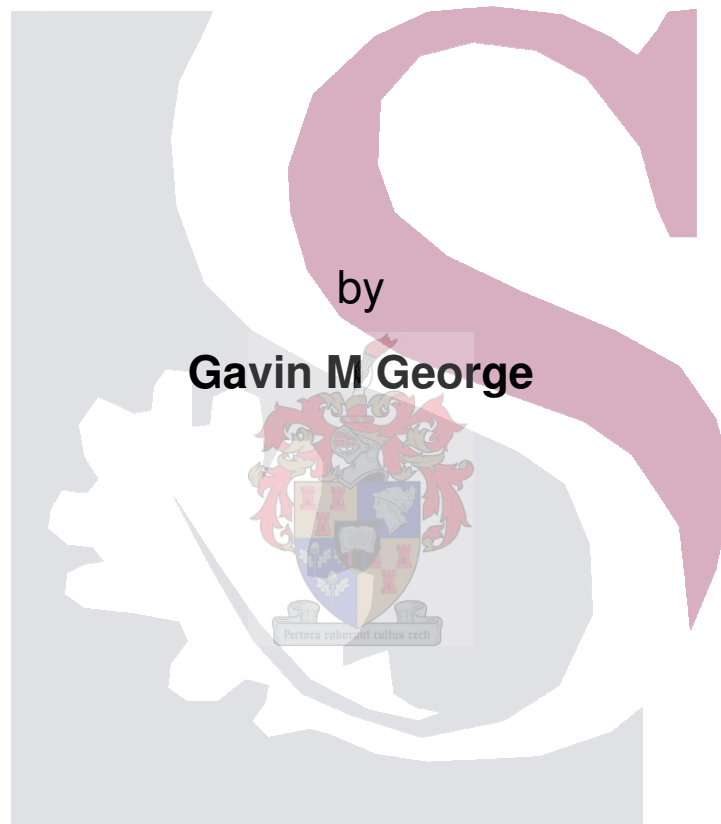


Virus Induced Gene Silencing for the Study of Starch Metabolism



Institute for Plant Biotechnology

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Declaration

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



Gavin George

03 February 2010

Date

Summary

Virus Induced Gene Silencing (VIGS) was optimized to allow for the study of starch metabolism. The plastidial inorganic pyrophosphatase gene, for which a mutant has never been identified, was studied using VIGS and it was found to have a broad role in this subcellular compartment. The accumulation of inorganic pyrophosphate limited the production of starch, carotenoids, chlorophyll, and increased the plants susceptibility to drought stress. These effects highlight the importance of this enzyme in maintaining a low intraplastidial concentration of PP_i providing an environment which facilitates these anabolic processes. Several genes involved in starch synthesis and degradation were also targeted with the aim of establishing a system of multiple gene silencing for the study of metabolic pathways. One, two and three genes were successfully silenced using this system which was validated based on previously published data. Interestingly, simultaneous silencing of the two isoforms of disproportionating enzyme led to a novel phenotype as a large reduction in starch instead of the expected increase was observed.

Biographical Sketch

Gavin George was born in Port Elizabeth on October 18th 1983. He matriculated from The Grey High School in 2001. Gavin moved to Stellenbosch the following year where he enrolled at the University. There he completed his Bachelors degree (BSc Animal Biotechnology) in 2004 followed by a BSc Honours in Biochemistry which was completed the following year. In 2006 he enrolled for a Masters degree at the Institute for Plant Biotechnology which was upgraded to a PhD in 2008.

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Preface

This Dissertation is presented as a compilation of five chapters. Chapters 1, 2, and 5 are written in such a way as to provide a literature background on the work presented in the experimental chapters. Chapters 3 and 4 represent the experimental work performed during the course of my PhD and will be submitted to Plant Physiology and in a revised version to FEBS Letters respectively.

Chapter 1 GENERAL INTRODUCTION AND PROJECT AIMS

Chapter 2 LITERATURE REVIEW

An in depth review of the state of the art of gene silencing technologies and how they can be applied to starch research

Chapter 3 RESEARCH ARTICLE

VIGS repression of plastidial inorganic pyrophosphatase leads to specific impairments in photosynthetic-related processes in *Nicotiana benthamiana*

Chapter 4 RESEARCH ARTICLE

Virus Induced Gene Silencing to Study Metabolic Pathways

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Chapter 1

General Introduction and Specific Project Aims

1.1 Introduction

Starch makes up one of the most abundant components of the world's crops in terms of carbohydrate production (Jobling, 2004). It is not only an important foodstuff, making up the primary source of carbohydrates in most diets, but it also has many diverse industrial applications. The vast majority of starch crop yield is consumed directly or indirectly as food or feed, but a significant proportion, approximately 250 Mt growing to 420 Mt in 2015, is also directed into industrial applications (Marz, 2006).

In terms of plant metabolism, starch is important for both short and long term storage of carbohydrates. Starch is stored in the plastids of both photosynthetic and non-photosynthetic organs including seeds, fruits, tubers, roots and leaves (Jobling et al. 2004). Its structure typically differs between species and even organs within the same plant (Kossmann and Lloyd, 2000). Leaf starch granules are generally smaller than those found in storage organs and its primary function in leaves is to act as a carbohydrate reserve during times of darkness (Czaja, 1969; Smith et al. 2005). This 'transitory starch' is broken down to glucose which can be fed into glycolysis, providing energy to cells when photosynthesis is limited. Long term storage of starch usually occurs in amyloplasts which are found in organs such as potato tubers, cassava roots, and developing cereal seed endosperm.

1.2 Specific Project Aims

Plastidial metabolism is key to the existence of plant life. The plastids themselves exist as self replicating sub-cellular organelles, which developed from endosymbiotic cyanobacteria around 1.5 billion years ago (Margulis, 1970, Tetlow et al. 2004; Hedges et al. 2004). While the most fundamental role of these organelles is the fixation of atmospheric CO₂ through photosynthesis, they are also involved in the production of starch, nitrogen assimilation, amino acid biosynthesis and the production of precursors for many plant hormones (Tetlow et al. 2004; Lichtenthaler, 1999). With this in mind we aimed to investigate some aspects of plastidial metabolism using virus induced gene silencing (VIGS) in *Nicotiana benthamiana* with a specific focus on, but not limited to, starch metabolism.

1.2.1 Virus Induced Gene Silencing repression of plastidial inorganic pyrophosphatase leads to specific impairments in photosynthetic-related processes in *Nicotiana benthamiana*

- In order to study the effects of reduced plastidial pyrophosphatase (psPP) activity we chose to repress it using a VIGS system in *Nicotiana benthamiana*

- A tomato EST (CLET20N17) was identified which shared a high level of homology to the identified Arabidopsis psPP
- In order to determine whether this sequence would be effective in down-regulating the gene in tobacco it was cloned into a modified tobacco rattle virus (TRV) and infiltrated into the plants using an Agrobacterium mediated system
- Using this system we aimed to fully characterise the plants for:
 - Activity of the endogenous psPP
 - Effects on starch metabolism which has a pyrophosphate generating reaction at the first committed step of its production
 - Effects on other metabolic pathways which also generate pyrophosphate including chlorophyll and carotenoid biosynthesis
- Using the data generated through this metabolic profile of the psPP silenced plants we aimed to gain a deeper understanding of the function of this important, and non-redundant enzyme in plant metabolism

1.2.2 VIGS to Study Metabolic Pathways

- While gene silencing in plants using VIGS is well established, it has yet to be determined whether the system can be used to target multiple genes simultaneously.
- The aim of this work was to first determine whether it was possible to target multiple genes using a number of techniques:
 - First by mixing a number of single gene targeting TRV constructs and co-infiltrating plants
 - Secondly, by building chimeric TRV constructs containing multiple fragments targeting different genes
- Once an effective method of targeting multiple genes had been established the system could be used for the study of complex biological pathways
- The starch metabolic pathway was chosen for the study and so it was aimed to manipulate the activity of several genes of known function and to determine what the effect on the plants would be if they were simultaneously down regulated
- To this end we targeted the DPE1, DPE2, and GWD, genes and characterised plants where the genes were targeted alone or with one or two of the others by:
 - The level of silencing by immunoblotting or activity assays
 - The starch content of each line of plants

- The levels of soluble sugars associated with starch degradation
 - Any other phenotypes that the plant lines developed
- The data we generate using these plant lines could then be compared to previously published information gathered from stable silenced and mutant plants to validate the technique which can then be applied to the study of other complex biological pathways

1.2 References

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Chapter 2

Literature Review

2.1 Carbon Partitioning in Leaves

Photosynthesis in plant leaves is a complex enzymatic pathway which allows for the conversion of carbon dioxide and water into carbohydrates. Indeed the autotrophic nature of plants allows for these carbohydrates, along with inorganic ions taken up through the roots, to be utilized for the production of nucleic acids, proteins, polysaccharides, lipids, and secondary metabolites satisfying the requirements of cellular metabolism. Photosynthesis is carried out in chloroplasts. Both the chloroplast and the cytosol contain metabolic intermediates which can feed into carbohydrate metabolism. These can be separated into two pools of carbon which include the triose phosphate/pentose phosphate pool and the hexose phosphate pool. The pentose phosphate pathway contains a number of sugar intermediates including ribulose 5-phosphate, ribose 5-phosphate and glycerate 3-phosphate (3-PGA). 3-PGA is produced through the carboxylation of ribulose-1,5-bisphosphate by ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) producing a six carbon sugar which immediately breaks down to two molecules of 3-PGA (Figure 1, Andrews and Lorimer, 1987). A balance between 3-PGA and triose phosphate is maintained by the enzyme triose phosphate dehydrogenase. The triose phosphate generated by this reaction is also maintained in balance with fructose-1,6-bisphosphate through its interconversion by the enzyme aldolase. While fructose-1,6-bisphosphate is technically a hexose phosphate it is considered to remain part of the triose/pentose phosphate pool due to its irreversible conversion to fructose 6-phosphate (F6P). In contrast the diversity of sugar intermediates found in the triose/pentose phosphate pool, the hexose phosphate pool contains only three: F6P, glucose 6-phosphate (G6P), and glucose 1-phosphate (G1P). These three sugars are maintained in equilibrium through the action of two enzymes, phosphogluco-isomerase (PGI) and phosphoglucomutase (PGM). As F6P is used as a substrate for the pentose phosphate pathway it forms the link between these two carbon pools.

