim, Germany): TBME, ethyl acetate, chloroform and hexane. Methanol was 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 \geq 97%), *trans*-lycopene (purity \geq 90%) and β -apo-caroten-8-al (purity \geq 96%; Fluka Chemie), astaxanthin (purity \geq 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.

3.2.3 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 HPLC analysis are listed in Table 1. All dilutions were made in ethyl acetate:methanol (1:4).

Table 1
The authentic standards used and their preparation for HPLC analysis.

Authentic standard	Solvent for stock solution	Stock concentration ($\mu\text{g}\cdot\text{mL}^{-1}$)	Working concentration ($\mu\text{g}\cdot\text{mL}^{-1}$)
Astaxanthin	chloroform:hexane (1:9)	100	10
β -Apo-caroten-8-al	ethylacetate:methanol (1:4)	100	10
<i>trans</i> -Zeaxanthin	chloroform	100	10
<i>trans</i> - β -Carotene	chloroform:hexane (1:9)	100	10
<i>trans</i> -Lycopene	chloroform	100	2

3.2.4 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. 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 μ L of 50 mM Tris-HCl (pH 8.0) containing 1M NaCl was added and mixed by inversion for 5 min. Chloroform (400 μ L) was added for partitioning of the carotenoids. The mixture was incubated for 10 min and centrifuged at 3000 g for 5 min at 4°C. After centrifugation, the lower chloroform phase was collected and the upper aqueous phase re-extracted with 400 μ L 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 μ L of a 1:4 ethyl acetate, methanol solution containing 0.1% (w/v) BHT and centrifuged at RT for 5 min at 12 000 g. Samples were shielded from strong light and kept on ice during all procedures.

3.2.5 *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 x 2.1 mm) and YMC30 guard cartridge (10 mm x 2.1 mm, particle size 3 μ m) 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% ddH₂O in methanol containing 0.05M 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 μ L.min⁻¹ at 25°C with an injection volume of 10 μ L 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 μ A. 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).

3.2.6 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.

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

The response factor of the *trans*-zeaxanthin authentic standard was used to quantify the xanthophyll pigments; namely *trans*- and *cis*-neoxanthin, *trans*-violaxanthin, *trans*-antheraxanthin and *trans*-lutein. 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). 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 mmol pigment per mol chlorophyll *a*.

3.2.7 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 ($RSD \leq 3\%$).

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.

3.3. Results and discussion

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

Separation of the various pigment constituents extracted from plant leaf tissues, on the C₃₀ 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 0-16% 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 temperature 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 the experimental section). 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 1 000-10 000 $\mu\text{g}\cdot\text{mL}^{-1}$ 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 $\mu\text{g}\cdot\text{mL}^{-1}$ 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 $\mu\text{g}\cdot\text{mL}^{-1}$ 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 $\mu\text{g}\cdot\text{mL}^{-1}$. Furthermore, its stability deteriorated rapidly between consecutive HPLC analyses after a period of ~36 hours, 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 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.

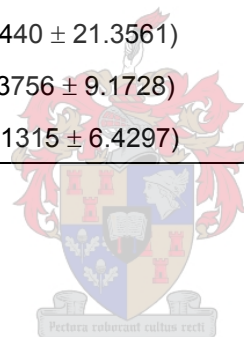
The regression equations and the limits of quantification for all the authentic standards used are detailed (Table 2). The response of astaxanthin, β -apo-caroten-8-al, *trans*-zeaxanthin, *trans*- β -carotene and *trans*-lycopene was linear over the range used for the standard curve and the relative 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).

Table 2
Parameters determined during validation of the developed methodology.

Compound	Validation Parameter					
	Recovery from column (%)	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
<i>trans</i> -Zeaxanthin	99.3	$y = (33.5090 \pm 0.1736)x + 48.5440 \pm 21.3561)$	0.9999	34.142	1.0	82
<i>trans</i> - β -Carotene	98.0	$y = (37.1497 \pm 0.1494)x + (13.3756 \pm 9.1728)$	1.0000	10.907	3.0	99
<i>trans</i> -Lycopene	86.6	$y = (171.6713 \pm 0.4480)x + (-6.1315 \pm 6.4297)$	1.0000	10.688	0.5	ND ^a

^a Not determined



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.

3.3.2 Extraction of major carotenoids from plant tissue

The extraction procedure recommended by Fraser and co-workers [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 in 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.

3.3.3 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 the experimental section) was developed on a RP-C₃₀ 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 typical chromatogram is illustrated in Fig 1A. The α values for all peaks were found to be greater than 1 (Table 3), indicating that a good selectivity of mobile phase to sample components was achieved.



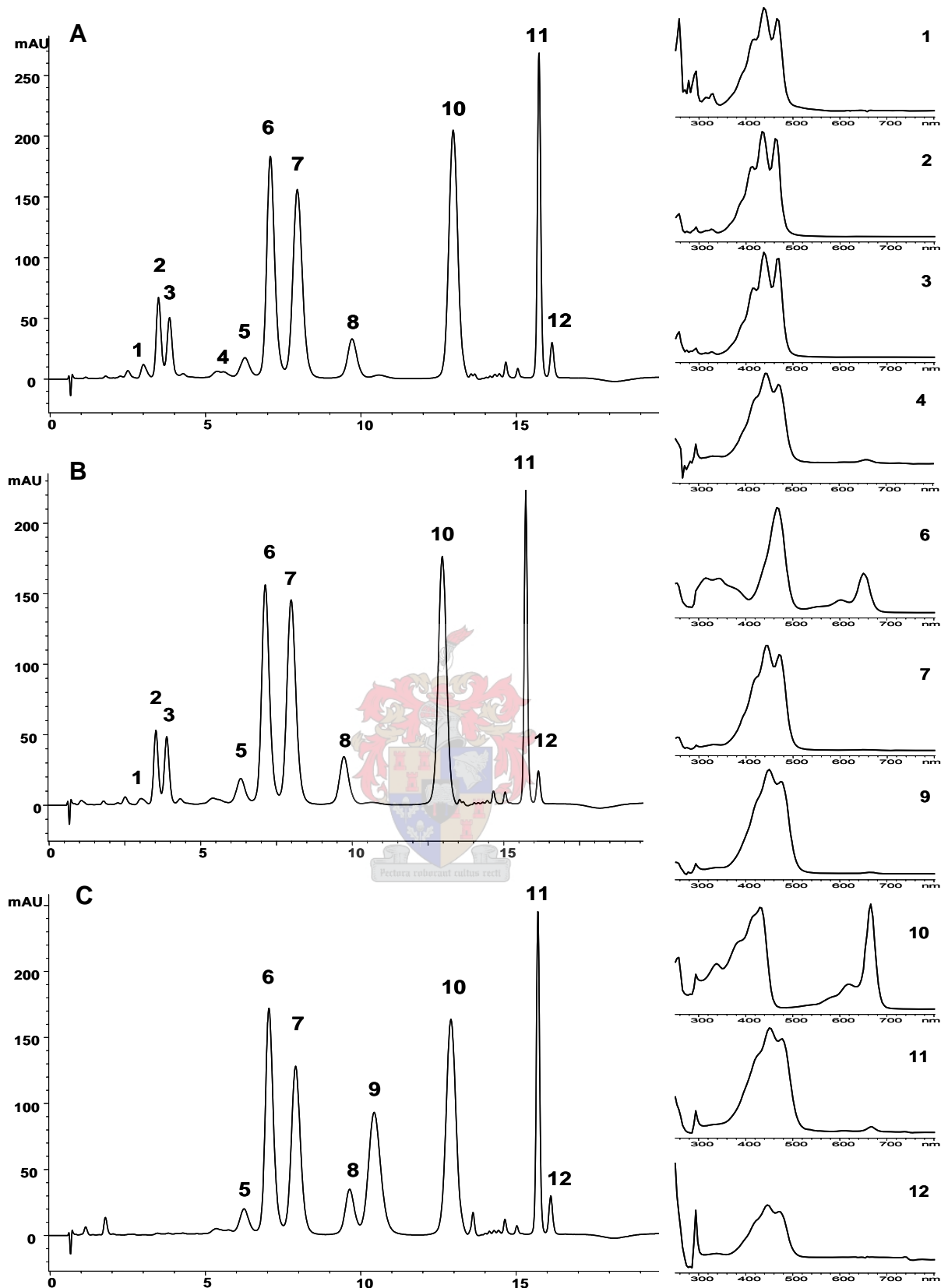


Fig. 2. HPLC chromatograms to profile carotenoids of *A. thaliana* WT and the *npq1* and *npq2* mutants with the visible absorbance spectrum of each major carotenoid indicated on the right hand side. A. *A. thaliana* WT, B. *A. thaliana npq1*, C. *A. thaliana npq2*. 1. *trans*-neoxanthin, 2. *trans*-violaxanthin, 3. *cis*-neoxanthin, 4. *trans*-antheraxanthin, 5. astaxanthin, 6. chlorophyll *b*, 7. *trans*-lutein, 8. *trans*-zeaxanthin, 9. β -apo-caroten-8-al, 10, chlorophyll *a*, 11, *trans*- β -carotene, 12, *cis*- β -carotene.

Table 3

Peak identification of plant carotenoids separated on a C₃₀ HPLC column and their spectral characteristics (in the eluting solvent) following DAD.

Peak No.	Compound	Molecular weight (average) ^a	Retention factor (κ)	Separation factor (α)	Spectral characteristics (nm)						%III/II ^b		APCI-MS (negative)
					Observed ^c			Reported			Observed ^c	Reported	<i>m/z</i>
1	<i>trans</i> -Neoxanthin	600.9	3.55	1.00	418	438	466	417	440	470 ^d	66	- ⁱ	- ⁱ
2	<i>trans</i> -Violaxanthin	600.9	4.29	1.16	414	438	470	415	436	466 ^e	80	90 ^e	600.0
3	<i>cis</i> -Neoxanthin	600.9	4.82	1.10	414	434	462	413	435	464 ^d	81	80 ^f	600.0
4	<i>trans</i> -Antheraxanthin	584.9	7.45	1.45	422	442	470	422	444	472 ^f	41	55 ^f	- ⁱ
5	Astaxanthin	596.4	8.49	1.12			470			478 ^f	0	0	596.0
6	Chlorophyll <i>b</i>	907.5	9.73	1.13		466	650		469	652 ^e	0	0	906.0
7	<i>trans</i> -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	<i>trans</i> -Zeaxanthin	568.9	15.00	1.09	(426)	450	474	(429)	448	473 ^e	18	22 ^e	568.14
10	Chlorophyll <i>a</i>	893.5	18.58	1.22		430	666		431	665 ^e	0	0	893.0
11	<i>trans</i> -β-Carotene	536.9	22.72	1.21	(426)	450	478	(429)	449	475 ^e	17	25 ^e	536.0
12	9- <i>cis</i> -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

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 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 2-6 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 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-ol 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 C₃₀ columns.

3.3.4 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.

Table 4

Compounds quantified by C₃₀ RP-HPLC of WT *Arabidopsis* and the *npq1* and *npq2* *Arabidopsis* mutants.

Peak No.	Compound	WT	<i>npq1</i>	<i>npq2</i>
1	<i>trans</i> -Neoxanthin ^a	6.95	4.05	ND ^c
2	<i>trans</i> -Violaxanthin ^a	31.57	31.43	ND ^c
3	<i>cis</i> -Neoxanthin ^a	29.22	32.12	ND ^c
4	<i>trans</i> -Antheraxanthin ^a	ND ^c	ND ^c	ND ^c
7	<i>trans</i> -Lutein ^a	154.88	183.06	121.85
9	<i>trans</i> -Zeaxanthin ^a	5.52	4.60	118.58
11	<i>trans</i> - β -Carotene ^a	89.47	96.20	79.28
12	9- <i>cis</i> - β -Carotene ^a	15.15	15.89	14.75
Total Carotenoids ^a		333.62	367.35	338.32
$\frac{(A + Z)}{(V + A + Z)}$		0.168	0.128	0.990
10	Chlorophyll a ^b	13.90	10.83	12.14
6	Chlorophyll b ^b	7.60	5.89	6.62
	Chlorophyll a/b	1.83	1.83	1.83

^a Expressed as mmoles carotenoid per mole chlorophyll a.

^b Expressed as μ moles chlorophyll per gram freeze dried tissue.

^c Not detected (Below the quantification limit).

3.4 Conclusion

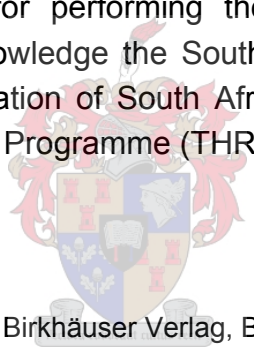
As mentioned previously, the reliable and reproducible quantification of carotenoids is a complex task. To date a range of different methodologies have been used for the extraction, storage and HPLC analysis of carotenoids from a wide range

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

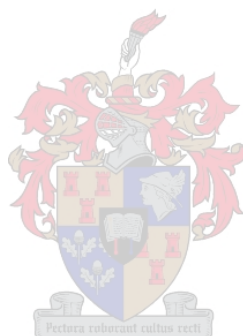
We would like to thank Dr. Marietjie Stander (Stellenbosch University, Stellenbosch, South Africa) for performing the HPLC-APCI-MS analysis of our samples. We gratefully acknowledge the South African Wine Industry (Winetech), the National Research Foundation of South Africa (NRF) and the Technology and Human Resources for Industry Programme (THRIP) for funding.

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RESEARCH RESULTS

Over-expression and analysis of two *Vitis vinifera* carotenoid biosynthetic genes in transgenic *Arabidopsis*



This chapter will form part of a manuscript prepared for eventual publication in
Plant Physiology

Over-expression and analysis of two *Vitis vinifera* carotenoid biosynthetic genes in transgenic *Arabidopsis*

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ABSTRACT

Plants have evolved mechanisms to efficiently protect the photosynthetic apparatus against the damaging effects of excess light. Carotenoids, especially the xanthophyll cycle pigments, play important roles in the dissipation of excess light energy as heat. The β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*) genes were isolated from *Vitis vinifera* L. cv Pinotage. Respectively, they encode proteins that convert β -carotene to zeaxanthin and zeaxanthin to violaxanthin. To determine the effect of these genes on the carotenoid concentrations and their roles in photoprotection, they were transformed into the model plant *Arabidopsis thaliana*. Several independent transgenic lines stably over-expressing the *VvBCH* and *VvZEP* genes were generated, harbouring one to multiple copies of the transgene. Homozygous populations of the transgenic lines, the untransformed control as well as two well characterised *Arabidopsis* mutants affected in the xanthophyll cycle, *npq1* and *npq2* were used to evaluate the effect of the over-expressed genes on pigment levels and photosynthetic parameters under normal and excessive light conditions. The transgenic lines over-expressing the *VvBCH* gene had elevated xanthophyll cycle pigments, specifically violaxanthin, which was available for de-epoxidation under a high light stress treatment, resulting in a two-fold increase in zeaxanthin compared to the wild type. The additional zeaxanthin under high light conditions however, did not affect the magnitude of non-photochemical quenching (*NPQ*) of chlorophyll fluorescence. In contrast, the *VvZEP* over-expressing lines had reduced zeaxanthin levels possibly due to faster epoxidation of zeaxanthin to violaxanthin than the de-epoxidation of violaxanthin to zeaxanthin. This resulted in decreased induction and final extent of *NPQ*, and significantly increased photoinhibition compared to the wild type under high light conditions. These lines demonstrated that accumulation of zeaxanthin does not necessarily lead to increased photoprotection and that even small decreases in zeaxanthin can result in significantly increased photoinhibition.