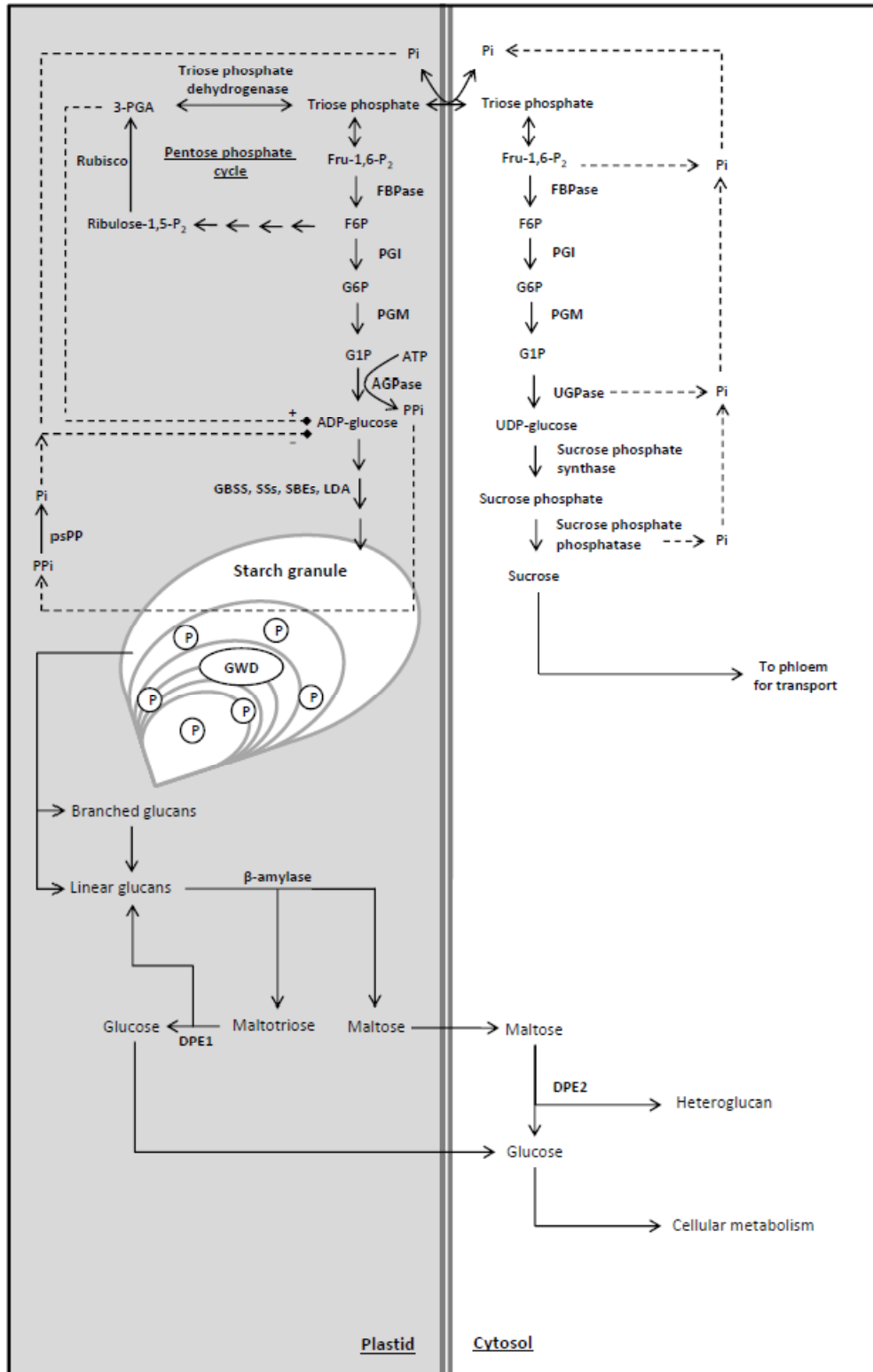


Figure 1 An overview of the carbon pools generated by photosynthesis in autotrophic tissues of plants as well as the general pathway of starch degradation

The sugar intermediates of these two pools are found in both the plastid and cytosol. These two compartments are isolated from one another by the chloroplast membranes and so exchanges between them must be mediated by sugar transporters. In leaves this occurs through the exchange of inorganic phosphate (P_i) for triose phosphate and the export of maltose from degraded starch. Such a link between the cytosolic and plastidial hexose phosphate pool allows for the coordination of photosynthesis with sucrose and starch metabolism.

Starch, produced in the plastid from the hexose phosphates, is made up of a great number of glucose molecules linked together into a densely packed and osmotically inert granule. The storage of carbohydrates as starch prevents the osmotic imbalance that would occur in the plastid if a comparable number of hexoses were retained as soluble sugars, and release P_i for further ATP production. During times of darkness, when carbon cannot be sourced from photosynthesis, starch is first phosphorylated and then broken down by a number of hydrolytic and glucotransferase enzymes to maltose and glucose which are exported into the cytosol where they are converted to hexose phosphates (Kossmann and Lloyd, 2000; Smith et al. 2005). In the cytosol, the hexose phosphates are converted into sucrose. The enzyme UDP-glucose pyrophosphorylase catalyses the production of UDP-glucose from G1P and UTP. The successive action of two more enzymes, sucrose phosphate synthase and sucrose phosphate phosphatase, forms a glycosidic bond between the UDP-glucose and F6P to produce sucrose. The glycosidic bond formed between the glucose and fructose molecules protects reactive groups of the sugars from oxidation making sucrose ideal for phloem transport to parts of the plant where it is needed.

At the beginning of the day triose phosphates are exported to the cytosol and are converted to sucrose to be transported to heterotrophic tissues (Riesmeier et al. 1993). When sucrose synthesis exceeds export the excess sugar is stored in the vacuole, but when that compartment becomes saturated the plant manufactures starch instead. Coordination between the rate at which starch and sucrose are synthesized and CO_2 is assimilated is required to prevent substrate inhibition of the pentose phosphate pathway (Stitt, 1993). If triose phosphate is exported too rapidly then chloroplastial ribulose-1,5-bisphosphate concentrations will become reduced, so limiting production of new photosynthate. On the other hand, if export of triose phosphate proceeds too slowly, P_i will become limiting and there will be an accumulation of hexose phosphates. One mechanism controlling the allocation of photosynthate can be seen from in the inhibition of cytosolic FBPase by fructose-2,6-bisphosphate ($Fru_{2,6}P_2$). FBPase plays a key role in directing carbon flux in the cytosol and when it is active it directs carbon towards sucrose synthesis. The enzyme

responsible for Fru2,6P₂ production is fructose 2,6 bisphosphate kinase/phosphatase (F2,6P₂K/P) a bifunctional enzyme that both manufactures and degrades F2,6P₂ (Stitt, 1990). The direction that it acts in depends on the levels of various metabolites in the cytosol. For example the phosphatase direction is stimulated by 3PGA and so when photosynthesis starts at the beginning of the light period and 3PGA is exported into the cytosol F2,6P₂ becomes rapidly degraded leading to a concomitant release from inhibition of the cytosolic FBPase and a stimulation of sucrose synthesis. The resulting rise in cytosolic G6P (one of the intermediates towards sucrose production) activates sucrose phosphate synthase (SPS) leading to faster sucrose synthesis which in turn deactivates SPS (Stitt et al. 1988). This leads to an accumulation of F6P and the subsequent activation of the kinase direction of F2,6P₂K/P producing Fru2,6P₂ so inhibiting FBPase leading to an accumulation of triose phosphate in the cytosol. Cytosolic triose phosphates inhibit their own export from the plastid leading to their accumulation in that compartment along with decreases of plastidial P_i. The increase of triose phosphate and the subsequent decrease in P_i in the plastid stimulates the activity of the first committed step in starch biosynthesis the ADP glucose pyrophosphorylase (AGPase) reaction and so directs the flow of carbon into starch rather than sucrose production. It is clear that the concentration of P_i in the cytosol and plastid plays a key role in the coordination of carbon metabolism, however, the role of inorganic pyrophosphate (PP_i) in these compartments remains unresolved.

2.2 PP_i metabolism

The compartmentation of the plastidial stroma from the cytosol allows for independent control over the concentrations of metabolites. This is displayed clearly with the very different concentrations of PP_i found between the two. The majority of PP_i found in plant cells is located in the cytosol (Weiner et al. 1987; Dancer and ap Rees 1989; Takeshige and Tazawa 1989; du Jardin et al. 1995; Farré et al., 2000, 2006). The plastidial concentration is maintained at a very low level through the action of a plastidial isoform of a pyrophosphatase enzyme (which degrades PP_i to 2 P_i) that constitutes the majority of the PPase activity in a plant cell (Gross and ap Rees 1986, Farré et al., 2006, Schulze et al. 2004). *Arabidopsis thaliana* contains six soluble pyrophosphatase (sPP) isoforms of which five (*AtPPa1*, *2*, *3*, *4* and *5*) are far more similar to each other are localized in the cytosol (Ergen, 2006) while *AtPPa6* is localized in the plastid (psPP; Schulze et al. 2004). PP_i has been proposed to act as an alternative energy source to ATP for some enzymatic steps (Stitt, 1998), however its precise role has not been well studied. Attempts have been made to do this by expressing

Escherichia coli inorganic pyrophosphatase (IPP) in different compartments (Sonnewald 1992, Farré et al. 2006, Geigenberger et al. 1998, Lee et al. 2005). Expression of this enzyme in the cytosol led to major effects on the accumulation of sugar and starch. In tobacco an eight fold increase in source leaf starch was observed while sucrose, fructose and glucose accumulated to between 12 and 68 times normal levels (Sonnewald 1992). A similar pattern of accumulation was observed in potato and *Arabidopsis* where increases in sucrose and starch were seen in the tubers (Geigenberger et al. 1998) and glucose and fructose were increased in the rosette leaves of *Arabidopsis* (Lee et al. 2005). When the IPP was targeted to the plastid only minor changes in metabolite levels were noted (Farre et al. 2006). These differences are probably explained by the relatively high PP_i levels found in the cytosol compared with the plastid (Gross 1986, Weiner 1987; Farré et al. 2001) indicating that the PP_i in the plastid is efficiently mobilized by the native sPP within that compartment. The gene encoding the plastidial sPP (psPP) was identified first in *Arabidopsis* (Schulze et al. 2004) and later in other species (Gómez-García et al. 2006). Several pathways within the plastid produce PP_i, for example the first committed step of starch synthesis leads to the production of PP_i by AGPase and this step is considered to be irreversible *in vivo* due to the high activity of the plastidial PPase (ap Rees et al. 1990). AGPase has been described as playing a regulatory role in starch biosynthesis as its activity is at a level not far above that required for flux through the pathway, is allosterically modulated and catalyses a non-equilibrium reaction (Stitt, 1993). It has been shown that the AGPase reaction becomes less thermodynamically favourable in the direction of ADP-glucose production when purified plant AGPases were measured under high PP_i conditions (Amir and Cherry, 1972). Furthermore, sodium fluoride has been shown to inhibit the action of soluble phosphatases and when potato tuber discs were incubated in media containing it starch accumulation was inhibited (Viola and Davies, 1991). When considering the overexpression studies, where sPP was targeted to the cytosol resulting in altered carbohydrate balance, and the likely effect that increased PP_i would have on AGPase activity it seems that maintenance of a low intraplastidial and higher cytosolic concentration is necessary for normal carbohydrate metabolism.

Other pathways within the plastid contain PP_i generating steps including chlorophyll and carotenoid biosynthesis. Chlorophyll *a* (Chl-*a*) is manufactured by chlorophyll synthetase within plastids, from chlorophyllide and ATP leading to the production of PP_i (Malkin et al. 2002). Chl-*a* then serves as a precursor for Chl-*b* in a non-PP_i generating reaction. Carotenoids are manufactured by the terpanoid biosynthetic pathway (Nambara et al. 2005), which involves a PP_i generating reaction catalyzed by phytoene synthase (Seo and Koshiba.

2002). It converts geranylgeranyl-pyrophosphate to phytoene in a two step reaction that is considered to be the rate limiting step in carotenoid biosynthesis (Hirschberg 2001). Phytoene is then converted to a number of carotenoids including β -carotene which itself is used to produce the xanthophylls zeaxanthin and violaxanthin. The carotenoids are involved in light harvesting and, more importantly, photoprotection of photosystem II (PS II) where they channel away excess quanta of energy from the reaction centre II (RCII) to quenching complexes (Nayak et al. 2002). With the starch, chlorophyll, and carotenoid biosynthetic pathways producing PP_i as a by product it is highly likely that a decrease in psPP activity will lead to broad changes in plastidial metabolism.

2.3 Starch Metabolism: Overview of the Enzymes involved in Synthesis and Degradation

While starch is almost solely comprised of chains of glucose units, an indefinite level of structural diversity in granules from different species has been observed (Czaja, 1969; Jobling, 2004). The reactions involved in catalyzing starch synthesis are fundamentally the same in all plants therefore the structural variability that exists (Table 1) must come from the relative proportions of the enzymes involved in starch metabolism, or differences in the substrate specificities of the enzymes between species. These changes affect the physical properties of the starches manufactured in the different species and so understanding the differences in enzymes between species helps to understand how they affect the starch structure. As most of the agriculturally produced starch is from maize, wheat, rice and potato the carbohydrate metabolism of these species has been studied extensively. This being said, some of the most important discoveries in starch metabolism have come from studies performed in model plant species such as *Arabidopsis* as well as the unicellular algae *Chlamydomonas reinhardtii*.

Table 1: Brief summary of the characteristics of starch obtained from the major starch yielding crops (adapted from Jobling et al. 2004 and Blennow et al. 2000)

Starch Characteristics	Maize	Wheat	Potato	Cassava
Type	Seed endosperm	Seed endosperm	Tuber	Root
Granule shape	Round, polygonal	Round, bimodal	Oval, spherical	Oval, truncated
Granule size (mm)	2–30	1–45	5–100	4–35
Phosphate (% w/w)	0.02	0.06	0.09	0.025
Protein (% w/w)	0.35	0.4	0.06	0.1
Lipid (% w/w)	0.7	0.8	0.05	0.1

Starch can be separated into two distinct fractions, amylose and amylopectin. In most starches amylopectin makes up the bulk of the granule. It has been shown in rice and potato that starch from leaf material contains less than 15% amylose, while that found in storage organs can contain between 11 – 37 % (Slattery et al. 2000). Higher ratios of amylose, up to 56%, have been achieved (discussed later) in transgenic potato (Schwall et al. 2000). Even more significant gains in total amylose have been observed in the maize amylose extender (*ae*), and low amylopectin starch (LAPS) mutants where it was increased to over 90% (Boyer et al. 1976; Sidebottom et al. 1998).

Both amylose and amylopectin are made up of linear chains of glucose molecules linked by α -1,4-glucosidic bonds. Within these chains, branch points occur which are characterized by an α -1,6-linkage. The primary difference between amylose and amylopectin is the number of these α -1,6,-glucosidic branch points. In amylose they occur approximately every 1000 glucose units whereas in amylopectin they occur about forty times more frequently (Hizukuri and Takagi, 1984). This results in amylose being a smaller polymer, with an approximate molecular weight of $5 - 10 \times 10^5$ Da, and predominantly linear whereas amylopectin occurs as a much larger, $1 - 10 \times 10^6$ Da, highly branched structure (Takeda et al. 1984; 1986). The two starch fractions also differ in their content of covalently bound phosphate. While

phosphate is nearly absent in amylose, in amylopectin there is approximately 1 to 30 nmol phosphate per mg starch, depending on the plant species (Hizukuri et al. 1970; Swinkels 1985).

2.3.1 Committing Carbohydrates to Storage: Enzymes Involved in Starch Synthesis

Starch synthesis is achieved through the action of several plastidially localized enzymes. In photosynthetic tissue the hexoses used to produce starch are created by photosynthesis. The first committed reaction in starch synthesis is the production of ADP-glucose and PP_i from G1P and ATP through the action of ADP-glucose pyrophosphorylase (Dickinson and Priess, 1969; Stark et al. 1992; Okita, 1992; Smith et al. 1997). Plastidial PP_i concentrations are maintained at a very low level through the action of an inorganic pyrophosphatase making the production of ADP-glucose thermodynamically highly favourable (Amir and Cherry, 1972). The ADP-glucose generated by this reaction is then utilized by the starch synthase (SS) enzymes to transfer the glucosyl moiety to the non-reducing end of a α -1,4-linked glucose polymer (Zhang et al. 2005). AGPase activity is tightly regulated in most plants through a mixture of allosteric stimulation and inhibition by 3-PGA and P_i respectively (Stark et al. 1992, Kleczkowski et al. 1993) and redox (Tiessen et al. 2002, Geigenberger et al. 2005).

ADP-glucose is utilised by starch synthases which form linear α -1,4-glucan chains. Starch synthase activity was identified not only in the soluble protein extracted from plastids but also bound to the starch granule (Denyer et al. 1995b). The common function of all the SS's is that they have the ability to transfer a glucose moiety from ADP-glucose to an existing α -1,4-glucan. There are five classes or families of SS's that have been identified through amino acid sequence analysis, the soluble SSI, SSII, SSIII, SSIV and the granule bound GBSS (Martin and Smith, 1995, Zhang et al. 2005). Plants from potato, pea, maize, rice, barley, sorghum and even *Chlamydomonas reinhardtii* deficient in GBSS activity produce amylose free starch indicating clearly that GBSS is responsible for its production (Hovenkamp-Hermelink et al. 1987; Denyer et al. 1995a; Wessler and Varagona, 1985; Sprague et al. 1943; Sano 1984; Rohde et al. 1988; Delrue et al. 1992). The soluble starch synthases have been demonstrated through mutational analysis to be involved in amylopectin synthesis. Plants lacking soluble isoforms produce amylopectin where the chain lengths within the amylopectin is altered (Delvalle et al. 2005, Fontaine et al. 1993, Roldán et al. 2007, Zhang et al. 2005). Specific SS isoforms are also thought to be involved in starch granule initiation as Arabidopsis plants mutated in both SSIII and SSIV isoforms are unable to manufacture starch (Zhang et al. 2008, Szydlowski et al. 2009).