Key words: *Arabidopsis*; xanthophyll cycle; zeaxanthin; chlorophyll fluorescence quenching; photoinhibition

4.1 INTRODUCTION

Light intensity may be in excess of the requirements for carbon reduction, causing damage to the photosynthetic apparatus and ultimately resulting in reduced photosynthetic efficiency (photoinhibition). Plants have evolved protective mechanisms against photoinhibition, for example by dissipating excess energy as heat, by neutralising triplet chlorophyll and reactive oxygen species formed in the chloroplast when the thylakoid is over-energised, or by rapidly repairing damage to the photosynthetic apparatus caused by the excess energy (Asada, 1994). Other protective mechanisms are increased photosynthetic (carbon reduction) capacity (Demmig and Björkman, 1987), reorientation and/or movement of chloroplasts within cells (Brugnoli and Björkman, 1992) and movement of whole leaves (Björkman and Demmig-Adams, 1994), changes in leaf reflectance (Ripley et al., 1999), and soluble antioxidants in the thylakoid membrane (α -tocopherol and ascorbate) (Fryer, 1992). Carotenoids have been shown to play important functional and structural roles in the light-harvesting complexes of photosystems (Siefermann-Harms, 1985), especially in photoprotection (Björkman and Demmig-Adams, 1994). The photoprotective roles of carotenoids include the quenching of triplet chlorophyll and singlet oxygen as well as the inhibition of lipid peroxidation (Cogdell and Frank, 1987; Frank and Cogdell, 1993; Demmig-Adams et al., 1996a; Frank and Cogdell, 1996).

The carotenoid, β -carotene, is essential for the assembly and photoprotection of photosystem II (PS II) reaction centers. The xanthophyll carotenoids, violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) are components of the xanthophyll cycle, they occur in the light harvesting complexes of PS II and PS I and play an important role in energy dissipation (Demmig-Adams and Adams, 1996b; 2000; Horton et al., 1996). Under conditions of excessive excitation energy (high light conditions and an acidified thylakoid lumen) V is de-epoxidised to Z, via A, by violaxanthin de-epoxidase which is a strong dissipator of excitation energy as heat. Under low light (neutral to only slightly acidic thylakoid lumen), Z is epoxidised to V by zeaxanthin epoxidase (Yamamoto et al., 1962). V is not an energy dissipator and passes the excitation energy on to chlorophyll and ultimately onto a reaction center.

Thermal dissipation of excessive light by zeaxanthin can be measured as non-photochemical quenching (*NPQ*) of chlorophyll fluorescence (Björkman and Demmig-Adams, 1995), although other processes and state transitions in the PS II reaction center also contribute to *NPQ*. The main component of *NPQ* is the pH-dependent (*qE*) type of quenching or the fast-relaxing component, involving the xanthophyll cycle. Yamamoto and Kamite (1972) have shown that the induction and final extent of *NPQ* in isolated thylakoids and detached leaves are dependent on the formation of zeaxanthin and antheraxanthin. Although the induction of *NPQ* is dependent upon on the formation of zeaxanthin and antheraxanthin, it has been shown that another carotenoid, lutein, also contributes to *NPQ* (Pogson et al., 1998). The mechanism by which these xanthophyll pigments promote *NPQ* is still unclear. Proposed mechanisms suggest that either direct transfer of excitation energy from

excited chlorophyll to zeaxanthin takes place (Holt et al., 2005) or zeaxanthin takes part in the formation of a quenching complex (Gilmore et al., 1995; Dreuw et al., 2003).

Several *Arabidopsis thaliana* and *Chlamydomonas* mutants that have altered NPQ have been isolated and characterised. These mutants have been used to confirm the role of the xanthophyll cycle in NPQ and also the involvement of other xanthophylls in NPQ (Niyogi et al., 1997a; 1997b). The *npq1* mutant is defective in the violaxanthin de-epoxidase enzyme which catalyses de-epoxidation of violaxanthin to zeaxanthin in the xanthophyll cycle. A *Chlamydomonas npq1* mutant has been shown to be partially defective in NPQ, whereas the *A. thaliana npq1* mutant exhibited greatly reduced NPQ, demonstrating that zeaxanthin formation is required for full NPQ development in *A. thaliana*. The *npq2* mutant defective in the gene for zeaxanthin epoxidase (catalyses the conversion of zeaxanthin to violaxanthin) accumulates zeaxanthin in the dark and in the light shows rapid initial NPQ induction compared to the wild type.

Plants stress, whether it is caused by salinity, drought, nutrient or other types of stress, is manifested ultimately as a light stress phenomenon, since the stressed plants are unable to efficiently transform light energy into carbon reduction. The xanthophyll cycle, with its photoprotective role, is therefore a promising target for genetic engineering to enhance environmental stress tolerance. Davison et al, (2002) showed that *A. thaliana* that over-expresses the endogenous *BCH* gene which codes for β -carotene hydroxylase that converts β -carotene to zeaxanthin, has a larger xanthophyll pool and shows less leaf necrosis and lipid peroxidation under high light and high temperature conditions than wild type plants. In tobacco, over-expression of the endogenous *ZEP* gene, coding for zeaxanthin epoxidase enzyme, leads to delayed seed germination and increased abscisic acid (ABA) levels (Frey et al., 1999). Transgenic tobacco with reduced violaxanthin de-epoxidase activity showed low NPQ and a greater susceptibility to photoinhibition (Sun et al., 2001; Verhoeven et al., 2001).

We investigated the physiological roles of β -carotene hydroxylase and zeaxanthin epoxidase under high light conditions by over-expressing the encoding genes from *Vitis vinifera* L cv Pinotage in *A. thaliana*. Stable populations of independent transgenic lines over-expressing the *VvBCH* or *VvZEP* genes were generated by selection and used for further analyses. We analysed the effect of the over-expression on pigment concentrations under low and high light. We investigated their effect on photosynthesis using chlorophyll fluorescence. The characterisation of these lines provides an important basis for further analysis into the physiological relevance and biotechnological importance of these genes and their encoded products.

4.2 MATERIALS AND METHODS

4.2.1 Microbial strains and culture conditions

All the microbial strains used in this study are listed in Table 1. The *Escherichia coli* strain DH5 α was cultured on Luria Broth (LB) media (0.6% (w/v) yeast extract, 1.2% tryptone, 1.2% sodium chloride) at 37°C using standard techniques (Sambrook et al., 1989), whereas the *Agrobacterium tumefaciens* strain GV3103 was cultured in LB media containing 1% galactose, 150 $\mu\text{g}\cdot\text{ml}^{-1}$ rifampicin and 50 $\mu\text{g}\cdot\text{ml}^{-1}$ gentamycin at 28°C. LB media was supplemented with the appropriate antibiotic for selection of transformants or to retain selective pressure, when appropriate.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Bacterial strains		
<i>E. coli</i> DH5 α	supE44/lacU169[ϕ 80/lacZM15 <i>hsdR17recAgyrA96thi-1relA1</i>]	Life Technologies (GIBCO/BRL), California, USA
<i>A. tumefaciens</i> GV3101	Disarmed, nopaline-type strain	Holsters et al., 1980
Plasmids		
pGEMT-VvZep	pGEMTeasy, containing the cDNA of the <i>zeaxanthin epoxidase</i> (VvZEP) gene of <i>V. vinifera</i> cv Pinotage.	Dr. P Young, Institute for Wine Biotechnology, Stellenbosch University.
pART7	Cloning vector, CaMV35S promoter, OCS 3' terminator	Gleave, 1992
pART27	Plant expression vector, kanamycin resistance marker	Gleave, 1992
pART-VvBCH	pART27 based, containing the cDNA of the <i>β-carotene hydroxylase</i> (VvBCH) gene of <i>V. vinifera</i> cv Pinotage, under the 35S CaMV promoter.	Dr. P Young, Institute for Wine Biotechnology, Stellenbosch University.
pART-VvZEP	pART27 based, containing the cDNA of the <i>zeaxanthin epoxidase</i> (VvZEP) gene of <i>V. vinifera</i> cv Pinotage, under the 35S CaMV promoter.	This study

4.2.2 DNA manipulations and construction of plant expression cassettes

Standard DNA techniques were used for plasmid DNA isolation, purification, manipulation and cloning of DNA fragments (Sambrook et al., 1989). Full cDNA sequences of two carotenoid biosynthetic genes; the *VvZEP* gene encoding the zeaxanthin epoxidase enzyme and the *VvBCH* gene encoding the β -carotene hydroxylase enzyme, both from *V. vinifera* cv Pinotage, were previously isolated in our laboratory (Young, 2004). The pART-VvBCH construct (Figure 1) was generated

through the subcloning of a 900 bp *SalI/SpeI* DNA fragment containing the sequence of the *VvBCH* gene (Genbank accession: AF499108) into the *XhoI/XbaI* sites of pART7 (Gleave, 1992). To facilitate the cloning of the *VvZEP*, a *SalI* restriction site was added to the 5' end of the ZEP_ATG_ *SalI* primer 5'-GTCGACATGGCTTCAGCAGTGTTTTATAG-3'. The pART-*VvZEP* construct (Figure 1) was prepared by subcloning a 1990 bp *SalI/SpeI* DNA fragment containing the sequence of the *VvZEP* gene (Young, 2004) into the *XhoI/XbaI* sites of pART7. All DNA restriction enzymes were obtained from Fermentas International INC (Burlington, Canada). The expression cassettes were excised from the constructed pART7 plasmids by *NotI* digestion and subcloned into the corresponding site of pART27, yielding pART-*VvBCH* and pART-*VvZEP*, respectively. The PCR fragments were confirmed through sequencing with T7 5'-TAATACGACTCACTATTAGGG-3' and SP6 5'-ATTTAGGTGACTATAGAA-3' primers, using an ABI Prism automated DNA sequencer from the Central Analytical Facility (CAF) at Stellenbosch University (results not shown).

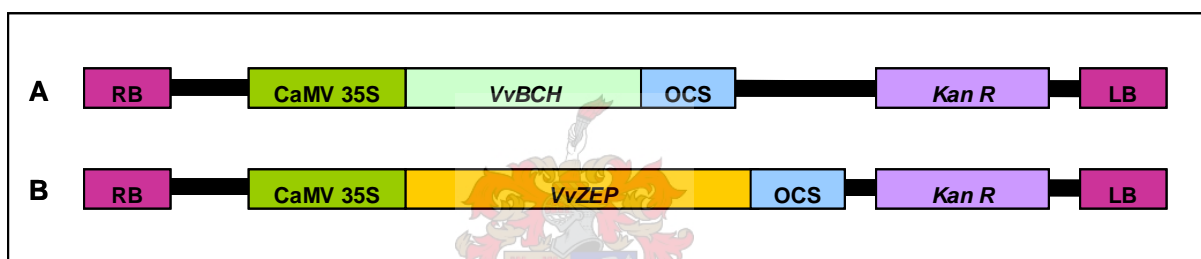


Figure 1. A schematic diagram of the pART27 cassettes transformed into *Arabidopsis thaliana* ecotype Columbia 0, containing the over-expressed β -carotene hydroxylase and zeaxanthin epoxidase encoding genes from *Vitis vinifera* cv Pinotage. The *VvZEP* and *VvBCH* genes were cloned into pART27 (Gleave, 1992) resulting in pART-*VvBCH* (A) and pART-*VvZEP* (B) constructs, respectively. The RB and LB corresponds to the T-DNA right and left borders, respectively, CaMV35S to the Cauliflower Mosaic Virus 35S promoter, OCS to the *Agrobacterium* nopaline synthase terminator, and Kan R to the *pnos-ntplI-nos3'* kanamycin resistance cassette.

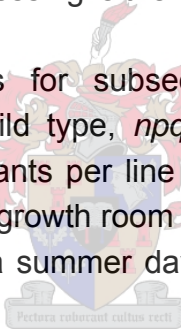
4.2.3 Plant material and *A. thaliana* transformation

The *A. thaliana* ecotype Columbia 0 wild type plants, the transgenic lines, as well as two *Arabidopsis* mutants *npq1* and *npq2* (*Arabidopsis* Biological Research centre, Ohio state University Co, USA) were grown in a 3:1 peat (Jiffy 7, Jiffy products International AS, Norway) and vermiculite mixture in a controlled growth room maintained at 23°C, 55% humidity, 16h: 8h light-dark cycle. Photon flux density in the light period was 43 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The pART-*VvZEP* or pART-*VvBCH* (Figure 1) constructs were mobilised into *A. tumefaciens* strain GV3103 by electroporation (Maltanovich et al., 1989). The transformation of *A. thaliana* was carried out via the floral dip method as described by Clough and Bent (1998). For each construct, six six-week old plants were used for the transformation. The dipping of the floral buds was repeated five days after the first dipping. The plants were covered for optimal

humidity at least 24 to 48 hours after dipping. Each of the dipped plants was allowed to self-pollinate and set seed. The seeds were harvested and surface sterilised using chlorine gas for up to three hours (Clough and Bent, 1998). The putatively transformed T1 generation seedlings were germinated on solid half Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with kanamycin sulphate ($35 \mu\text{g ml}^{-1}$) for selection of transgenic seedlings. The seedlings were grown in a tissue culture room at 25°C and a photon flux density of $43 \mu\text{mol.m}^{-2}.\text{s}^{-1}$.

After two weeks the putatively transformed plants were transferred to the growth room where they were allowed to self-pollinate and set seed. Plants were watered every four days and a nutrient solution (Nitrosol, FLEURON® Universal Selected Services, Braamfontein, South Africa) was applied every three weeks according to the manufacturer's specifications. PCR analyses were conducted to evaluate transgene insertion and segregation using the PL-5' Scr 5'-CCTTCGCAAGACCCTTCCTC-3' and the following reverse primers for the two genes, BCH-TM-3' 5'-CTCGAGTCATGAAGAGTCAGATGATTTAATTC-3' and ZEP Stop 5'-TCAAACCGCCTGGAAGAGCT-3'. PCR positive transgenic plants were carried through to the next generation. Homozygous T3 generation transgenic lines, stably inheriting and expressing either the *VvZEP* gene or the *VvBCH* gene were used in all further analyses.

To apply a light stress for subsequent pigment pool and chlorophyll fluorescence analyses of the wild type, *npq1* and *npq2* and the homozygous T3 *VvZEP* and *VvBCH* lines, six plants per line were used for the experiment. These plants were transferred from the growth room to outdoors for 30 min, four weeks after sowing, to natural sun light on a summer day at 30°C and a photon flux density of $2100\text{-}2200 \mu\text{mol.m}^{-2}.\text{s}^{-1}$.



4.2.4 Germination tests

Seeds were harvested from untransformed control, *npq1* and *npq2* and transgenic plants (homozygous T3 generation) of the same age and grown in the same conditions. After surface sterilisation with chlorine gas, approximately three hundred seeds of each plant line were sown in triplicate onto half strength MS medium, containing kanamycin sulphate ($35 \mu\text{g.ml}^{-1}$), as well as the same medium without antibiotics. Seeds were then placed in a growth room where the conditions were as previously described; seed germination was scored every day. Mean germination values were calculated for all lines. For lines where the seed germination values approached 100% the stable integration and expression of the transgenes were determined via Southern and Northern analyses. Only plant lines that showed stable gene integration and expression after 3 generations were used to investigate pigment pool concentrations and photosynthetic capacity.

4.2.5 DNA isolation and PCR screening of transformants

PCR screening was used to screen the putative transgenic lines and the subsequent generations. Genomic DNA for PCR analysis was prepared by macerating 5 mm² leaf tissue in 100 µl water. One µl aliquot was used in the PCR screening reactions, using the primers already described to amplify an ≈1 kb *VvBCH* and ≈2 kb *VvZEP* fragment, respectively. The PCR amplifications were performed using BioTaq™ DNA polymerase and buffer (Bioline, London, UK).

PCR amplification typically consisted of 1× Expand high fidelity PCR buffer with MgCl₂, 1.6 µmol of each primer and 20 ng of the appropriate DNA template. The PCR amplifications were performed in 50 µL reaction mixtures. The amplification program typically started with a denaturing step at 94°C for 5 min followed by 35 cycles, each consisting of a 35 s denaturing step at 94°C, a 35 s annealing at the appropriate melting temperature of the primers, a 40 s per 1 kb length of the fragment to be amplified at 72°C, and a final elongation step at 72°C for 5 min.

4.2.6 Southern blot analysis

Southern blot analysis was conducted on the putative transgenic lines as well as the T3 homozygous transgenic lines, to confirm and analyse the transgene integration patterns of the *VvZEP* and *VvBCH* genes.

Genomic DNA isolations were carried out according to McGarvey and Kaper (1991), using 800 µL extraction buffer consisting of 3% CTAB (w/v), 1.4 M NaCl, 0.02 M EDTA and 1 M Tris-HCL (pH 8.0). Approximately 1 µg gDNA was digested to completion with either *EcoRV* or *StuI*. The *EcoRV* enzyme recognises one site within the pART-*VvBCH* cassette and *StuI* enzyme recognises one site within the pART-*VvZEP* cassette. The *EcoRV* enzyme recognises two sites within the pART-*VvZEP* cassette, resulting in a band of known size (1.9 kb) and additional bands. These digestions were separated on a 0.8% (w/v) agarose gel and blotted onto positive Hybond-N nylon membranes (Roche Molecular biochemicals, Ingelheim, Germany) which were then hybridised with DIG labelled cDNA probes. The *VvBCH* and *VvZEP* DIG labelled probes were PCR labelled using the DIG dNTP labelling mixture (Roche Molecular Biochemicals, Ingelheim, Germany) according to the manufacturer's specifications. For the labelling reactions the PL-5' Scr in combination with BCH-TM-3' and ZEP Stop primers were used for the full cDNA of *VvBCH* and *VvZEP*, respectively. Chemiluminescent detection of the *VvBCH* or *VvZEP* genes proceeded as described by the supplier (Roche Molecular Biochemicals, Ingelheim, Germany).

4.2.7 Northern blot analysis

Northern blot analysis was conducted on the putative transgenic lines as well as the T3 homozygous transgenic lines to evaluate the gene expression of the *VvZEP* and *VvBCH* genes. Small scale RNA isolations were carried out according to the TRIzol®

method (GibcoBRL-LifeTechnologies, California, USA), with an all-in-one extraction buffer containing 1 % SDS (w/v), 0.3 M LiCl, 10 mM Na₂EDTA, 1% Na-deoxycholate (w/v), 1% Nonidet P-40 (v/v), 0.2 M Tris-HCL pH 8.5, 1% sodium metabisulphate (w/v) and 5 mM thiourea. One ml extraction buffer and 0.2 ml chloroform was added to ground leaf tissue (10 mg) and centrifuged for 10 min at 12 000 g and 4°C. The supernatant was transferred to a new microcentrifuge tube and the total RNA was precipitated by adding 500 µl of isopropanol. The pellet was collected via centrifugation for 15 min at 12 000 g and washed with 70% ethanol.

RNA was separated by electrophoresis on a denaturing 1.2% (w/v) formaldehyde-containing agarose gel and blotted onto a positive Hybond-N nylon membrane (Roche Molecular biochemicals, Ingelheim, Germany) using the technique described by Sambrook et al. (1989). Hybridisation of the DNA probes to the immobilised RNA was performed at 50°C overnight in a high sodium dodecyl sulphate (SDS) buffer containing 7% SDS (w/v), 4.5% skim milk (w/v), 0.1% N-lauroylsarcosine (w/v), 50% formamide (v/v) and maleic acid buffer (pH 8). After hybridisation the membranes were washed twice with 2 × tri-sodium citrate (SSC) containing 0.1% SDS (w/v) for 20 min followed by washing the membrane twice with 0.2 × SSC containing 0.1% SDS (w/v) for 20 min at 68°C. Chemiluminescent signal detection was carried out according to the manufacturer's specifications. The transcript sizes were confirmed by comparing co-migration of a high range RNA ladder (0.20 kb to 6 kb, Fermentas International INC, Burlington, Canada). The relative expression of the transgenes were determined using the AlfaEase® FC software (Alpha Innotech Corporation, San Leandro, CA), by comparing the chemiluminescent signal with that of the 28S RNA loaded on the RNA gels.

4.2.8 Evaluation of pigment pools with HPLC in the wild type, mutant and transgenic *Arabidopsis* lines

Leaf tissue (2 rosette leaves per plant) was harvested from all six plants of the controls (wild type, *npq1* and *npq2*) and the homozygous T3 transgenic lines before and after the high light treatment frozen in liquid nitrogen and freeze-dried overnight. These leaves were pooled per transgenic line and the pigments extracted according to Fraser et al., (2000). Two milligrams of freeze dried material was extracted by gently mixing it 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 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 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 ethylacetate:methanol solution containing 0.1% (w/v) BHT and centrifuged at RT for

5 min at 12 000 g. Samples were shielded from strong light and kept on ice during all procedures.

The carotenoid pigments were separated by reverse phase HPLC according to the method described in the technical report in Chapter 3 of this thesis on an Agilent 1100 series HPLC system (Agilent technologies, California, USA). An YMC30 column (100 mm x 2.1 mm) with a YMC30 guard cartridge (10 mm x 2.1 mm, particle size 3 μm) both from YMC Europe GmbH (Schermbach, Germany) were used. Chemstation software for LC3D (Rev.A.10.01[1635]; Hewlett-Packard, Waldbronn, Germany) was used for data processing. The binary mobile phase consisted of 3% ddH₂O in methanol containing 0.05 M ammonium acetate (solvent A) and 100% TBME (solvent B). Both solvents contained 0.1% (w/v) BHT and 0.05% triethylamine. A flow rate of 500 $\mu\text{L}\cdot\text{min}^{-1}$ 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 elution of the various carotenoid pigments was followed at 450 nm and 470 nm.

4.2.9 Evaluation of photosynthetic capacity of the wild type, mutant and transgenic *Arabidopsis* lines with chlorophyll fluorescence measurements

For each transgenic line, the mutants and the controls, chlorophyll fluorescence was measured on 9 leaves (three rosette leaves per plant, three plants per line) using a FMS1 pulse modulated fluorimeter (Hansatech instruments LTD, UK). The leaves were dark adapted for 30 min prior to all measurements. F_0 (minimum fluorescence in the dark-adapted state) was measured before applying a saturating pulse of white light (8000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 0.8 s) to determine F_m (maximum fluorescence in the dark-adapted state). After a further 2 minutes in the dark the leaf was exposed to a stepwise increase of actinic light, from 21 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to 1305 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR (photosynthetically active radiation). A 2 minute equilibration period was allowed at each PAR level and then F_s (fluorescence yield at the particular PAR), F'_m (maximum yield in the light-adapted state, by applying a saturating light pulse) and F'_0 (minimum fluorescence in the light-adapted state, measured after switching off the actinic light and applying 3 seconds of far red light).

From these basic fluorescence measurements the following parameters were calculated, according to Genty et al. (1989) and Van Kooten and Snel (1990): non-photochemical quenching ($NPQ = (F_m/F'_m) - 1$), efficiency of PSII photochemistry or the Genty parameter ($\Phi_{PSII} = (F'_m - F_s)/F'_m$), efficiency of open reaction centers (F'_v/F'_m), photochemical quenching (qP) and relative electron transport rate (ETR). NPQ consists of, the fast-relaxing qE (fast-relaxing NPQ , or NPQ_F) and the slow-relaxing qT and qI (slow-relaxing NPQ , or NPQ_S). The two components were determined from NPQ relaxation kinetics in the dark following the exposure to the highest light level, using the procedure suggested by Maxwell and Johnson (2000).

F_m was measured periodically for 25 minutes by applying a saturating light pulse. Back-extrapolation of the last three (log) F_m values to the time when the actinic light was switched off yielded F'_m , the F_m value that would have occurred had NPQ in the light consisted of only the slow-relaxing component. NPQ_F was then calculated as $(NPQ_F = (F_m/F'_m) - (F_m/F_m))$ and NPQ_S as $(NPQ_S = (F_m - F'_m)/F'_m)$.

4.2.10 Statistical analysis

The significances of the differences in pigment concentrations and chlorophyll fluorescence parameters between wild type, *npq1* and *npq2* and the transgenic lines were assessed by analysis of variance and the Tukey Honest Significant Difference method.

4.3 RESULTS

4.3.1 *A. thaliana* transformation and regeneration

A. tumefaciens was used to mobilise the pART-VvBCH and pART-VvZEP constructs to *A. thaliana* ecotype Columbia-0. In total 50 and 67 putative transgenic lines were generated for the pART-VvBCH and the pART-VvZEP constructs, respectively. All the putative transgenic plants were subsequently subjected to northern blot analysis to confirm transgene expression.

4.3.2 Verification of transgene expression of the putative transgenic lines

Northern blot analysis confirmed the expression of the *VvBCH* gene in only 30 of the 50 putative transgenic plants (Figure 2). The membranes were probed with the full cDNA DIG-labelled *VvBCH* sequence. A single hybridisation band at approximately 900 bp, was observed for the *VvBCH* transformants, which corresponds to the size of the *VvBCH* gene (Figure 2). The hybridisation signals were normalised to the 28S RNA band with the Alfa ease system. The relative expression of the *VvBCH* gene appear to differ considerably between the different transgenic plant lines if loading differences are taken into account by comparing rRNA levels on the RNA gels (qualitative data).

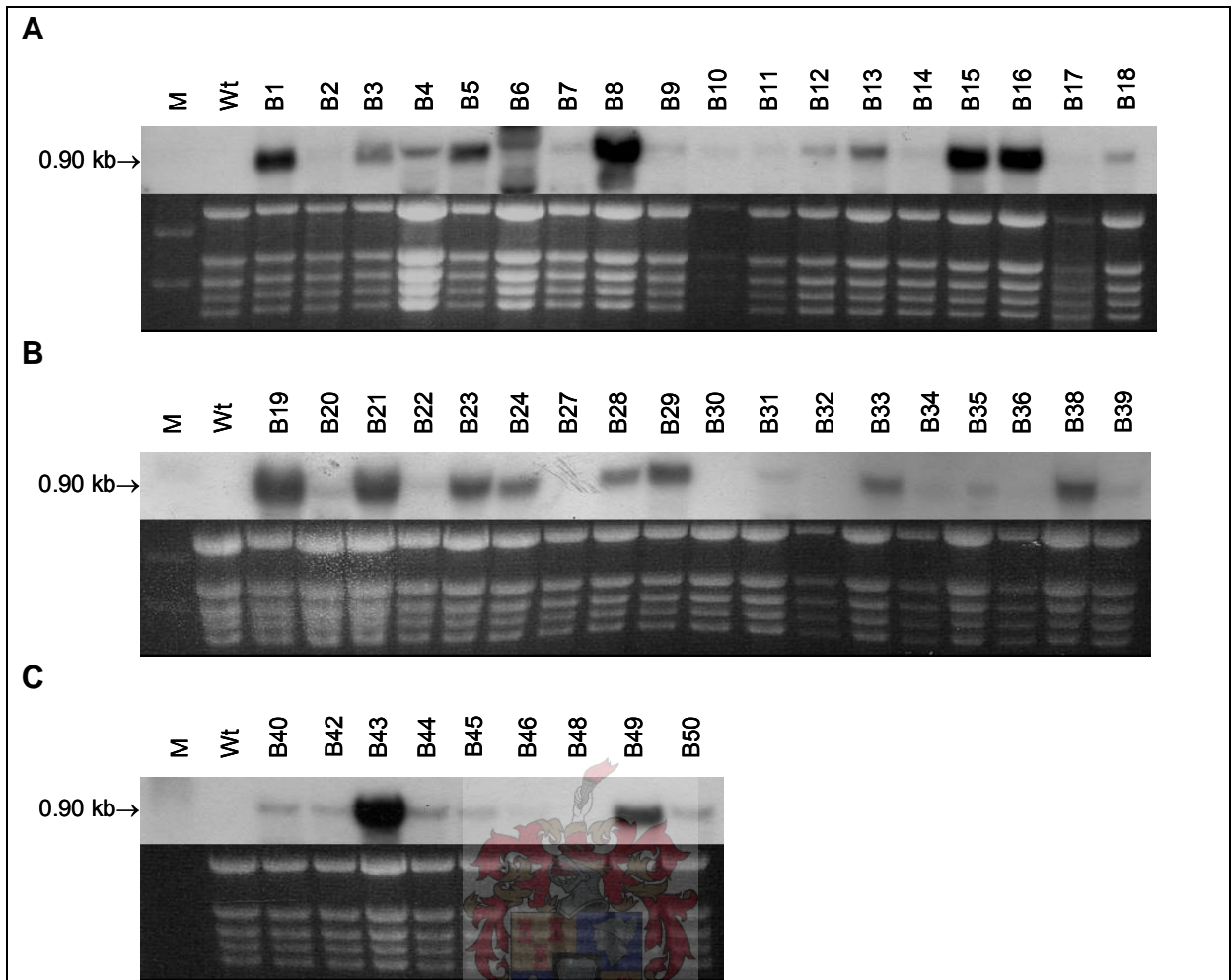


Figure 2. Northern blot analyses (top) conducted on the total RNA (bottom) isolated from 50 putative transgenic *Arabidopsis* plant lines over-expressing the β -carotene hydroxylase gene from *V. vinifera* L. cv Pinotage (*VvBCH*). For A-C: lane M represents the RNA marker and lane Wt represents the wild type (untransformed *A. thaliana* control). The numbers in the subsequent lanes represents the transgenic *A. thaliana* plant lines transformed with the *VvBCH* (B) gene.

Active transcription of the *VvZEP* gene was confirmed for 27 of the 67 putative transgenic plants. The *VvZEP* probe detected a single signal of approximately 1.98 kb, which corresponds to the size of the *VvZEP* sequence (Figure 3). Like the *VvBCH* gene, the *VvZEP* gene was differentially expressed in the transgenic plant lines if loading differences were taken into account by comparing rRNA levels on the RNA gels (qualitative data).