Starch branching enzymes (SBE) are responsible for the creation of branch points in the chains made by SS enzymes. They achieve this by cleaving an α -1,4-linkage and re-attaching it elsewhere on the chain to form an α -1,6-linkage (Borovosky et al. 1976; Borovosky et al, 1979). Generally mutations in *BE* genes, or repression of BE activity in transgenic plants leads to the production of high amylose starch in storage organs (Burton et al. 1995; Larsson et al. 1996; Fisher et al. 1996; Dumez et al. 2006). Arabidopsis contains three genes, *BE1 – 3*, coding for SBEs and all are part of the SBEII family (Dumez et al. 2006). On a sequence basis, *BE1* is very different to the other *BEs* in Arabidopsis and indeed in all other plants. Mutation of this *BE* alone or in the mutant background of *be2* and *be3* resulted in no observable effects on starch. In contrast *be2* and *be3* single mutants in Arabidopsis led to small changes in the chain length distribution of amylopectin mirroring the effect seen in rice *amylose extender (ae)* (SBEIIb) mutants (Dumez et al. 2006; Nishi et al 2001). Double mutants of *be2* and *be3* produced plants with no starch at all and an accumulation of water soluble glucans (WSG), mainly maltose (Dumez et al. 2006).

The Arabidopsis genome encodes four proteins which are involved in debranching which themselves fall into two categories, limit dextrinases (LDA) and isoamylases (ISA) (Lloyd et al. 2005; Streb et al. 2008). Arabidopsis contains three isoforms of the isoamylases, ISA1 – 3, and one LDA all of which have the ability to cleave α -1,6-glycosidic linkages (Nakamura, 1996). ISA1 and ISA2 form a heterotetramer, known as Iso1, and reduction in the activity of one isoform reduces the activity of the other due to the instability of the iso1 complex (Smith et al. 2005). It has been shown in maize, rice, Arabidopsis, *C. reinhardtii* and potato that a reduction in ISA1 leads to a reduction of granular starch (James et al. 1995; Nakamura et al. 1997; Zeeman et al 1998; Dauville et al. 2001; Bustos et al. 2004). Identical results were obtained through the attenuation of ISA2 in Arabidopsis (Delatte et al. 2005). The absent starch is replaced with a glycogen-like polymer, known as phytoglycogen, which consists of short chain length, DP less than 10, which are highly branched. Some residual starch remains in Iso1 mutants which shows a modified amylopectin distribution suggesting the involvement of the ISA1 and ISA2 complex in starch synthesis rather than degradation (Ball et al. 1996).

Starch phosphorylase catalyses the reversible release of G1P from the terminal residue of α -1,4-linked glucan chains (Zeeman et al. 2004). Although the intraplasmidial concentrations of G1P and P_i suggest its primary role falling into starch degradation, recent evidence from *C. reinhardtii* implicates it in starch synthesis when carbon flux in this direction is high (Dauvillée et al. 2006). In Arabidopsis it was shown that the plastidial isoform of starch phosphorylase has a high affinity for small unbranched maltodextrins, however, plants

carrying mutations in the gene showed little effect on starch synthesis or degradation (Zeeman et al. 2004). It is thought that the starch phosphorylase enzymes are responsible for the recycling of linear maltooligosaccharide (MOS) generated by the ISA enzymes during rapid starch synthesis or that they operate with the ISAs by trimming glucans to allow for efficient crystallisation of the starch.

2.3.2 Starch Phosphorylation: Glucan Dikinases are Required for normal Rates of Starch Degradation

While glucose is by far the largest constituent of the starch granule there are also small amounts of lipids, protein (such as the GBSS discussed earlier), and phosphate (Morrison, 1995; Schoch, 1942; Posternak, 1951). The type and level of phosphorylation in starch is an important determinant of the physico-chemical properties of the starch and so of the industrial applications it can be used for. Depending on the source, starch phosphate can exist as inorganic phosphate (Pi), in phospholipid complexes or as a covalently bound monoester (Schoch, 1942). In starches where phosphate exists mainly as a phospholipid, such as those derived from cereal endosperm, increasing phosphate results in decreasing viscosity and clarity. On the other hand, phosphate bound as a monoester, such as in potato tuber starch, correlates with increased viscosity and clarity. Cereal endosperm contains high levels of lipids which easily complex with the less structured amylose fraction of starch. The incorporation of phosphate esters in starch was more difficult to explain until recently. It was found that such covalently bound phosphate is limited to the C3 and C6 positions of the glucose molecules of amylopectin (Ritte et al. 2002, 2006; Tabata and Hizukuri, 1971; Kötting et al. 2005). Conversely, the amylose fraction contains negligible amounts. The level of covalently bound phosphate in amylopectin varies between plant species. Potato starch contains one of the highest levels of phosphorylation where approximately 1 in 300 glucose residues are phosphorylated, compared to wheat endosperm starch where this value is closer to 1 in 10 000 (Lorberth et al. 1998; Lim and Sieb, 1993).

The two enzymes that catalyze the phosphorylation of glucose molecules in amylopectin are known as α -glucan, water dikinase (GWD) and phosphoglucan, water dikinase (PWD) which attach phosphates to the C6 and C3 positions respectively (Ritte et al. 2006). Potato starch contains a relatively high ratio of phosphorylated glucose moieties and of this phosphate the majority, approximately 70 – 80%, is bound to the C6 while the rest is at the C3 position (Tabata and Hizukuri, 1971; Lim and Sieb 1993; Blennow et al. 2000).

GWD and PWD enzymes are both monomeric, multi-domain proteins which catalyse similar reactions. While GWD is approximately 155 kDa in size the PWD is slightly smaller at 130

kDa (Baunsgaard et al. 2005; Kötting et al. 2005; Lorberth et al. 1998; Yu et al. 2001). This difference is largely attributed to differences in the N-terminal carbohydrate binding domain which is highly dissimilar between the two (Machovič and Janeček, 2006a, b). GWD first autophosphorylates a histidine residue (Ritte et al. 2000, 2006; Mikkelsen et al. 2004) using the β -phosphate of ATP and then this phosphate is transferred to the C6 position of a glucose residue on the starch granule by the following reactions:



While the PWD enzyme shares a similar activity to GWD it was found that PWD activity was only measurable on native starches if they were pre-phosphorylated by GWD suggesting sequential activities for the two enzymes *in vivo* (Ritte et al. 2006, Kötting et al. 2005). The reaction for PWD has been, therefore, proposed to be:



Where $m \geq 1$

Attenuation of the activity of either GWD or PWD results in a decrease in bound phosphate at the C6 and/or C3 position and this in turn generates a repression of starch degradation in the plants (Baunsgaard et al. 2005; Lorberth et al 1998; Yu et al. 2001; Kötting et al. 2005; Nashilevitz et al. 2009). The starch excess (SEX) phenotype observed in plant leaves (Lorberth et al., 1998; Yu et al, 2001; Naschiviletz et al, 2009) lacking GWD is generated through reduced breakdown of starch due to a lower level of phosphorylation. This is not to say that the starch phosphorylation only occurs during breakdown of starch, indeed, it occurs during both net synthesis and degradation albeit more rapidly during the latter (Nielsen et al.

1994; Ritte et al. 2004). Lack of GWD also leads to a repression of starch degradation in cold stored potatoes, where starch is broken down in tubers stored at low temperatures, and in tomato pollen (Lorberth et al. 1998; Nashilevitz et al. 2009). PWD mutants also produce a SEX phenotype in Arabidopsis leaves although it is not as severe as that produced by GWD mutants (Kötting et al. 2005; Baunsgaard et al. 2005).

It has been suggested that GWD has a high affinity for the highly ordered maltodextrins found in starch and that it catalyses the initial phosphorylation event which destabilizes the outer surface of the granule (Hejazi et al 2008). This was tested with the use of crystallized maltodextrins (MD) which were produced to mimic highly ordered amylopectin, more so than that found in native granules. It was shown that GWD from potato tubers phosphorylated the MDs at a higher rate than that seen in less ordered native starch. When these MDs were pre-solubilized with heat treatment the GWD lost its ability to phosphorylate them (Hejazi et al. 2008). The situation is similar for PWD which was also tested on the MDs. It could also phosphorylate the glucans when in their crystal form but only when the MDs had been pre-phosphorylated through the action of GWD. These experiments were extended to MDs which were produced to resemble both A- and B-type allomorphs of starch (Hejazi et al. 2009). The A-type MDs resemble the starch found in maize which is more compact than that of B-type, typically found in potato tuber starch. Potato GWD was found to be more efficient at phosphorylating the A-type crystals than B-type however this did not translate into a higher rate of solubilisation, indeed, in the presence on GWD alone the B-type MDs were solubilized four times more efficiently than the A-types. Solubilisation of B-type MDs in this way was shown to be a progressive process where the outer layers are accessed first before the enzymes can access the inner layers (Hejazi et al. 2009). Together the GWD and PWD enzymes were able to effect the solubilisation of the MDs without the influence of any other enzymes involved in starch degradation. Taken together these data show that GWD and PWD act most efficiently on the more highly ordered regions of starch to phosphorylate the glucan molecules. This in turn causes the partial solubilisation of the outer layers of the starch granule, or complete solubilisation of MDs which have no α -1,6-branched glucan structures, which can then be further broken down by the hydrolytic enzymes such as β -amylase.

The GWD and PWD are two of three glucan-related dikinases identified in Arabidopsis (GWD: At1g10760; PWD: At5g26570; GWD2: At4g24450) (Fettke et al. 2009). They all possess an N-terminal carbohydrate binding domain, a C-terminal nucleotide binding domain and a histidine residue which is required for phosphorylation of glucans but while GWD and

PWD have been shown to be present in the plastids the third isoform is localized to the cytosol (Lorberth et al. 1998; Fettke et al. 2009; Mikkelsen et al. 2004; Glaring et al. 2007).