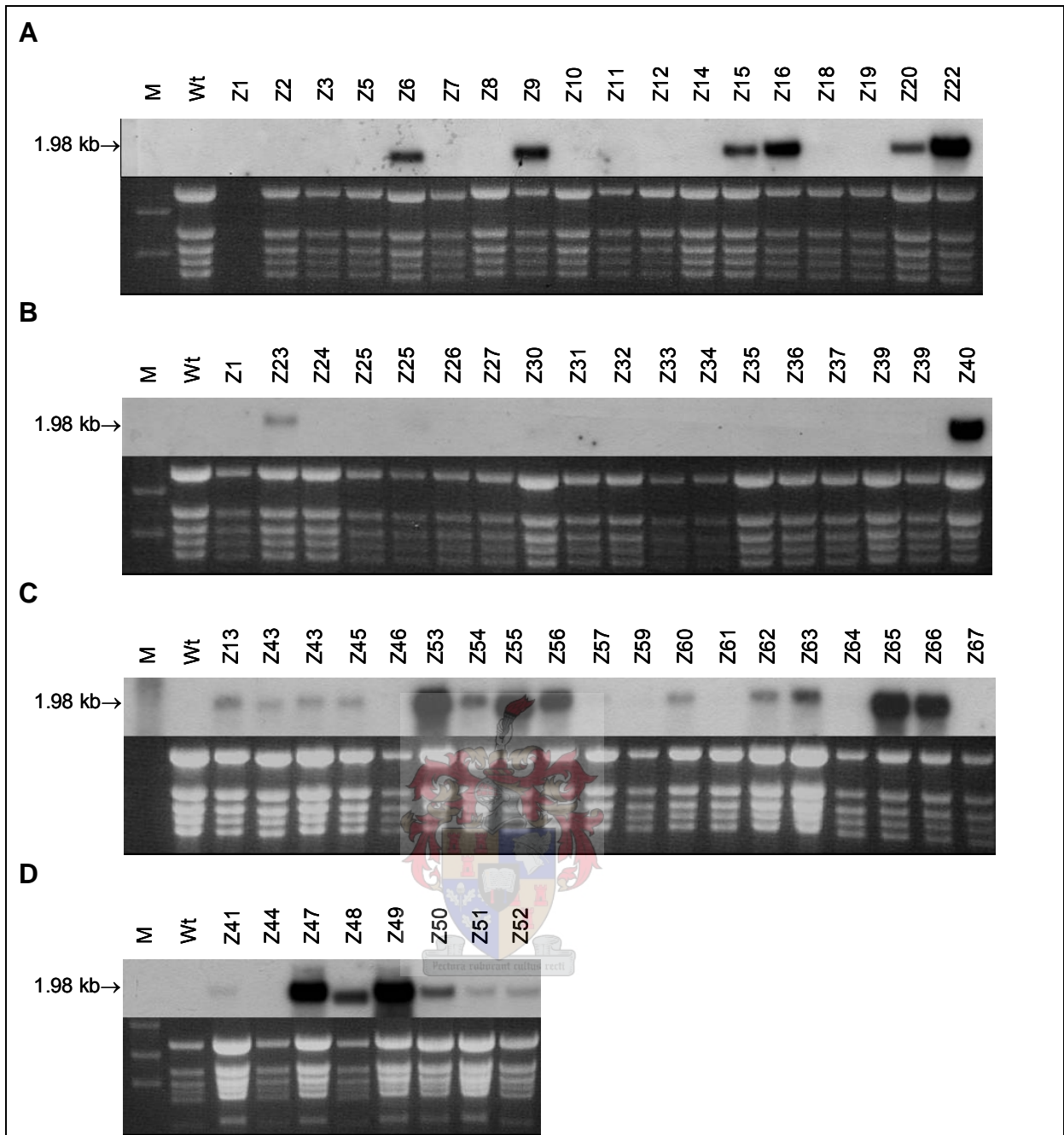


Figure 3. Northern blot analyses (top) conducted on the total RNA isolated (bottom) from 67 putative transgenic plants over-expressing the zeaxanthin epoxidase gene from *V. vinifera* cv Pinotage (*VvZEP*). For A-D: lane M represents the RNA marker and lane Wt represents the wild type (untransformed *A. thaliana* control). The numbers in the subsequent lanes represents the transgenic *A. thaliana* plant lines transformed with the *VvZEP* (Z) gene.

Twenty four of the *VvBCH* lines and twenty seven *VvZEP* lines expressing the respective transgenes were subjected to Southern blot analysis.

4.3.3 Confirmation of transgene integration of the transgenic lines

Integration of the *VvBCH* gene into the *Arabidopsis* genome was verified in only 24 primary transformants that showed expression of this transgene. Genomic DNA isolated from putative *VvBCH* transgenic plantlets was digested with *EcoRV*, which

recognises one site within the pART-VvBCH cassette. Each hybridisation band therefore represented a copy of the gene integrated (Figure 4), when probed with the same ≈ 900 bp cDNA digoxigenin-labelled fragment corresponding to the coding region of the *V. vinifera* L. *VvBCH* gene. The independent lines showed between one to seven copies of the transgene integrated. No clonal copies between the *VvBCH* lines were observed, or a correlation between the copies of the transgene integrated and the expression levels thereof.

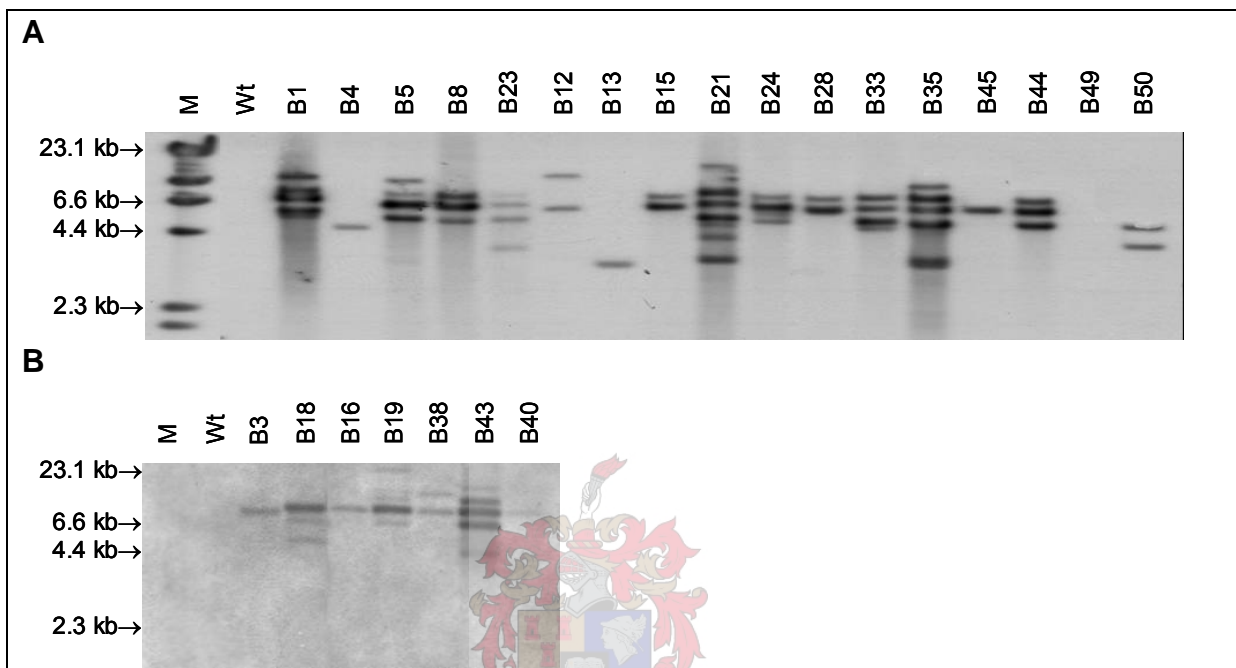


Figure 4: Southern blot analysis of genomic DNA, digested with *EcoRV* of independent *Arabidopsis* transgenic lines transformed with the β -carotene hydroxylase gene from *V. vinifera* L. cv Pinotage (*VvBCH*). For A-B: The λ (*HindIII*) marker (M), with fragment sizes indicated in kb, as well as the wild type (Wt, untransformed *A. thaliana* control). The numbers in the subsequent lanes represents the transgenic *A. thaliana* plant lines transformed with the *VvBCH* (B) gene.

Like the *VvBCH* lines, the integration of the *VvZEP* gene into the *A. thaliana* genome could be verified in only 26 Northern positive transgenic lines (Figure 5). Genomic DNA isolated from putative *VvZEP* transgenic plantlets was digested with either *StuI* or *EcoRV*. *StuI* recognises only one site, whereas *EcoRV* recognises two sites within the pART-*VvZEP* cassette. This resulted in a single hybridisation band of known size (1.9 kb) for *EcoRV* and each additional band representing a copy of the gene integrated (Figure 5). The *StuI* enzyme produces hybridisation bands for each of the copies integrated. Like the *VvBCH* lines, the *VvZEP* lines displayed integration of between one and five copies of the transgene. No clonal copies were observed for the *VvZEP* lines and there was no correlation between the number of copies integrated and the expression levels.

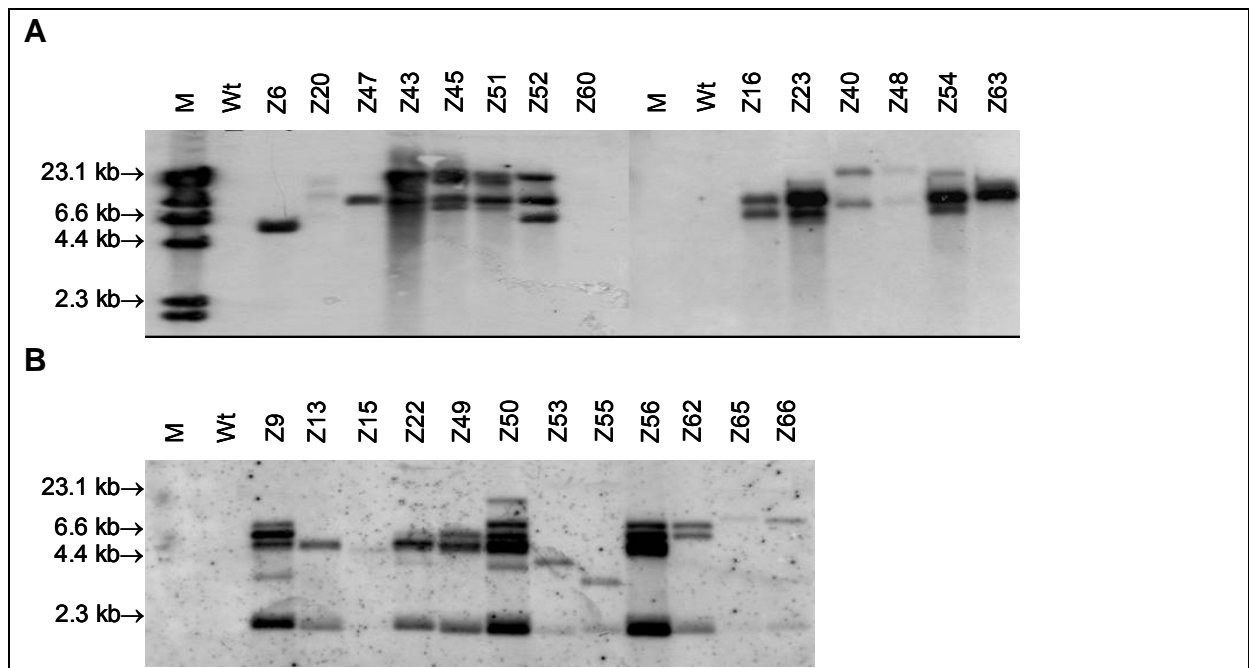


Figure 5: Southern blot analysis of genomic DNA, digested with (A) *Stul* and (B) *EcoRV* of independent *Arabidopsis* transgenic lines transformed with the zeaxanthin epoxidase gene form *V. vinifera* cv Pinotage (*VvZEP*). For A-B: The λ (*HindIII*) marker (M), with fragment sizes indicated in kb, as well as the wild type (Wt, untransformed *A. thaliana* control). The numbers in the subsequent lanes represents the transgenic *A. thaliana* plant lines transformed with the *VvZEP* (Z) gene.

4.3.4 Establishment of homozygous transgenic populations stably expressing the respective transgenes

The percentage germination of 100 to 150 seeds per line was scored after 10 days. All the 24 *VvBCH* and 26 *VvZEP* lines displayed germination rates between 75% to 100% germination. Fifteen lines for each of the transgenes showed 100% germination and these lines were selected as the homozygous population.

Ten representative plants from each of the homozygous lines for each of the transgenes being studied were used to determine if they stably maintained and expressed the transgene through each generation (Figures 6, 7 and 8). Interestingly, of the initial 15 homozygous Northern positive *VvBCH* lines, only 8 lines continued to express the gene in the T_3 -generation; and an additional 7 lines were found to produce an on-off expression pattern between plants within a single line or altered integration profile. Figure 6 illustrates a typical on-off expression and altered integration profile in a *VvZEP* and *VvBCH* line, respectively. Thus between the putative primary *VvBCH* transgenic line (T_1) and the subsequent T_3 generation, 16 lines were either silenced with respect to transgene expression or the integration pattern altered.

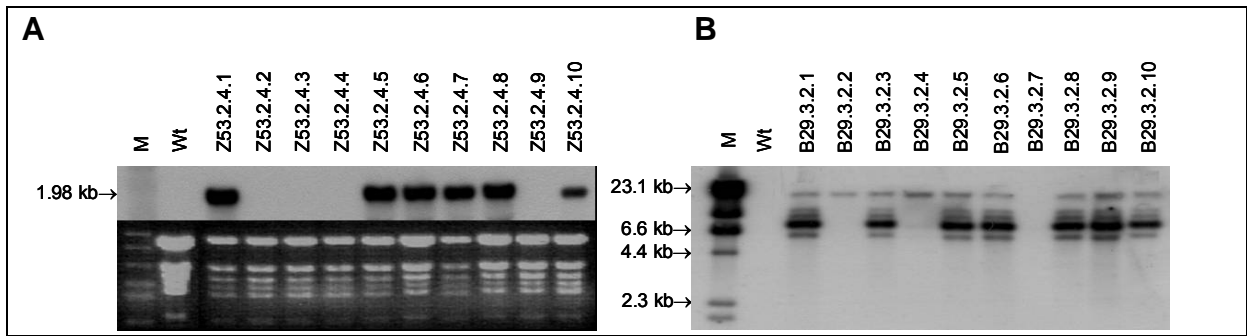


Figure 6. (A) Northern blot analyses (top) conducted on the total RNA isolated (bottom) from 10 plants from one independent *VvZEP* line, illustrating the on-off expression within this homozygous line and (B) Southern blot analysis of genomic DNA, digested with *EcoRV* of 10 plants of one independent *VvBCH* line, illustrating the altered integration profile within this homozygous line. For A-B: high range RNA ladder (M) (A) and λ (*HindIII*) marker (M) (B), with fragment sizes indicated in kb, as well as the wild type (Wt, untransformed *A. thaliana* control). The numbers in the subsequent lanes represents the transgenic *A. thaliana* plant lines.