2.3.3 Starch Degradation: Amylases Catalyze Glucan Hydrolysis

In dicotyledonous species, such as *Arabidopsis* and potato, once the surface of the starch granule has been destabilized through phosphorylation it is thought that enzyme attack on the glucan chains liberates them for further enzymatic processing. Here the primary route of enzymatic processing after phosphorylation is via the β -amylases.

The α -amylases play an important role in starch breakdown in endosperm of germinating cereal seeds. This was clearly shown when repression of a rice α -amylase caused a distinct delay in seed germination (Asatsuma et al. 2005). The rice and barley genomes encode ten α -amylases which fall into two families, AMY1 and AMY2 (Huang et al. 1992; Bak-Jensen et al. 2007). Isoforms from other families are involved in germination of barley seeds (Bak-Jensen et al. 2007). The barley AMY1 enzymes preferentially targets starch while the AMY2 enzymes have a higher affinity for linear glucans indicating differential roles in starch breakdown (Søgaard and Svensson, 1990). Three α -amylases are predicted to exist in *Arabidopsis*, AMY1 – 3, of which only AMY3 is localized in the chloroplast (Lohmeier-Vogel et al. 2008). Interestingly, neither individual mutants for the α -amylases nor a triple mutant for all three revealed any effect on starch breakdown (Yu et al. 2005). It has been suggested that instead of AMY3 being directly involved in the hydrolysis of glucans it acts as a scaffold and regulatory molecule during degradation (Lohmeier-Vogel et al. 2008).

Linear glucans released from starch breakdown can be acted upon by β -amylase (BAM) to produce maltose (Lao et al. 1999). The *Arabidopsis* genome encodes nine β -amylase genes which have been shown to fall into four phylogenetic sub-families (I-IV) (Fulton et al. 2008). The BAM enzymes are grouped where BAM5 and -6 are part of sub-family I, BAM1 and -3 are in II, BAM4 and -9 in III, and BAM2, -7 and -8 are in sub-family IV (Smith et al. 2004). To determine the roles of the β -amylases in starch metabolism it was first necessary to establish which of them were localized in the plastid. Based on the predicted presence of transit peptides allowing for plastidial localization and fluorescent localization studies it was determined that BAM1 to 4 were present in this compartment (Fulton et al. 2008). Repression of one of the BAM isoforms in potato (homologous to BAM3 in *Arabidopsis*) led to a reduction in the rate of starch breakdown in leaves indicating that it is an important step in starch degradation or that it might act together with debranching enzymes as an initial step in this process (Scheidig et al. 2002). This study was repeated in *Arabidopsis* where repression of BAM3 through RNAi also led to a SEX phenotype (Kaplan and Guy, 2005).

Arabidopsis mutants of these BAMs showed that BAM3 is partially redundant for BAM1 but important for starch degradation. Mutants for either BAM1 or 3 resulted in a mild SEX phenotype while a double mutant of the two revealed a much stronger excess of starch with a concomitant decrease in maltose. In Arabidopsis, neither reduced BAM2 activity alone nor in combination with mutations in other BAM isoforms produced a discernable phenotype. BAM4 and 9 (sub-family III) share amino acid substitutions at points which are strictly conserved in the other BAM isoforms (Fulton et al. 2008). Both these isoforms show no β -amylase activity but interestingly Arabidopsis mutants of BAM4 show a starch excess phenotype (Fulton et al. 2008). Mutants of BAM4 in a *bam1/bam3* mutant background show an even greater SEX phenotype than the double mutant alone. The precise role of BAM4 has yet to be elucidated but it seems to have a regulatory role in the breakdown of starch *in vivo*. Of the remaining BAM enzymes, BAM5 (which makes up a large portion of the total activity in Arabidopsis), has been shown to be targeted to the phloem and mutants of the gene show no effect on starch metabolism (Wang et al. 1995; Laby et al. 2001). The β -amylase enzyme itself hydrolyses, like α -amylase, only α -1,4-glycosidic linkages and not α -1,6-branch points, furthermore, the β -amylases hydrolysis can only proceed on unphosphorylated portions of a glucan chain (Takeda and Hizukuri, 1971). It is thought that once the outer surface of the starch granule has been partially solubilized through the action of GWD and PWD, there will be a certain number of exposed linear glucans which can be acted on by the BAM enzymes (Zeeman et al. 2007). Once these have been acted on, short branch points remain which the BAM cannot traverse. These branch points could then be eliminated through the action of the debranching enzymes.

2.3.4 Linearization of Glucans: Debranching Enzymes Required for Complete Processing of Starch Breakdown Products

The Arabidopsis genome encodes four proteins which are involved in debranching which themselves fall into two categories, limit dextrinases (LDA) and isoamylases (ISA) (Lloyd et al. 2005; Streb et al. 2008). Arabidopsis contains three isoforms of the isoamylases, ISA1 – 3, and one LDA all of which have the ability to cleave α -1,6-glycosidic linkages (Nakamura, 1996). The attenuation of LDA alone has no effect on starch degradation in Arabidopsis and studies on the ISAs are made more complicated by the fact that they are also involved in starch synthesis (Delatte et al. 2006; Wattedled et al. 2005). In both Arabidopsis and potato, ISA1 and ISA2 form a heterotetramer, known as Iso1, and reduction in the activity of one isoform reduces the activity of the other due to the instability of the iso1 complex (Smith et

al. 2005; Hussain et al. 2003). It has been shown in maize, rice, Arabidopsis, *C. reinhardtii* and potato that a reduction in ISA1 leads to a reduction of granular starch (James et al. 1995; Nakamura et al. 1997; Zeeman et al 1998; Dauville et al. 2001; Bustos et al. 2004). Identical results were obtained through the attenuation of ISA2 in Arabidopsis (Delatte et al. 2005). The absent starch is replaced with a glycogen-like polymer, known as phytoglycogen, which consists of short chain length, DP less than 10, which are highly branched. Some residual starch remains in Iso1 mutants, which shows a modified amylopectin chain length distribution suggesting the involvement of the ISA1 and ISA2 complex in starch synthesis rather than degradation (Ball et al. 1996). Similar results were obtained in maize and rice where loss of either ISA1 or ISA2, known as *sugary1* (*su1*) mutants, led to altered chain length distribution of amylopectin and, like Arabidopsis, accumulation of phytoglycogen (James et al. 1995; Kubo et al. 1999; Dinges et al. 2001;). In potato reduction in ISA1 or -2 led to a small amount of phytoglycogen production but also to the generation of a higher number of much smaller starch granules with no effect on the total starch content (Bustos et al. 2004). Mutants in the third isoform of isoamylase, ISA3, do not accumulate phytoglycogen but are strong candidates to be involved in starch breakdown (Delatte et al. 2006). It has been shown to have a diurnal expression pattern which is similar to other enzymes involved in starch degradation furthermore, it was shown that Arabidopsis mutants of ISA3 demonstrate a distinct starch excess phenotype (Wattebled et al. 2005; Smith et al. 2004; Smith et al. 2005). Interestingly, double mutants for *isa3/lda* in Arabidopsis revealed a 92% reduction in the total starch content, showing that these two enzymes have partially redundant functions in starch metabolism (Wattebled et al. 2005). The relative importance of the two enzymes is different in cereal species. Loss of LDA in maize showed a starch excess phenotype indicating a more important role in starch degradation in these species (Dinges et al. 2003). Furthermore, it was shown that LDA mutations in an ISA mutant background led to the increased production of phytoglycogen indicating that in cereal species the LDA is also involved in starch synthesis. It seems that the ISA1 and ISA2 are required for normal starch synthesis in Arabidopsis, where they act to properly position branch points that allow for the formation of crystalline starch (Streb et al. 2008). While many of the studies into the initial reaction of starch degradation have been complicated by the redundant nature of the isoforms involved it seems clear that once phosphorylation of the starch has occurred the combination of β -amylase with ISA3 and LDA produces linear glucans which form the substrate for the downstream degradative enzymes.

2.3.5 Processing for Export: Enzymes Involved in the Final Stages of Starch Breakdown

A minor product of β -amylase processing is maltotriose which is processed efficiently by a disproportionating enzyme, DPE1. It was first isolated from potato tubers and was later characterized in Arabidopsis, pea, wheat endosperm and even the thermophilic bacteria *Thermotoga maritima* (Peat et al. 1956; Lin and Preiss, 1988; Kakefuda, and Duke, 1989; Bresolin et al. 2006; Liebl et al. 2004). The *Chlamydomonas* mutant, *Sta11*, has also been shown to be a lesion in the DPE1 gene (Wattebled et al. 2003). Arabidopsis plants with reduced DPE1 activity accumulate far more maltotriose, as well as maltotetraose, and maltoheptaose during starch breakdown (Critchley et al. 2001). While maltotriose is the major substrate for DPE1 it has also been shown to utilize higher order malto-oligosaccharides (MOS) (Lin and Preiss, 1988). DPE1 does not hydrolyze α -1,4-linkages, instead it liberates glucose while transferring the remaining maltosyl units to free MOS in the plastid. For example, when maltotriose is the substrate, maltopentaose and glucose will be the products (Lin and Preiss, 1988). It is thought that through this process glucose is generated as the primary product which is subsequently exported to the cytosol driving DPE1 to replenish higher order MOS (Lin and Preiss, 1988; Okita et al. 1979; Lloyd et al. 2005). The combined action of β -amylase and DPE1 produces maltose and glucose, both of which can be exported from the plastid and into the cytosol by their respective transporters (Weber et al. 2000; Niittylä et al. 2004).

Through the use of affinity labelling, a putative glucose transporter was discovered on the inner membrane of the plastid (Weber et al. 2000). This transporter had a higher affinity for glucose than any other sugar. In a later study, it was shown that glucose export is an active process which is coupled to proton transport (Servaites and Gieger, 2002). The maltose exporter was recently discovered by screening Arabidopsis *sex* mutants for a maltose excess (MEX) phenotype (Niittylä et al. 2004). A lesion was identified at the At5g17520 locus in the Arabidopsis genome which causes an approximate 40 times accumulation of maltose and a starch excess in comparison with wild-type plants. To confirm that the MEX1 protein in Arabidopsis coded for a transporter, *E. coli* maltose transport mutants were complemented with the MEX1 protein and grown on minimal media with added maltose. The mutants alone could not import maltose into the cytosol and so failed to grow, however, the complemented mutants could transport maltose over the membrane showing that MEX1 is indeed a trans-membrane transporter of maltose (Niittylä et al. 2004). With the identification of a maltose transporter, the conjecture surrounding the fate of maltose has been mostly resolved. It appears that it is exported to the cytosol and that from here it is further metabolized by

another enzyme, disproportionating enzyme 2 (DPE2). This is a transglucosidase which transfers one glucose molecule from maltose to a polymer, liberating the second one which can be converted to sucrose or enter glycolysis. (Lloyd et al. 2004; Chia et al. 2004; Lu and Sharkey, 2004). The precise nature of the polymer acceptor is unknown, although it is possible that it is a cytosolically localized polyglycan (Smith et al. 2005)

The second disproportionating enzyme was identified through sequence examination of the Arabidopsis genome and while there was homology between the two they were found to be distinct enzymes with differing functions (Chia et al. 2004). Potato plants were generated with RNAi constructs targeting DPE2 which showed a distinct starch excess phenotype in the leaves (Lloyd et al. 2004). The accumulation of starch in both control and DPE2 silenced plants was unaffected indicating that the starch excess phenotype arose from a slower net rate of degradation rather than an increased rate of synthesis. As was observed for MEX1 mutants, DPE2 silenced plants accumulated maltose to much higher levels than those found in control plants (Niittylä et al. 2004; Lloyd et al. 2004; Chia et al. 2004; Lu and Sharkey, 2004). During the night there was also a concomitant decrease in the level of cytosolic sucrose in the DPE2 mutant plants showing that it is involved in the conversion of maltose into utilizable sugars (Chia et al. 2004; Lu and Sharkey, 2004). The DPE2 enzyme was tested with maltose and other higher order MOS which revealed that maltose was indeed the substrate of the enzyme. *In vitro* experiments showed that DPE2 transfers one glucosyl unit from maltose to glycogen and liberates the other as glucose which can be fed directly into glycolysis (Lloyd et al. 2004; Steichen et al. 2008). While there is still debate as to the sub-cellular location of the DPE2 enzyme it is clear that whether it is plastidial or cytosolic it is involved in the final stages of starch breakdown. The glucose released by the action of DPE2 can either be fed into cytosolic glycolysis directly or can be exported from the plastid and then utilized.

2.4 Analysis of Enzyme Function: Development of Gene Silencing Technologies in Plants

There are many techniques available to determine the metabolic role of a specific gene product. A traditional biochemical approach would be to identify and purify the protein to homogeneity using chromatographic methods. The pure protein can then be characterized based on its substrate specificity, products generated as well as its dependency on cofactors and inhibitors. This information can then be used to infer the proteins position within a biological pathway. With the plethora of DNA sequences becoming available, the fastest way to deduce protein function is to compare the gene sequence to that of a homologous gene which produces a characterized protein. This is however limited to proteins that show a high

level of homology to one that is known and, even then, may be inaccurate in the organism under study. While such techniques have proven to be extremely useful in the characterization of proteins they can be limited in that they provide little information on the functional *in vivo* role of an enzyme and the biological pathways in which they are indirectly involved.

2.4.1 Using genetic engineering to modify gene transcription

A better way to elucidate the function of a gene product is to alter its activity in the organism where it occurs and then, through subsequent analysis, determine which pathways and metabolites become altered. To achieve this there are two highly effective methods which yield complementary information. The first is the heterologous expression of a gene, leading to the increase in activity of an enzyme. This can be used to overcome the transcriptional control of the plants native enzyme or, to introduce a novel activity into an organism which does not possess that enzyme. Often, however, enzyme activities are present in sufficient amounts to catalyse the maximum possible flux through a pathway. As such, supplementation of the activity would have no effect on the pathway and the role of the enzyme would be determined by reducing its activity.

It is also possible to reduce the transcription of a gene by inserting sequences of that gene in other parts of the genome driven by a promoter. For example expression of all, or part, of a gene in antisense orientation has been shown in bacteria, *Drosophila melanogaster*, mammalian cells, and plants to lead to down regulation of the gene product (Green et al. 1986; Cabrera et al. 1987; Yokoyama and Imamoto, 1987; Van der Krol et al. 1988).

A second type of silencing is called co-suppression and differs from antisense in that the gene to be targeted is expressed in sense orientation. This method was discovered in experiments designed to increase expression of a native Petunia gene, chalcone synthase in order to increase pigmentation in Petunia (Napoli et al. 1990). Unexpectedly, instead of an increase in expression of the gene, a decrease was observed in expression of both genes. Furthermore, the transgenic plants often reverted to a normal phenotype which was characterized by mRNA production from both genes. This process could be initiated by only a single copy of a gene and was independent of the promoter driving the *trans* gene (van der Krol et al. 1990). In a later study it was found that while one transgene was sufficient to cause co-suppression, a dosage effect was observed in that the more transgenes present in the genome the greater the elicited silencing effect (Jorgensen et al. 1996). Interestingly, when isolated nuclei of sense suppressed plants were analyzed, no reduction in mRNA accumulation was observed indicating that silencing occurred post-transcriptionally (de

Carvalho et al. 1992; Jorgensen, 1995). This contradicted previous findings where transgene copy number was positively correlated with expression level (Hobbs et al. 1993), however, a large scale analysis of transgenes in *Arabidopsis* showed that expression did in fact increase with copy number, but only up to a threshold number of inserts (which was gene specific) at which point genome surveillance systems identify the transcripts targeting them for silencing (Schubert et al. 2004).

These observations led to research to understand the mechanism(s) behind this post-transcriptional gene silencing (PTGS). PTGS is characterized by the specific degradation of mRNA produced from both the endogenous gene and transgene when the two are expressed simultaneously, regardless of whether the transgene is in sense or antisense orientation (Waterhouse et al. 1998). Indeed, when both a sense and antisense gene were introduced into a single tobacco genome targeting a viral protease, the silencing efficiency was increased 5- to 10 fold.

Similar experiments performed using the nematode *Caenorhabditis elegans* showed that the injection of either sense or antisense RNA into the worms caused a level of silencing, but the effect was potentiated by at least two orders of magnitude when double stranded RNA was used (Fire et al. 1998).

In plants, little literature exists showing that direct application of double stranded RNA (dsRNA) to be as effective in eliciting silencing as was demonstrated in *C. elegans*. While dsRNA applied to tobacco leaves was shown to interfere with viral replication it has still remains to be shown that RNA applied in this way can have an effect on an endogenous gene (Tenllado et al. 2004). This being said, dsRNA can be produced *in vivo* through the use of inverted gene repeats separated by an intron which can be spliced out in the nucleus (Smith et al. 2000). This technique proved to be far more efficient than sense, antisense, or both combined in the same plant, most likely due to the fact that the intron is cut out by the spliceosome, annealing two perfect repeat sequences to form dsRNA. When sense and antisense strands are expressed from distal locations on the genome, the single strands are distributed more randomly reducing the chance of them coming into contact and exposing them to nucleolytic attack. The use of intron-spliced inverted repeat sequences has all but become the standard method for eliciting silencing in most plant species where an insertion mutant is unavailable.

Post transcriptional gene silencing, in one form or another, has been observed in species spanning a wide variety of kingdoms. First observed as co-suppression in plants, quelling in the fungus *Neurospora crassa*, and RNA interference (RNAi) in many invertebrate and

vertebrate animals, it became clear that all these systems shared the same underlying mechanism (For reviews see Vaucheret et al. 1998; Cogoni and Macino, 1997; Cogoni and Macino, 2000).

2.4.2 General pathway and the enzymes involved in gene silencing

The pathway leading to RNA silencing is generally accepted to be separated into three discrete (initiation, effector and amplification) stages (Hannon, 2002). The initiation of RNA silencing has been found to be triggered by double stranded RNA (Fire et al. 1998). While sense and antisense strands of RNA are moderately effective in eliciting silencing, this is mainly due to their complementation of endogenous mRNA molecules or through processing into dsRNA through the action of the RNA-dependant RNA polymerase (RdRP) (Dalmay et al. 2000). The dsRNA is recognized by a highly specific ribonuclease, Dicer, which falls into the RNaseIII ribonuclease family (Bernstein et al. 2001). It acts to degrade long dsRNA molecules into 21 - 25 nucleotide fragments known as small interfering RNAs (siRNAs). Cleavage by the dicer enzyme produces double stranded siRNAs with a 5'-phosphate and a two nucleotide 3' overhang (Elbashir et al. 2001). The population of siRNA molecules created are available for integration into a large multimeric nuclease complex which can target and cleave single stranded RNA with high specificity. This enzyme complex that associates with siRNA's is known as the RNA-induced silencing complex (RISC) (Hammond et al. 2000). Upon integration into the RISC the siRNA acts as a guide, targeting the complex in a sequence specific manner to homologous RNA (Nykänen et al. 2001). Here, the endonucleolytic cleavage of the target mRNA occurs, effectively disabling it. Discrepancies between the initiating RNA molecules, used to trigger interference, and siRNA's recovered after silencing suggests that a mechanism exists to bolster and amplify the siRNA population (Sijen et al. 2001). This amplification was shown to be part of a cyclic process where siRNA primed mRNA is made double stranded through the action of the RDRP. In this way it provides new substrate RNA for cleavage by Dicer and expands the targetable area of mRNA.