Only 5 of the homozygous *VvBCH* lines stably maintaining and expressing the *VvBCH* gene were selected for further analyses (Figure 7), namely: B3.4.5, B13.4.3, B16.4.3, B23.5.2 and B45.10.1.

A similar silencing phenomenon was observed for the *VvZEP*-expressing lines. Of the initial 15 homozygous northern positive lines, only 8 lines continued to express the transgene, while 10 lines exhibited an on-off expression pattern or a different integration profile between plants within a single line as illustrated in Figure 6. Like the *VvBCH* lines, 19 lines were silenced between the putative primary transgenic lines (T_1) and the subsequent T_3 generation. Only 5 homozygous *VvZEP* lines stably maintaining and expressing the *VvZEP* gene were selected for further analyses (Figure 8), namely: Z40.8.1, Z47.4.1, Z49.8.1, Z55.4.1 and Z66.8.3.

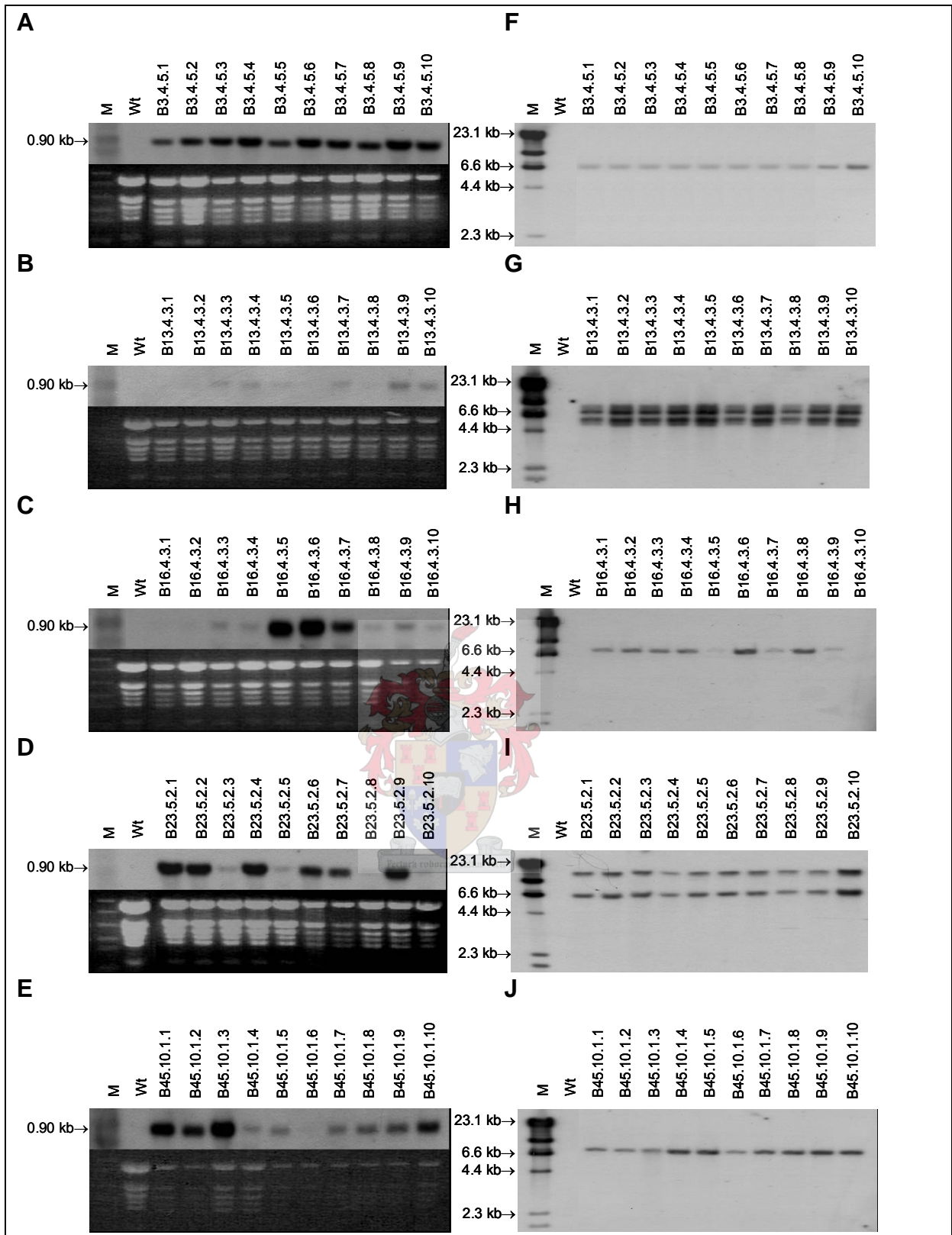


Figure 7. (A-E) Northern blot analyses (top) conducted on the total RNA isolated (bottom) and (F-J) southern blot analysis of genomic DNA, digested with *EcoRV* of populations of independent *Arabidopsis* transgenic lines transformed with the β -carotene hydroxylase gene form *V. vinifera* cv Pinotage (VvBch). For A-J: high range RNA ladder (M) (A-E) and λ (*HindIII*) marker (M) (F-J), with fragment sizes indicated in kb, as well as the wild type (Wt, untransformed *A. thaliana* control). The numbers in the subsequent lanes represents the transgenic *A. thaliana* population of a specific transgenic line transformed with the VvBCH (B) gene.

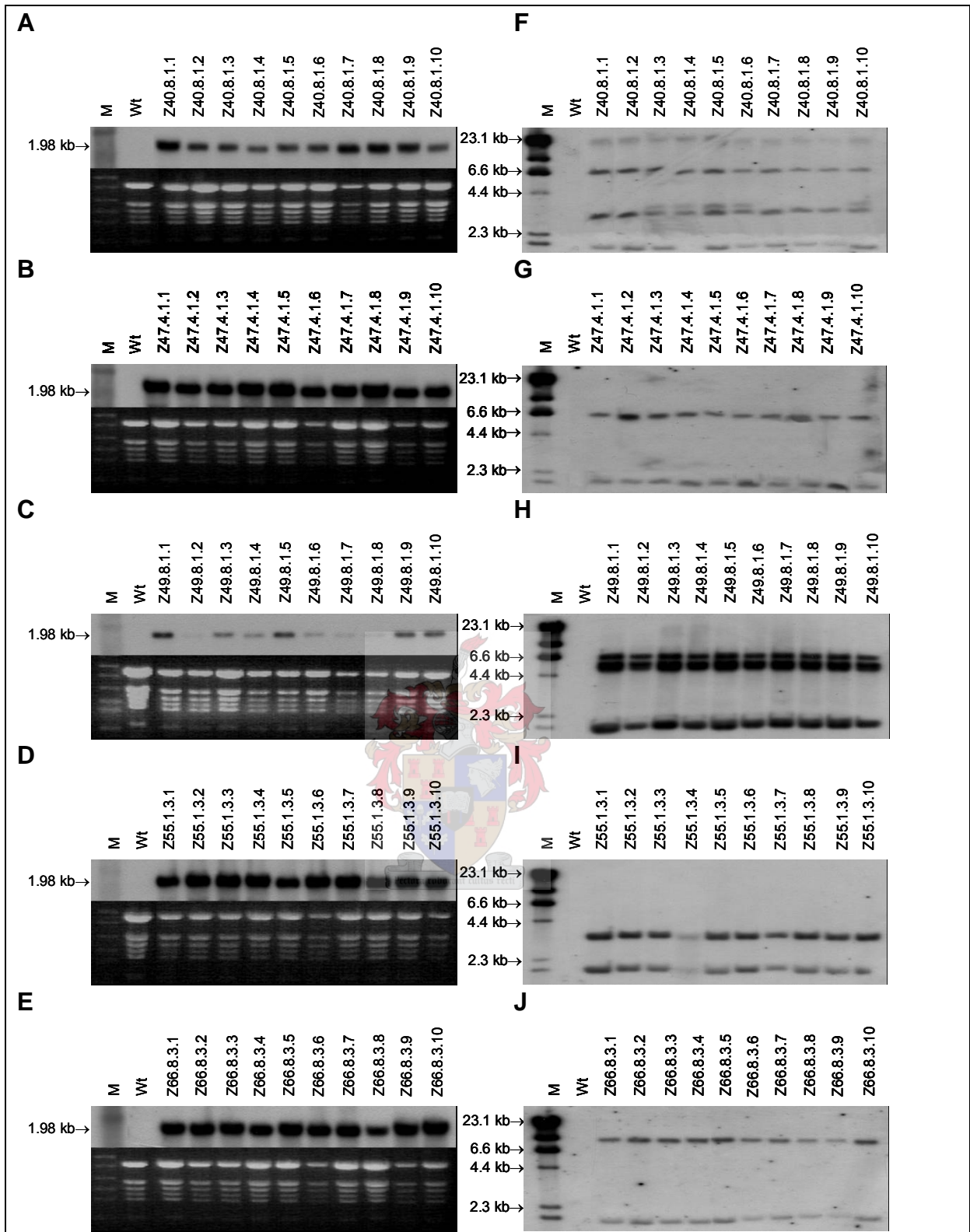


Figure 8. (A-E) Northern blot analyses (top) conducted on the total RNA isolated (bottom) and (F-J) southern blot analysis of genomic DNA, digested with *EcoRV* of populations of independent *Arabidopsis* transgenic lines transformed with the zeaxanthin epoxidase gene form *V. vinifera* cv Pinotage (*VvZEP*). For A-J: high range RNA ladder (M) (A-E) and λ (*HindIII*) marker (M) (F-J), with fragment sizes indicated in kb, as well as the wild type (Wt, untransformed *A. thaliana* control). The numbers in the subsequent lanes represents the transgenic *A. thaliana* population of a specific transgenic line transformed with the *VvZEP* (Z) gene.

4.3.5 Effect of gene expression on phenotype and growth

To assess if over-expression of *VvBCH* and *VvZEP* had any effect on the phenotype and growth of the transgenic lines, we examined the germination rate and over-all phenotype. All lines were maintained under controlled conditions. Germination of the homozygous seed progeny was analysed over 7 days (Figure 9).

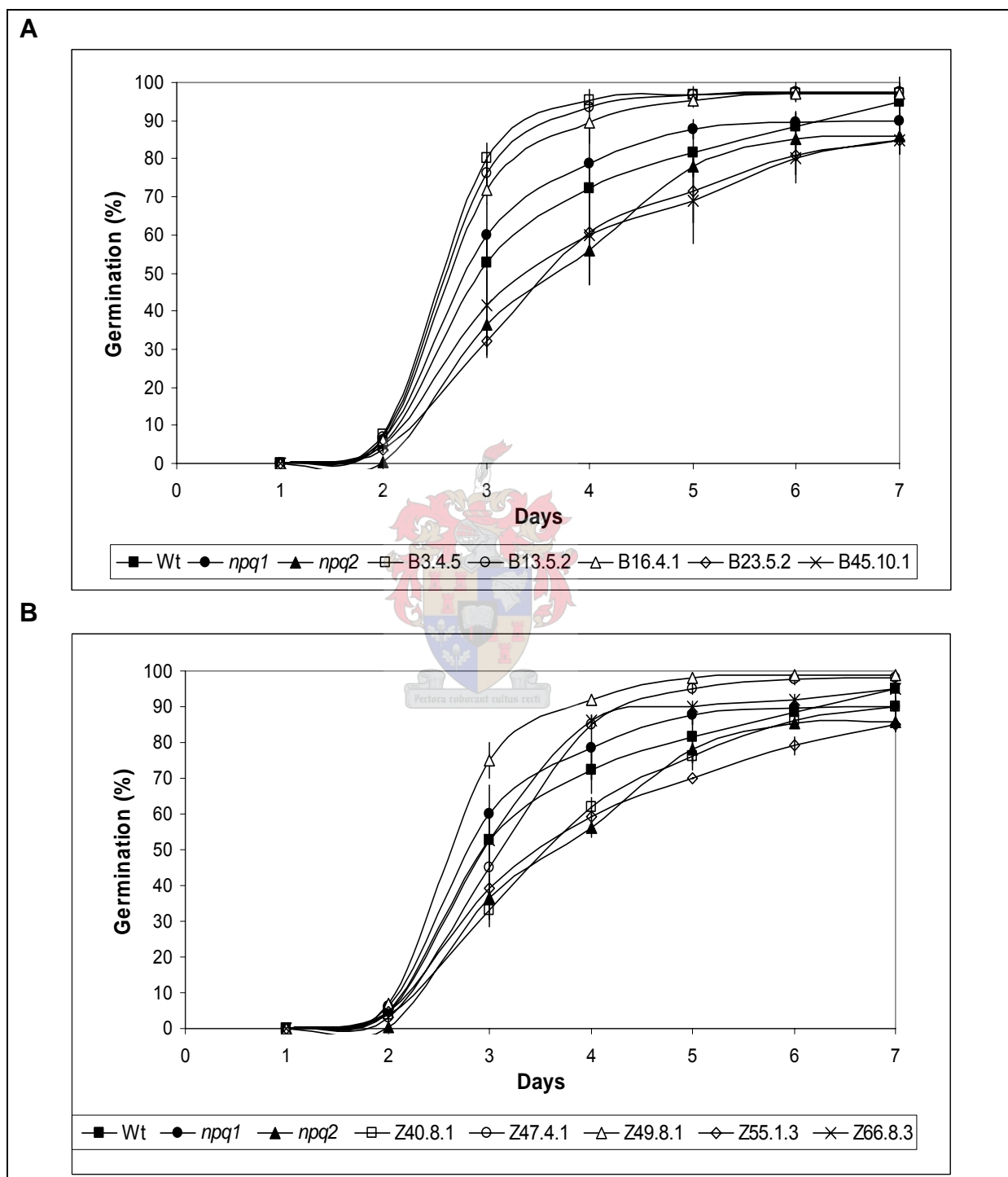


Figure 9. Percentage seed germination scored over 7 days for the wild type (Wt, untransformed *A. thaliana* control, *npq1* and *npq2* mutants and transgenic lines over-expressing the (A) β -carotene hydroxylase (*VvBCH*) gene and (B) zeaxanthin epoxidase (*VvZEP*) form *V. vinifera* L. cv Pinotage.

The VvBCH and VvZEP lines showed similar germination rates compared to the wild type and the *NPQ* mutants. There were small differences in the seed germination rates between individual lines over-expressing the *VvBCH* gene (Figure 9). The same was true for the *VvZEP* lines (Figure 9). Seed germination ranged from 85% to 95% after six days.

No obvious differences in the coloration, size or fertility of well-watered wild type, *npq1* and the respective transgenic lines could be observed (Figure 10). Leaves from the *npq2* mutant showed dark green leaf coloration, as well as an overall stunted and 'wilty' phenotype as has been previously described (Niyogi et al., 1998).