2.4.3 Virus Induced Gene Silencing

It has been long known that plants infected with a virus showed a level of resistance or cross-protection to subsequent infections by similar viruses (McKinney, 1929). With the advent of genetic modification the knowledge of cross-protection prompted successful attempts to confer resistance by introducing viral genetic material into plants, a technique known as pathogen derived resistance (Beachy, 1997). The resistance acquired by the plants was found to be due to post-transcriptional gene silencing (PTGS) of the viral genome. Indeed, it has been speculated that PTGS may have originally evolved as a

primitive mechanism of anti-viral defence (Burch-Smith et al. 2004). The fact that the PTGS mechanisms innate to plants could silence viral genes meant that the possibility existed that viral genes could trigger silencing of endogenous plant genes. It was shown that a recombinant virus carrying a plant gene triggered the specific and targeted down-regulation of the homologous endogenous mRNA of the plant that was infected (Kumagai et al. 1995). The ability to silence endogenous plant genes using viruses became known as Virus Induced Gene Silencing (VIGS) (Baulcombe, 1999). While the potential value of VIGS in plant research was clear, the problem still existed that symptoms of the disease accompanied the viral infection. The earliest VIGS vectors were based on tobacco mosaic virus (TMV) and, while it was highly infectious, it caused severe symptoms in the host plant which made it less than ideal for physiological studies (Kumagai et al. 1995). In an effort to reduce the background symptoms of viral infection and to expand the targetable host range of VIGS, several vectors have been produced. A potato virus X (PVX) was produced which was more stable than TMV but which displayed a reduction in the targetable host range (Ruiz et al. 1998). Tomato golden mosaic virus, a barley stripe mosaic virus, and cabbage leaf curl viruses have also been utilized, the latter two with the intention of targeting genes in barley and Arabidopsis respectively (Peele et al. 2001; Holzberg et al. 2002; Turnage et al. 2002). To date however, the most effective VIGS vector has proven to be based on a tobacco rattle virus (TRV; Liu et al. 2002).

The TRV VIGS vector overcame many of the initial problems associated with viral silencing of genes. It has a broad host range, very mild symptoms of infection, has been shown to move systemically throughout the aerial parts of the plant and is stable enough to produce consistent results between experiments (Burch-Smith et al. 2004; Liu et al. 2002; Ratcliff et al. 2001). The genome of the wild type TRV comprises of two positive single stranded sense RNAs called RNA1 and -2 (MacFarlane, 1999). The RNA1 portion of the genome produces four proteins, two replicases, a movement protein, and a cysteine-rich protein and alone has the ability to replicate and move systemically through the plant. The RNA2 portion encodes a coat protein and two non-structural proteins. To allow for their use as VIGS vectors cDNA clones of the viruses were transferred into T-DNA vectors making it possible to infect plants using an Agrobacterium mediated system (Liu et al. 2002). In order to facilitate the insertion of heterologous gene fragments into the virus, the non-structural genes of the RNA2 were replaced with a multiple cloning site. In addition, the viral cDNAs were placed under the control of two 35S CaMV promoters (Liu et al. 2002). These two plasmids containing the TRV cDNA are called TRV1 and TRV2, respectively. This system allows for Agrobacterium T-DNA transfer of the viral cDNA into the plant genome where the ssRNA of the virus can be

produced. The RNA1, moving independently through the plant, can form the full virus when it comes in contact with RNA2. As the virus moves through the plant, the PTGS system in the plant recognizes the virus and initiates a silencing response during which the transgene carried by the virus will trigger the silencing of any endogenous genes of the same sequence.

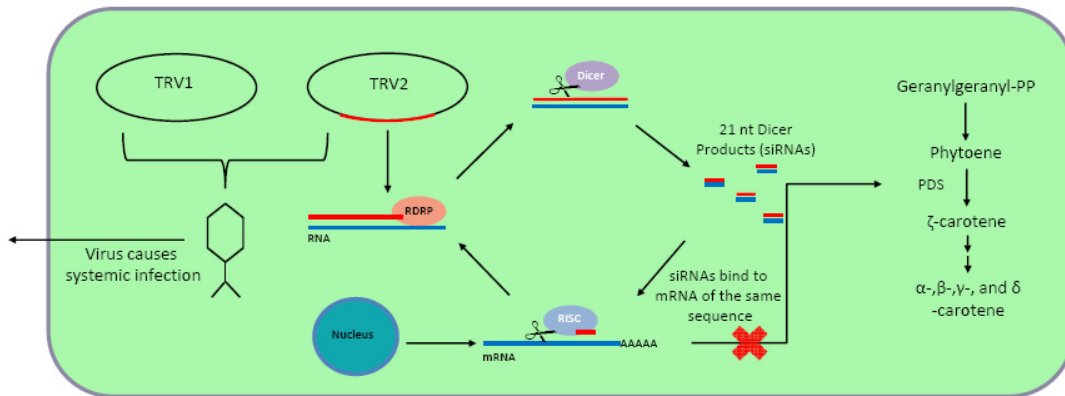


Figure 2 Schematic representation of the VIGS biological pathway. Transcription of the gene fragment contained in TRV2 binds to endogenous mRNA or is recognised as aberrant by the RdRP and made double stranded. The dsRNA is cleaved by Dicer to produce 21 nt fragments which are incorporated into the RISC, targeting homologous mRNA. The cleaved mRNA is again recognised as aberrant by the RdRP for amplification of the silencing response.

VIGS is an excellent system for rapid and specific gene repression studies in plants. The requirement and cost of producing VIGS infected plants is extremely low in comparison to all other silencing techniques discussed above. The production of chemical, radiation, or T-DNA insert mutants for a specific gene requires thousands of plants and laborious screening and the mutations mapped. VIGS only requires a small number of plants and, when fully optimized, doesn't require screening or mapping of the mutation. Sense, antisense and hairpin-RNA silencing, to a large degree, also overcome some of these limitations but still require screening. VIGS has a further advantage over these techniques in that no transgenesis is required to cause silencing. Indeed from the identification of a gene sequence, the repression of its product in plants can be done in less than a month. Furthermore, because VIGS is initiated after germination of the plant it is possible to use it for the targeting of essential or deleterious genes.

The fact that VIGS can be performed so rapidly makes it an excellent tool for starch research. While starch may initially seem to be a relatively simple molecule, made up solely of glucose, there is an amazing structural and physico-chemical diversity between species and even organs within the same plant. This diversity within starch structure is, to a large extent, attributed to the complement of enzymes involved in its synthesis and degradation. Within these suits of enzymes there exists a high degree of redundancy which can often complicate their study. In the chapters that follow it will be shown how VIGS systems can be used to effectively study this complex pathway in the leaves of *Nicotiana benthamiana*.

The synthesis and degradation of starch is a very complicated but essential part of plant metabolism. As discussed above, there are many enzymes involved in the process storing and remobilizing energy into and out of starch. While much progress has been made in this area of research many questions still remain especially when it comes to the specific interactions of the identified enzymes. In the next chapter I will discuss the effects on starch and general metabolism when an enzyme, plastidial pyrophosphatase, is silenced. This enzyme breaks down pyrophosphate into inorganic phosphate which can be exported from the plastid and is thought to maintain an intra-plastidial environment which is favourable for starch synthesis. Chapter 4 deals with the establishment of a VIGS system able to silence multiple genes simultaneously. This technique is then used to study the complex interactions of starch degradative enzymes and shows how the use of this technique can reveal unexpected phenotypes.

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Chapter 3

Research Article

Virus induced gene silencing repression of plastidial inorganic pyrophosphatase leads to specific impairments in photosynthetic-related processes in *Nicotiana benthamiana*

3 Virus induced gene silencing repression of plastidial inorganic pyrophosphatase leads to specific impairments in photosynthetic-related processes in *Nicotiana benthamiana*.

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Abstract

Pyrophosphate metabolism, although poorly understood, has profound effects on the primary plant metabolism. Here we report on the transient down regulation of the native inorganic pyrophosphatase (psPP) in *N. benthamiana* targeted to the plastid. Physiological and metabolic perturbations in the leaves were specific to plastidial localized pathways that were generally reliant on fast and efficient pyrophosphate-dissipation. Plants lacking plastidial soluble psPP were characterized by increased pyrophosphate levels and also significant reductions in starch, chlorophyll, carotenoid and abscisic acid biosynthesis. Interestingly several phenylpropanoid compounds were significantly increased in these plants. In contrast, constituents like sucrose, glucose and fructose levels remained unaltered. Furthermore, plants with lowered psPP exposed to mild drought stress conditions showed a moderate wilting phenotype and reduced vitality under these conditions. These results were discussed within the context of biosynthetic pathways affected, demonstrating the importance of plastidial PP_i metabolism.

As first author of this paper I was responsible for the experimental planning, and the majority of the experiments described in this paper. Margaretha van der Merwe was responsible for the GCMS analysis and phytohormone profiling, as well as assisting with pyrophosphate determinations.

3.1 Introduction

Pyrophosphate (PP_i) is generated as a by-product in the activation or polymerization steps of nucleic acid, carbohydrate, protein, amino acid, fatty acid and carotenoid biosynthesis (Geigenberger et al., 1998; Stitt, 1998; Rojas-Beltrán et al., 1999; Farré et al., 2001; Sonnewald, 2001; López-Marqués et al., 2004). Its removal is essential to prevent the inhibition of thermodynamically unfavourable reactions (Geigenberger et al., 1998; López-Marqués et al., 2004) and/or to generate ATP (Pérez-Castiñeira et al., 2001). PP_i is generally removed by inorganic pyrophosphatases which hydrolyse PP_i to inorganic phosphate (Pi). Inorganic pyrophosphatases are ubiquitous in plant cells and found both as soluble forms in the cytosol and plastid, as well as membrane-bound forms on the tonoplast (Rea and Poole, 1993; Baltscheffsky et al., 1999; Maeshima, 2000), mitochondria (Vianello and Macrí, 1999) and chloroplast (Jiang et al., 1997).