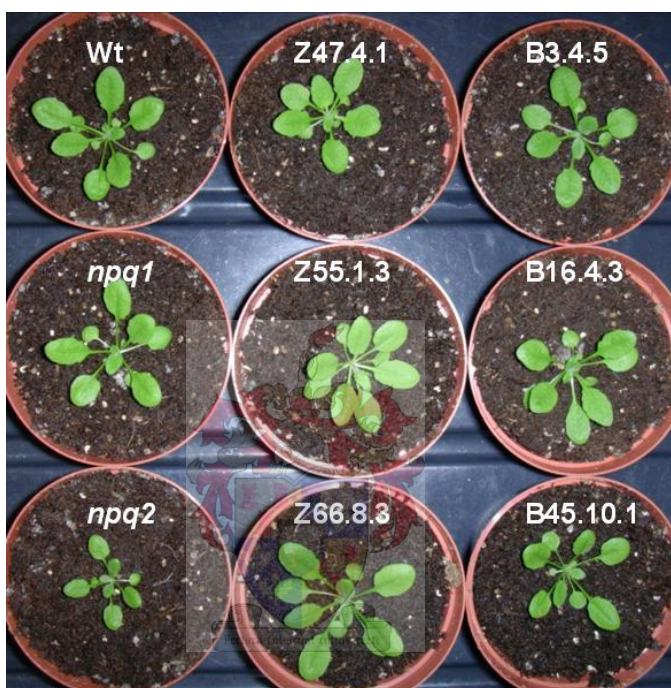


Figure 10. Morphological representation of some of the *A. thaliana* lines used during this study. A representative plant for each of the lines are shown; wild type (Wt, untransformed *A. thaliana* control), *npq1* and *npq2* mutants and the transgenic lines over-expressing the β -carotene hydroxylase (B3.4.5, B16.4.3 and B45.10.1) and zeaxanthin epoxidase (Z47.4.1, Z55.1.3 and Z66.8.3) genes from *V. vinifera* L. cv Pinotage.

4.3.6 Quantification of carotenoids and chlorophylls from leaves

The pigment concentrations for each line were determined on pooled leaf samples of the population of a line. The wild type, *npq1* and *npq2* samples came from single lines and thus had no replication. The five independent *VvBCH* and five independent *VvZEP* lines were used in these analyses. Pigment concentrations for these lines under both low and high light conditions are given in Tables 2 and 3 and were obtained from plants under both low and high light conditions.

There were no striking differences in the chl *a* and chl *b* concentrations between the transgenic lines and the wild type, except in the *npq1* mutant where the levels of both pigments were greater than the upper 95% limits of those in the *VvBCH*

lines under low light conditions. In all instances, exposing the leaves to high light caused a decrease in chl *a* and chl *b* concentrations (Table 2). Across all the control and transgenic lines, this effect of high light was significant at $P=0.008$ (chl *a*) and $P=0.05$ (chl *b*). The chl *a*:*b* ratio was between 2.1 and 2.3 and did not differ between the lines.

Table 2. HPLC analysis of pigment¹ composition of the *Wt*, *npq1* and *npq2* mutants and the transgenic lines over-expressing the zeaxanthin epoxidase (*VvZEP*) and the β -carotene hydroxylase (*VvBCH*) genes from *V. vinifera* L. cv. Pinotage, before (low light, LL) and after exposure to high light (HL) for 30 min.

Line	Chl <i>a</i> ¹		Chl <i>b</i> ¹	
	LL ²	HL ²	LL ²	HL ²
Wt	12.1	9.2	5.4	4.4
<i>npq1</i>	13.3	12.3	6.0	5.3
<i>npq2</i>	11.3	8.1	5.0	3.5
<i>VvZEP</i>	12.1 (10.8-13.4)	10.5 (9.3-11.8)	5.4 (4.7-6.1)	4.8 (4.0-5.5)
<i>VvBCH</i>	11.1 (9.3-12.8)	9.9 (7.7-12.2)	4.8 (3.8-5.9)	4.7 (3.8-5.6)

¹ Chlorophyll *a* and *b* is expressed in $\mu\text{g mg}^{-1}$ dried weight (DW). The data represent the mean of three extractions.

² Two leaves of six plants per line were pooled before (LL, $43 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) and after exposure to high light (HL, $\approx 2100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) for 30 min. Brackets indicate the 95% confidence interval

A change in the partitioning of the respective xanthophyll cycle pigments, comprising violaxanthin, antheraxanthin and zeaxanthin was observed under low light conditions. This was reflected in the entire xanthophyll cycle pool being partitioned to violaxanthin (Table 3). In none of the control and the transgenic lines antheraxanthin was observed, and only the *npq2* mutant accumulated zeaxanthin, at low light. High light induced the formation of zeaxanthin in the wild type, *npq2* mutant and the transgenic lines except the violaxanthin de-epoxidase deficient mutant *npq1*. Only the *npq1* and *npq2* mutants were unable to form antheraxanthin under high light.

Table 3. HPLC analysis of carotenoid content of the wild type (Wt), *npq1* and *npq2* mutants and the transgenic lines over-expressing the zeaxanthin epoxidase (*VvZEP*) and the β -carotene hydroxylase (*VvBCH*) genes from *V. vinifera* L. cv Pinotage, before (low light, LL) and after exposure to high light (HL) for 30 min.

Line	Lutein ¹		β -carotene ¹		Neoxanthin ¹		Violaxanthin ¹		Antheraxanthin ¹		Zeaxanthin ¹	
	LL ²	HL ²	LL ²	HL ²	LL ²	HL ²	LL ²	HL ²	LL ²	HL ²	LL ²	HL ²
Wt	211	234	194	239	39	41	53	19	0	12	0	24
<i>npq1</i>	212	217	193	179	43	40	55	52	0	0	0	0
<i>npq2</i>	168	170	207	196	0	0	0	0	0	0	167	152
<i>VvZEP</i>	211 (201-220)	214 (206-221)	184 (175-193)	172 (163-182)	39 (35-44)	38 (34-42)	48 (44-52)	21 (17-24)	0	8 (6-10)	0	16 (8-23)
<i>VvBCH</i>	208 (183-234)	225 (185-265)	166 (148-184)	157 (126-188)	42 (38-47)	45 (39-50)	79 (54-104)	26 (22-30)	0	15 (9-21)	0	41 (24-58)

¹ Pigments are expressed relative to chlorophyll a ($\text{mmol mol}^{-1} \text{chl a}$). The data represent the mean of three extractions.

² Two rosette leaves of six plants per line were pooled before (LL, $43 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) and after exposure to high light (HL, $\approx 2100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) for 30 min. Brackets indicate the 95% confidence interval.

The total xanthophyll cycle intermediate pool was not affected by the light treatment but there were conspicuous differences in the total pool size between the various lines. Under both low and high light, the mean total pool of xanthophyll cycle pigments was about 50% greater for the *VvBCH* lines, which over-expressed the β -carotene hydroxylase, than for the wild type, *npq1* mutant and the *VvZEP* lines. The *npq2* mutant had the largest pool of xanthophyll pigments, in the form of zeaxanthin. The levels were twice those produced in the *VvBCH* lines. In *npq2* there was no zeaxanthin epoxidation and no violaxanthin and hence no possible ABA formation (for this reason the *npq2* mutant is sometimes referred to as the “ABA mutant”). Similarly, the *npq2* mutant did not have any neoxanthin, which is also formed from violaxanthin. The wild type, *npq1* mutant and the transgenic lines had similar concentrations of neoxanthin, which were unaffected by the level of light. The *VvBCH* lines had a low β -carotene concentration compared with the wild type, *npq1* and *npq2* mutants, reflecting an over-expression of β -carotene hydroxylase in *VvBCH*.

All the above differences in carotenoid pigment concentrations in the various lines, and on the different effects of high light on the pigment concentrations between lines, match those expected from the genetic difference (addition of the exogenous genes) between the lines. What was surprising is that the enhanced production of xanthophyll cycle pigments in the *VvBCH* lines was not accompanied by a lower lutein concentration compared to the other lines, since over-expression of the β -carotene hydroxylase might be expected to divert lycopene metabolism toward the formation of xanthophyll cycle carotenoids at the expense of lutein synthesis. The low lutein concentration of the *npq2* mutant (about 20% lower than all the other lines and considerably below the lower 95% confidence limits of the *VvBCH* and *VvZEP* lines) was also surprising, since accumulation of zeaxanthin in *npq2* might be expected to cause accumulation of β -carotene and divert carbon flow towards lutein at the lycopene bifurcation.

4.3.7 Fluorescence characteristics during illumination of the transgenic lines

The relationship between the chlorophyll fluorescence parameters and photosynthetically active radiation (PAR) is shown in Figures 11 to 15, and a between-line comparison of the parameters given in Table 4.

The most striking differences in the chlorophyll fluorescence parameters involved *npq2* and *npq1* mutants as well as the *VvZEP* lines. The *VvBCH* lines did not differ from wild type for any of the parameters.

The *npq2* mutant had a significantly lower maximum quantum yield (dark-adapted F_v/F_m) than the other lines (Table 4). In fact, it was the only line where maximum quantum yield differed significantly from wild type. The reason for the lower F_v/F_m in *npq2* was a smaller F_m , and hence smaller F_v ; F_0 did not differ from the Wild type. This suggests inactivation of the PS II reaction centers by non-radiative dissipation processes and, in fact, non-photochemical quenching at the lowest light

level tested (NPQ_{21}) was nearly four times greater for *npq2* than for any of the other lines, accordant with the high constitutive levels of zeaxanthin found in *npq2* in the dark. As PAR increased, NPQ of all the lines increased but at about $150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR it increased less steeply for *npq2* than for wild type (Figure 11), leading to the situation where at high light the difference between the two lines was not significant. The other striking feature of the NPQ :PAR curves was that for both the *npq1* mutant and the *VvZEP* lines, NPQ increased much less steeply as PAR increased up to about $500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ than it did for the other genotypes. Hence, the level of NPQ induced by light was considerably lower for the *npq1* mutant and the *VvZEP* lines than that observed in the other three lines (Figure 11).

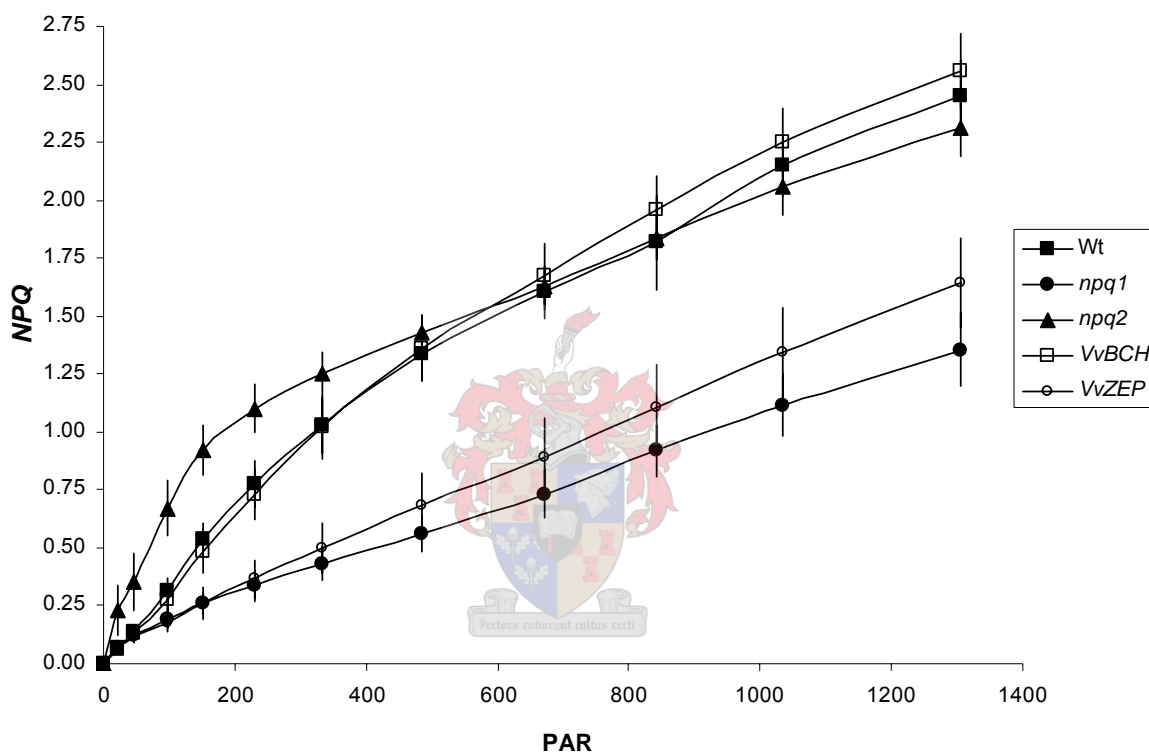


Figure 11. Non-photochemical quenching (NPQ) induction followed over a range of PAR values for the wild type (Wt, untransformed *A. thaliana* control), *npq1* and *npq2* mutants and the transgenic lines over-expressing the β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*) genes from *V. vinifera* L. cv Pinotage.

The effective quantum yield of PS II photochemistry (the quantum yield in the light, Φ_{PSII21} and $\Phi_{PSII1305}$) was also lower for the *npq2* mutant than for wild type and this translated into significantly lower relative electron transport rates (Figure 12 and 13). For instance, relative ETR_{1305} of the *npq2* mutant was only 66% of that of wild type (Table 4). Photochemical quenching (qP , an indication of the proportion of open reaction centers) and the quantum yield of open reaction centers ($\Phi_{PSII}/qP = F'_v/F'_m$) were both also lower for the *npq2* mutant than for wild type (Figures 14 and 15).

The difference in chlorophyll fluorescence parameters between the lines is best illustrated by the differences in the elevation of the light curves for the different

lines at moderately low PAR values, between about 200 and 500 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Figures 11-15). In this PAR range, for all the parameters the curves for the *npq1* mutant and the *VvZEP* lines were at similar elevations, which were different to the wild type and the *VvBCH* lines. For Φ_{PSII} and ETR the elevation of the curve for the *npq2* mutant was similar to the *npq1* mutant and the *VvZEP* lines. For *qP* it was similar to wild type and the *VvBCH* lines. The elevation of the *NPQ:PAR* curve for the *npq2* mutant was higher, and that of the F'_v/F'_m curve lower, than for all the other lines. These differences are illustrated by a comparison of fluorescence parameter values at 332 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR (Table 4). The wild type and the *VvBCH* lines group together, with similar values for all the parameters. The *VvZEP* lines and the *npq1* and *npq2* mutants all have lower Φ_{PSII} than the wild type/*VvBCH* group. The *VvZEP* lines and the *npq1* mutant have lower *qP* and *NPQ*, but a higher F'_v/F'_m than both the wild type and the *VvBCH* lines. The *npq2* mutant has a similar *qP*, but higher *NPQ* and lower F'_v/F'_m than the wild type and the *VvBCH* group. A similar grouping is shown by the fast and slow components of *NPQ* relaxation in the dark after the measurement at 1305 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR. For the *npq1* mutant and the *VvZEP* lines a considerably greater proportion of the *NPQ* was due to slowly-relaxing quenching than was the case for the wild type, *npq2* mutant or the *VvBCH* lines.

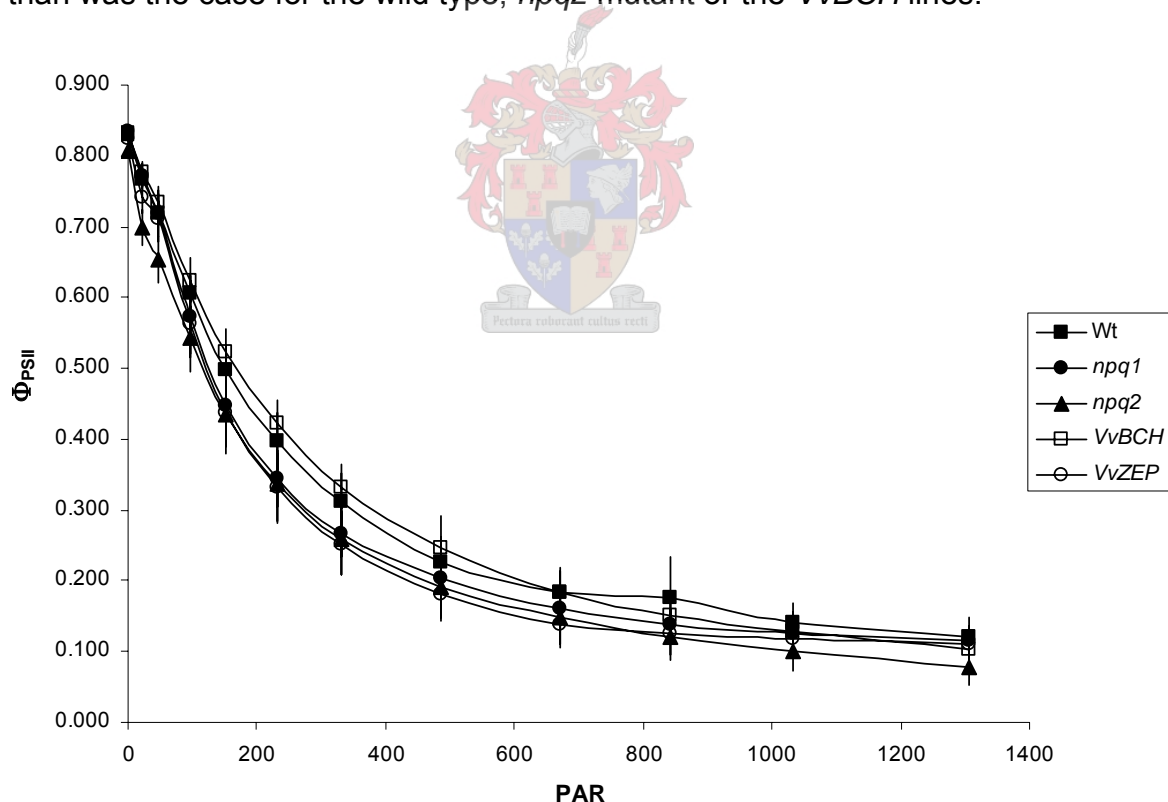


Figure 12. Quantum yield of PS II (Φ_{PSII}) followed over a range of PAR values for the wild type (Wt, untransformed *A. thaliana* control), *npq1* and *npq2* mutants and the transgenic lines over-expressing the β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*) genes from *V. vinifera* L. cv Pinotage.

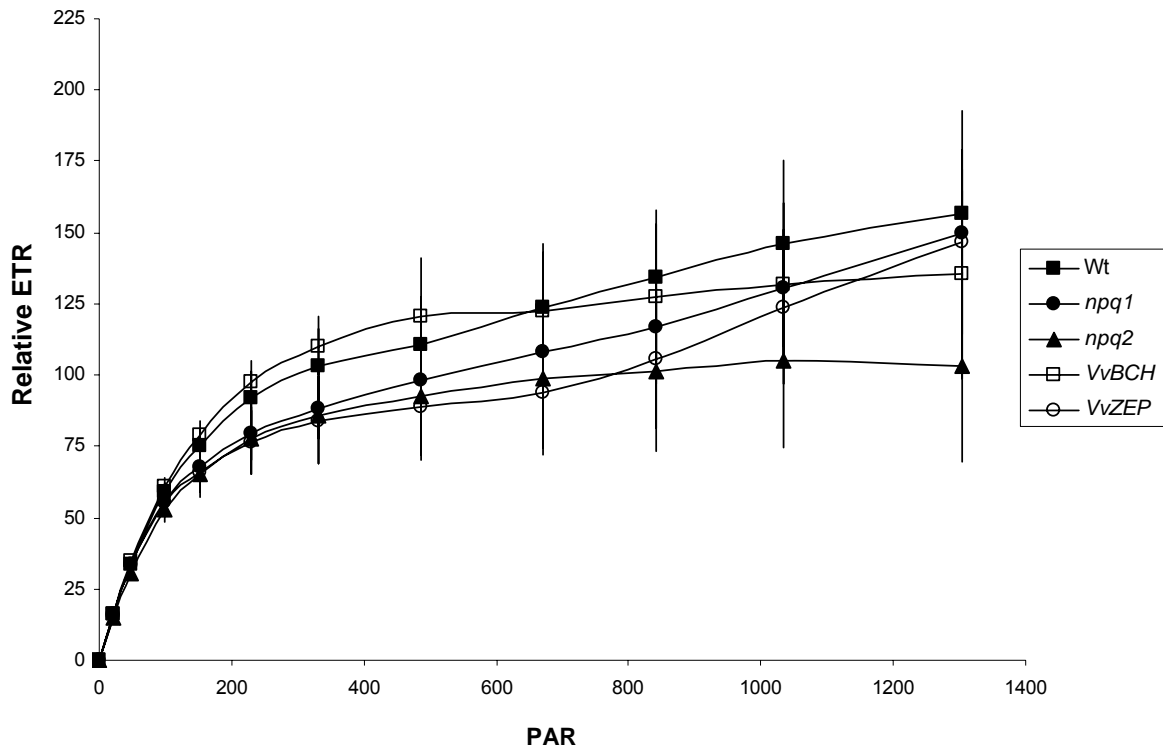


Figure 13. Relative electron transport (*ETR*) followed over a range of PAR values for the wild type (Wt, untransformed *A. thaliana* control), *npq1* and *npq2* mutants and the transgenic lines over-expressing the β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*) genes from *V. vinifera* L. cv Pinotage.

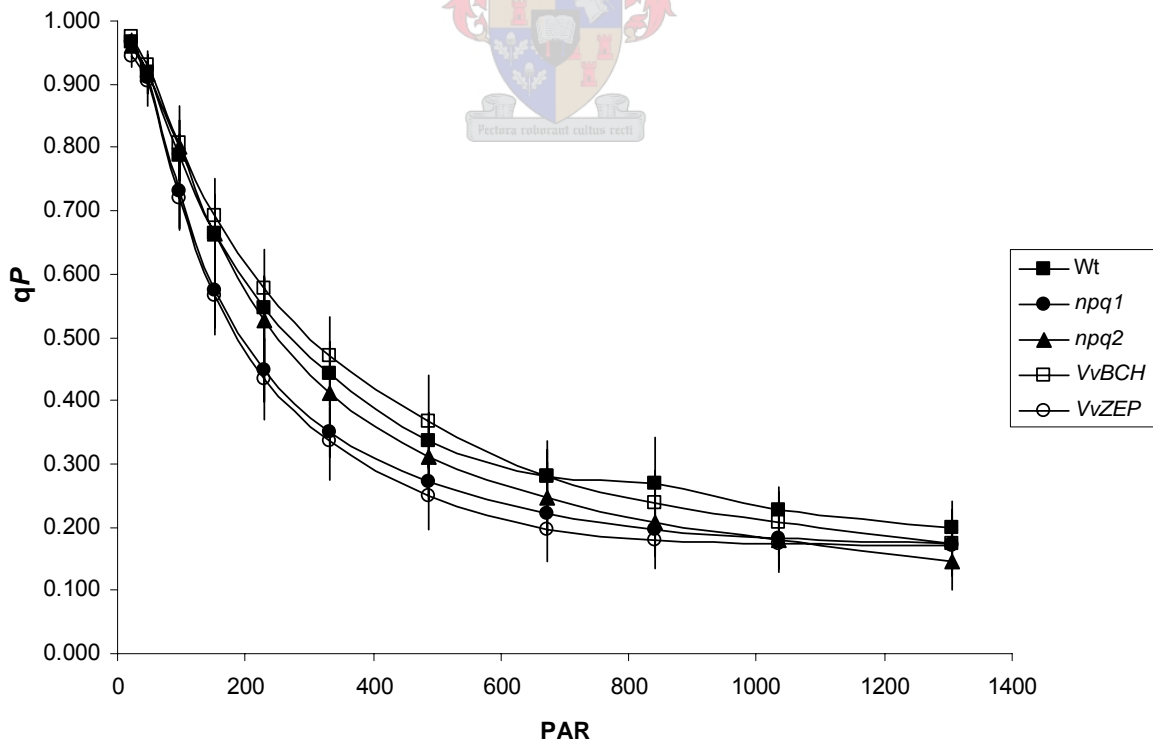


Figure 14. Photochemical quenching (*qP*) followed over a range of PAR values for the wild type (Wt, untransformed *A. thaliana* control), *npq1* and *npq2* mutants and the transgenic lines over-expressing the β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*) genes from *V. vinifera* L. cv Pinotage.

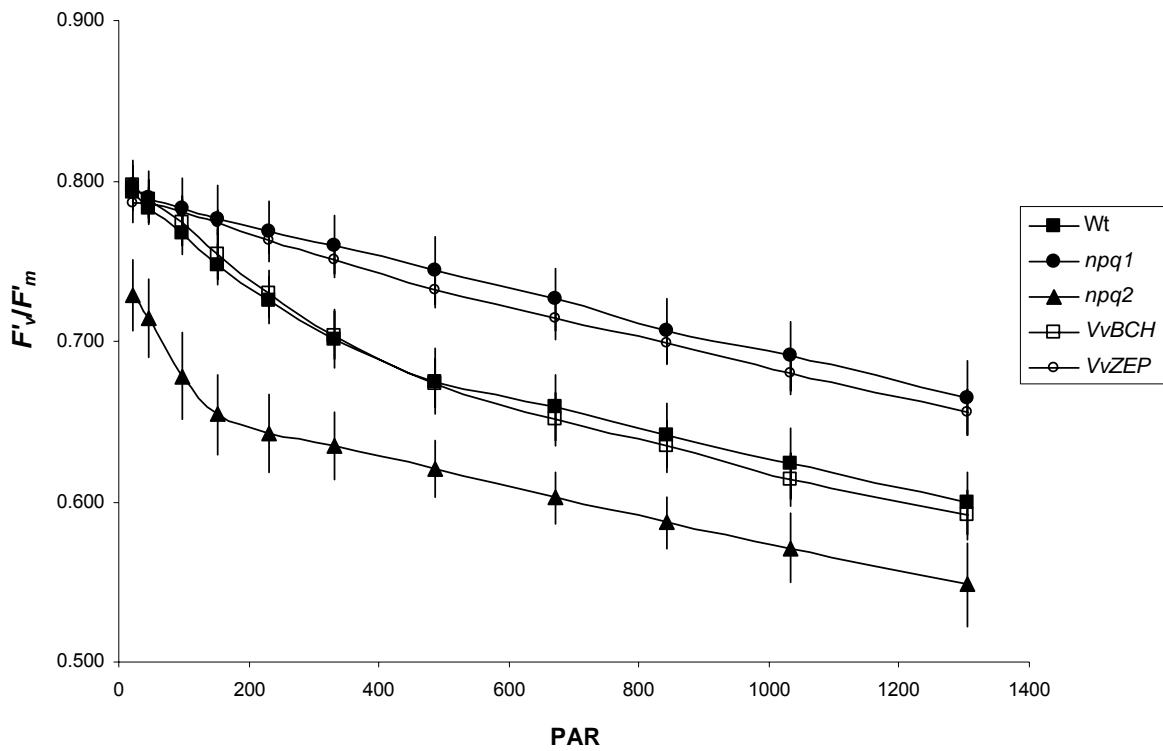


Figure 15. Efficiency of open reaction centers (F_v/F_m) followed over a range of PAR values for the wild type (Wt, untransformed *A. thaliana* control), *npq1* and *npq2* mutants and the transgenic lines over-expressing the β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*) genes from *V. vinifera* L. cv Pinotage.

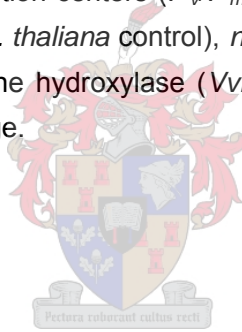


Table 4: The fluorescence characteristics of the wild type (Wt, untransformed *A. thaliana* control), *npq1* and *npq2* mutants and the transgenic lines over-expressing the β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*) from *V. vinifera* L. cv. Pinotage.

Line	F_v/F_m	$\Phi_{PSII1305}$	qP_{1305}	F'_v/F'_{m1305}	NPQ_{21}	NPQ_{1305}	Rel ETR ₁₃₀₅	$\Phi_{PSII332}$	qP_{332}	F'_v/F'_{m332}	NPQ_{332}	NPQ_S	NPQ_F
Wt	^{ab} 0.831 ±0.002	^a 0.120 ±0.009	^a 0.199 ±0.013	^b 0.600 ±0.006	^a 0.064 ±0.005	^{ab} 2.49 ±0.06	^a 157 ±11	^{bc} 0.315 ±0.013	^a 0.448 ±0.016	^b 0.702 ±0.006	^b 1.039 ±0.039	^a 29 ±1	^a 71 ±1
<i>npq1</i>	^a 0.835 ±0.002	^a 0.115 ±0.004	^{ab} 0.174 ±0.006	^c 0.660 ±0.009	^a 0.064 ±0.004	^c 1.39 ±0.06	^a 150 ±5	^{ab} 0.270 ±0.011	^{bc} 0.357 ±0.014	^c 0.757 ±0.006	^a 0.444 ±0.027	^c 67 ±2	^c 33 ±2
<i>npq2</i>	^b 0.805 ±0.003	^b 0.079 ±0.008	^b 0.145 ±0.014	^a 0.548 ±0.008	^b 0.231 ±0.034	^b 2.27 ±0.05	^b 104 ±11	^a 0.259 ±0.016	^a 0.406 ±0.022	^a 0.634 ±0.007	^c 1.233 ±0.034	^a 29 ±2	^a 71 ±2
<i>VvZEP</i>	^c 0.832 ±0.001	^{ab} 0.110 ±0.004	^{ab} 0.168 ±0.007	^c 0.657 ±0.003	^a 0.068 ±0.004	^c 1.63 ±0.04	^{ab} 144 ±6	^a 0.251 ±0.008	^c 0.335 ±0.011	^c 0.751 ±0.002	^a 0.497 ±0.025	^b 56 ±1	^b 44 ±1
<i>VvBCH</i>	^a 0.825 ±0.001	^{ab} 0.103 ±0.005	^{ab} 0.174 ±0.008	^b 0.589 ±0.004	^a 0.068 ±0.003	^a 2.59 ±0.04	^{ab} 134 ±7	^c 0.331 ±0.007	^a 0.472 ±0.008	^b 0.701 ±0.004	^b 1.041 ±0.032	^a 28 ±2	^a 72 ±2

Superscript indicates the significance at $p < 0.05$, while subscript indicates the PAR value.

4.4 DISCUSSION

Several transgenic *Arabidopsis* lines overexpressing the β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*) genes from *V. vinifera* were generated. In addition to the untransformed wild type (Wt), two *NPQ* mutants, *npq1* and *npq2* were used as controls throughout this study. The integration and expression of the transgenes in these primary transgenic lines revealed that one to several copies of the transgenes were integrated and that the transgene expression differed considerably between these lines. Furthermore, only 16% and 12% of the initial primary transformants were successfully propagated to a homozygous T3-generation for the *VvBCH* and *VvZEP* lines, respectively. The constitutive over-expression of these transgenes did not however, alter plant growth or vigour of the primary transgenic plants and the resulting homozygous T3-generation. As reported previously, one *Arabidopsis* mutant control line, *npq2*, showed a stunted and 'wilty' phenotype in accordance with previous reports (Marin et al., 1996). In contrast to previous experiments using over-expression of the *VvZEP* gene in *N. plumbaginifolia* (Frey et al., 1999) no delayed seed germination could be detected in the *VvZEP* lines. These dissimilarities could possibly be related to species-specific differences between the model plant systems used or different growth conditions.

The pigment concentrations observed for the *NPQ* mutant control lines were in accordance with previous results which show the *npq2* mutant, defective in the epoxidation of zeaxanthin to violaxanthin, accumulated high levels of zeaxanthin and no epoxy-xanthophylls (Duckham et al., 1991; Rock and Zeevaart, 1991; Niyogi et al., 1998), while the *npq1* mutant (defective in violaxanthin de-epoxidase) was unable to de-epoxidase violaxanthin to zeaxanthin under high light conditions (Niyogi et al., 1998). The missing epoxy-xanthophyll pigments in the *npq2* mutant appear to be replaced by an equimolar increase in zeaxanthin, such that there was little to no variation in total carotenoid content per mole of chlorophyll *a* between the wild type and *npq2*. The *npq2* mutant had the largest pool of xanthophyll pigments in the form of zeaxanthin, about twice that of *VvBCH* lines. This suggests that in the lines where zeaxanthin epoxidation occurs, some of the violaxanthin is further metabolised to other, non-xanthophyll cycle compounds. The most probable one is ABA, which is formed from violaxanthin. The *VvZEP* lines, similar to the *npq1* mutant control line, had similar violaxanthin concentrations to the wild type under low light conditions. However, the *VvZEP* lines were able to convert violaxanthin to zeaxanthin under high light conditions, but not to the same levels as the wild type. This could indicate that the epoxidation reaction (catalysed by *VvZEP*) is outstripping the de-epoxidation reaction under high light conditions. The *VvBCH* lines showed a marked increase in the xanthophyll cycle pool both under low and high light conditions, compared to the wild type. Furthermore, the β -carotene concentrations in these lines were also

decreased compared to the wild type, indicating elevated β -carotene hydroxylase activity possibly due to over-expression of the *VvBCH* gene.

The wild type and *VvBCH* lines had almost identical efficiencies of PS II photochemistry, degree of PS II reaction center openness and magnitude of non-photochemical dissipation of excitation energy. *VvZEP*, *npq1* and *npq2* mutants had lower PS II efficiency in the light than did Wt and *VvBCH* lines. In the *VvZEP* lines and *npq1* mutant this was associated with a lower proportion of open reaction centers than in the wild type, whereas the *npq2* mutant maintained the same degree of reaction center openness as the wild type (Figure 14). The lack of zeaxanthin epoxidase activity in the *npq2* mutant translated into high constitutive levels of zeaxanthin (the concentration in the dark). Similarly to previous reports (Niyogi et al., 1998), high levels of zeaxanthin resulted in higher *NPQ* in the *npq2* mutant compared to the wild type in low and moderate light. However this difference disappeared at high light, where zeaxanthin formation induced considerable *NPQ* in the wild type. In contrast, the *npq1* mutant and the *VvZEP* lines showed considerably lower *NPQ* across the whole PAR range. This corresponds well with previous results obtained for the *npq1* mutant (Niyogi et al., 1998) and transgenic tobacco lines (violaxanthin de-epoxidase antisense suppression) (Verhoeven et al., 2001). The impaired *NPQ* observed in the *npq1* mutant and *VvZEP* lines was the reason for the greater degree of reaction center closure (less RC openness) in the light. The slow relaxing component of *NPQ* was much greater for the *npq1* mutant and *VvZEP* lines than for the wild type, *npq2* mutant and *VvBCH* lines, suggesting that the lowered photochemical efficiency and RC openness in the *npq1* mutant and *VvZEP* lines was caused by photodamage of the PS II reaction centers, rather than photoprotection mechanisms such as high energy state quenching or state transitions between PS I and PS II. In contrast, the contributions of fast-relaxing processes to *NPQ* were practically identical in the wild type, *npq2* mutant and *VvBCH* lines, and were considerably higher than for the *npq1* mutant or *VvZEP* lines, suggesting that photoprotective, rather than photoinhibitory mechanisms were the major components of non-photochemical energy dissipation in the wild type, *npq2* mutant and the *VvBCH* lines. Since all three lines were able to build up substantial pools of zeaxanthin in the light this suggests that xanthophyll cycling was an important component of photoprotection. Interestingly, although slow-relaxing processes (photoinhibition) contributed most of the *NPQ* in the *npq1* mutant and *VvZEP* lines, there was a significant difference between the two lines in the degree to which it contributed. For the *npq1* mutant, where no zeaxanthin was produced even at high light, slow-relaxing quenching was a significantly greater component of *NPQ* than in the case of the *VvZEP* lines, where zeaxanthin formation did occur in the light but where the zeaxanthin pool was kept low due to rapid zeaxanthin epoxidation.

In conclusion, we have successfully over-expressed two carotenoid biosynthetic genes in transgenic *A. thaliana*. The over-expression of these genes leads to significant alterations in the contribution of the individual carotenoids to the

total carotenoid as well as xanthophyll cycle pool. These changes, specifically the increased xanthophyll pool in the *VvBCH* lines, did not offer additional photoprotection to the LHC's or RC's. However, the reduced zeaxanthin levels in the *VvZEP* lines resulted in increased photoinhibition. These results are consistent with previous experiments involving the xanthophyll cycle (Niyogi et al., 1998; Verhoeven et al., 2001) and provide valuable fundamental knowledge for genetic engineering involving carotenoid metabolism. Further experiments, including hormone profiling and lipid peroxidation of these transgenic lines could provide additional information regarding the biotechnological importance for the enhancement of plant responses to abiotic stresses.

4.5 ACKNOWLEDGEMENTS

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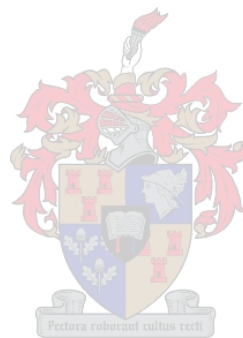
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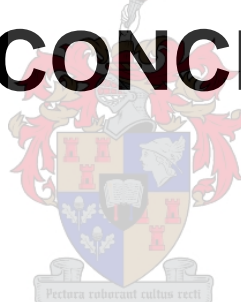
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GENERAL DISCUSSION AND CONCLUSION



5.1 GENERAL DISCUSSION AND CONCLUSION

Excess light can damage the photosynthetic apparatus and can lead to a decrease in photosynthetic capacity and ultimately to photoinhibition. For this reason, plants have developed a number of photoprotective mechanisms of which, the dissipation of excitation energy non-radioactively as heat is possibly the most effective. This mechanism involves specific carotenoids namely, the xanthophyll cycle pigments, and can be quantified as non-photochemical quenching (*NPQ*) of chlorophyll fluorescence. *Arabidopsis* and *Chlamydomonas* mutants, with altered *NPQ* function, have been isolated and have provided essential information regarding the role of the xanthophyll cycle and its enzymes in *NPQ* (Marin et al., 1996; Niyogi et al., 1997; Bugos et al., 1998; Niyogi et al., 1998). Due to the importance of the xanthophyll cycle as a photoprotective mechanism, it is a promising target for genetic engineering to enhance stress tolerance.

In this study the *A. thaliana* model system was used to evaluate the *in planta* physiological effect(s) and potential biotechnological application of two carotenoid biosynthetic genes, β -carotene hydroxylase (*VvBCH*) and zeaxanthin epoxidase (*VvZEP*), isolated previously in our laboratory from *Vitis vinifera* L. (Young, 2004). *A. thaliana* ecotype Columbia 0 plantlets were transformed with either the *VvBCH* or *VvZEP* gene under the control of the strong constitutive cauliflower mosaic virus promoter. In addition to the untransformed wild type, two *NPQ* mutants, *npq1* (defective in violaxanthin de-epoxidase) and *npq2* (defective in zeaxanthin epoxidase), were used as controls throughout this study. These mutants have been well described (Niyogi et al., 1998) according to their pigment profiles, their capacity for *NPQ* induction and relaxation and subsequently their photosynthetic capabilities under ambient and high light conditions. Putative positive transgenic plantlets were generated and propagated to a homozygous T3-population. Of the initial putative transgenic plants obtained stable integration and expression was shown in only 16% and 12% of the *VvBCH* and *VvZEP* lines, respectively. The over-expression of these genes did not result in obvious phenotypical differences when compared to the wild type, however the *npq2* mutant confirmed previous findings and showed a stunted and 'wilty' phenotype (Marin et al., 1996; Niyogi et al., 1998). The transgenic lines, the untransformed control and the mutants used in this study provided us with relevant genetic material to answer two very basic questions: (i) does the over-expression of either of these genes alter the overall pigment content and individual pigment levels; and (ii) does the over-expression of these genes lead to any change in photosynthetic capacity and parameters under normal and stress conditions and can these physiological changes be correlated to any of the pigment changes in *Arabidopsis*?

One of the most important outcomes of this study was that a new high performance liquid chromatography (HPLC) method was developed for quantitative

profiling of the major carotenoids found in plant tissues, with emphasis placed on those of the xanthophyll cycle. The optimisations to this method also included evaluation of very basic aspects linked to authentic standard and sample preparation, handling and storage. A step-by-step protocol has thus been developed and described (Chapter 3) and has been shown to be particularly useful to profile and quantify eight carotenoids from green leafy material. To this end, a C30 column and binary mobile solvent system (97% methanol and water: 100% TBME) were used for a combination isocratic or gradient elution program. Eight of the major carotenoids as well as chlorophylls (*a* and *b*) were resolved and identified using an array of techniques with particular reference being made to the significant baseline separation of lutein and zeaxanthin as well as the *cis*- and *trans*-forms of violaxanthin and neoxanthin. This method was subsequently used to determine if the over-expression of the grapevine genes in *Arabidopsis* caused shifts in the pigment pools and individual pigment quantities; the method also corroborated previous results regarding the pigment changes in the *Arabidopsis* mutants. The pigment profiling results provided a very important framework to evaluate and correlate any possible physiological effects linked to the over-expression of the genes.

The carotenoid content observed for the *NPQ* mutant control lines used during this study was well in accordance with previous results (Niyogi et al., 1998). The *npq2* mutant demonstrated the inability to produce detectable amounts of the epoxy-xanthophylls, violaxanthin and neoxanthin, resulting in the accumulation of zeaxanthin. Furthermore, these high levels of zeaxanthin were found to cause an initial rapid induction of *NPQ* at low to moderate light intensities compared to that of the wild type. However, maximum *NPQ* reached in this mutant at high light was similar to that of the wild type. Similarly, the carotenoid content of the *npq1* mutant control line was comparable to that reported by Niyogi et al. (1998). This mutant was also found to be unable to convert violaxanthin to zeaxanthin under high light conditions. The absence of zeaxanthin in this mutant caused severe inhibition of *NPQ* over the whole PAR (photosynthetically active radiation) range and resulted in increased photoinhibition. Furthermore, the efficiency of photosystem II (PS II) photochemistry was shown to be lower compared to that of the wild type.

The over-expression of *VvBCH* resulted in an increased pool size of xanthophyll cycle pigments (violaxanthin, antheraxanthin and zeaxanthin) and reduced β -carotene levels under both high and low light conditions. This was expected due to the hydroxylation of β -carotene to zeaxanthin by the heightened levels of β -carotene hydroxylase. These differences in carotenoid content did not however affect the efficiency of PS II photochemistry or *NPQ* induction and relaxation, when plantlets were exposed to excess light energy. As with the induction of *NPQ* in the *npq2* mutant, the increased zeaxanthin levels in these transgenic lines did not offer any additional photoprotection. This was further illustrated by the similar contribution of the fast relaxing component of *NPQ* (NPQ_F , photoprotection via the xanthophyll pigment, zeaxanthin) observed in the *npq2* mutant and the *VvBCH* lines

compared to the wild type. In all of these lines photoprotection, rather than photoinhibition (slow relaxing component of *NPQ*) was the major component of *NPQ*. This would suggest that additional zeaxanthin does not necessarily enhance photoprotection, however it may protect the thylakoid membrane against lipid peroxidation. This hypothesis has been tested by Davison et al. (2002) where it was shown that increased zeaxanthin levels in transgenic *Arabidopsis* over-expressing the native β -carotene hydroxylase reduced lipid peroxidation under high light conditions.

The *VvZEP* over-expressing lines showed reduced levels of zeaxanthin in high light conditions, probably due to the competition between the epoxidation and de-epoxidation reactions. Like with the *npq1* mutant, the reduced levels of zeaxanthin resulted in significantly reduced *NPQ* compared to the wild type, a phenomenon also observed through antisense suppression of *VDE* in tobacco (Verhoeven et al., 2001). Furthermore, photoinhibition (*NPQ_S*) was found to be significantly higher in the *npq1* mutant and the *VvZEP* lines than the wild type. The enhanced photoinhibition together with a lowered photochemistry and enhanced reaction center closure suggests that a greater degree of photodamage occurred in these lines compared to the wild type. This was also true for the *npq1* mutant, however observed photoinhibition in the *VvZEP* lines appeared to take place to a lesser extent. This may suggest that the zeaxanthin present in these lines, even though it was low amounts, did at least offer some photoprotection.

In conclusion, the characterisation of these lines has provided valuable information regarding the physiological effects of the *VvBCH* and *VvZEP* gene products *in planta* in response to high light. These findings demonstrate that increased zeaxanthin levels (observed in the *VvBCH* lines) do not necessarily enhance a plants stress tolerance to high light and that even a small reduction in zeaxanthin amounts (observed in the *VvZEP* lines) can result in a significant increase in photoinhibition (photodamage). These results confirm the importance of the xanthophyll cycle in photoprotection. It also show that the carotenoid pathway is tightly regulated and that flux control is a key aspect when manipulation to this pathway is considered. To this regard, it is necessary to ensure that no gene product exhausts the main substrates in this pathway. Fundamental knowledge of the physiological effect(s) of single gene integrations is important to evaluate the various contributions of the pathway members, but it also shows that it may be necessary to manipulate more than one gene to achieve an optimal effect. Furthermore, it may be worthwhile to investigate other compounds that may be affected by over-expressing these genes. The carotenoid pathway is studied intensively by scientists from various fields and fundamental knowledge regarding the pathway genes and the roles of their encoded products, such as was generated in this study, will ultimately define the biotechnological and other applications that can be derived from this central metabolic pathway in plants.

5.2 LITERATURE CITED

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