In *Arabidopsis thaliana* six soluble pyrophosphatase (sPP) isoforms have been identified to date (Schulze et al., 2004). Five of these (*AtPPa1*, 2, 3, 4 and 5) are far more similar to each other than to *AtPPa6* (Schulze et al. 2004), and are localized to the cytosol (Ergen, 2006). In potato two sPP genes, *StPPa1* and -2 which are most similar to *AtPPa1*, have also been identified and have been demonstrated to be present in the cytosol using immunogold labelling (Rojas-Beltrán et al. 1999). In addition to sPPs, several other cytosolic enzymes can remove PP_i , including the soluble enzymes pyrophosphate:fructose 6-phosphate phosphotransferase (PFP) and UDP-glucose pyrophosphorylase (UGPase). Due to a lack of adverse phenotypic alterations found when altering the expression of these enzymes, a considerable redundancy in cytosolic PP_i catabolism is evident (Hajirezaei et al., 1994; Paul et al., 1995).

Studies aimed at elucidating the biological role of PP_i in plants, through heterologous expression of a cytosolically targeted *Escherichia coli* inorganic pyrophosphatases (IPP), demonstrate that this leads to major effects on partitioning between sugar and starch (Sonnewald, 1992; Geigenberger et al., 1998; Farré et al., 2000; Lee et al. 2005). In contrast, when the same *E.coli* IPP was targeted to the plastid, only minor changes in metabolite levels were observed (Farré et al. 2006). Considering that the majority of sPP activity resides in the plastid (Gross and ap Rees 1986, Weiner et al. 1987, Gómez-García et al. 2006), the most likely explanation for this observation is the efficient removal of plastidial PP_i . This hypothesis is supported by the relatively low PP_i levels found within the plastid (5 - 15% of the total) compared to the cytosol (68 - 78% of the total) (Weiner et al. 1987; Farré et al., 2006). In *Arabidopsis* a single isoform, *AtPPa6*, with an N-terminal extension predicted to

encode for a plastidial transit peptide, has been identified (Schulze et al., 2004). Subsequent plastidial import experiments (Schulze et al., 2004) and GFP localization studies (Ergen, 2006) have confirmed its localization to this subcellular compartment. Plastidial PP_i generation occurs during several biosynthetic pathways within the chloroplast, for example starch, chlorophyll, carotenoid and xanthophyll biosynthesis (Figure 1) and it is hypothesized that these pathways could be severely inhibited if PP_i is not effectively removed within the plastid.

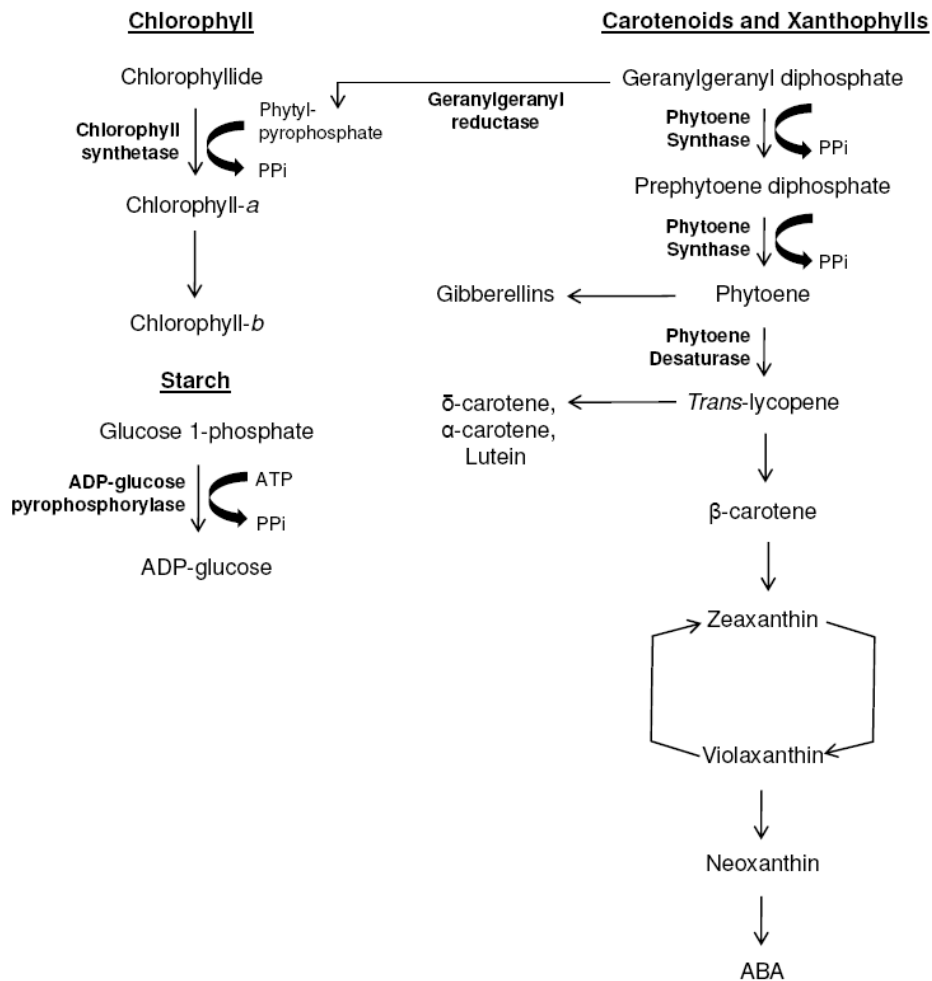


Figure 1 Schematic representation of known plastidial reactions generating pyrophosphate (PP_i). Chlorophyll synthetase utilizes chlorophyllide and phytol-pyrophosphate to produce chlorophyll-a and releases PP_i during chlorophyll biosynthesis. During carotenoid and xanthophyll biosynthesis, phytoene synthase produces phytoene from geranylgeranyl diphosphate in a two step reaction which releases two PP_i molecules. In addition, geranylgeranyl diphosphate can be converted to phytol-pyrophosphate by geranylgeranyl reductase which can then be utilized in the chlorophyll synthetase reaction. Phytoene serves as a precursor to many other

metabolites, including gibberellins, carotenoids, xanthophylls and abscisic acid (ABA). Lastly, ADP-glucose pyrophosphorylase, one of the key enzymes in starch biosynthesis, produces PP_i as a by-product in the conversion of glucose 1-phosphate to ADP-glucose.

Here we demonstrate, through a transient platform and metabolite profiling, that virus induced gene silencing (VIGS) repression of the native plastidial sPP (psPP) gene in *Nicotiana benthamiana* leaves leads to a reduction in starch, carotenoid and chlorophyll levels. This impairment also lead to plants which are less able to cope with drought stress due to an inability to synthesize abscisic acid (ABA) and thus regulate stomatal opening, and suggested a distinct role for soluble plastidial pyrophosphatase activity in coordinating photosynthetic carbon assimilation and plant performance.

3.2 Methods

Plant material and growth conditions

Nicotiana benthamiana seeds were surface-sterilized by a series of successive 70% (v/v) ethanol, 1% (v/v) sodium hypochlorite, and sterile water washes. Seeds were allowed to germinate on 0.4% (w/v) agar (Plant Gel, Highveld Biotechnology, South Africa) supplemented with 4.32g.L⁻¹ Murashige and Skoog basal salt media and 15% (w/v) sucrose. The plants were grown in a 16h/8h light/dark regime at 24°C. At the four leaf seedling stage, plants were transferred to 1L tissue culture containers with the same growth media constituents for ten days before being subjected to VIGS infiltration.

VIGS plasmid construction, transfection and plant generation

Construct design of tobacco rattle virus vectors (pTRV1, pTRV2, and pTRV2-PDS) for virus induced gene silencing (VIGS) have been previously described (Liu et al., 2002a). The tomato EST CLET20N17 was obtained from the Clemson University Genomics Institute and was used to produce a VIGS vector able to repress plastidial psPP activity (TRV2-psPP). This was done by isolating a *KpnI/BamHI* fragment from CLET20N17 and ligating it into the same restriction sites in pTRV2. For infiltration of *N. benthamiana* all vectors were transformed into *Agrobacterium tumefaciens* (strain GV2260) by the freeze-thaw method (Höfgen and Willmitzer, 1988). Transformed *A. tumefaciens* were grown in liquid LB media containing the appropriate antibiotics (25 µg.ml⁻¹ rifampicin, 50 µg.ml⁻¹ kanamycin, 100 µg.ml⁻¹ carbenicillin, 50 µg.ml⁻¹ streptomycin) and 100µM acetosyringone. The bacterial cells were subsequently pelleted at 4000g for 5 min, 4°C and re-suspended in infiltration media (10mM MgCl₂, 10mM MES, 100µM acetosyringone) to an OD₆₀₀ of 0.5. Seedlings (ca. 4 – 5

weeks after germination and 50mm in height) were removed from the tissue culture containers and placed inside a 60ml syringe containing 20ml of the *Agrobacterium* suspension (containing a 1:1 mix of TRV1 and either TRV2 or TRV2-psPP). The air volume was adjusted to 20ml before the nib of the syringe was stoppered. While the leaves of the plant were submerged, the air volume was increased to 40ml, corresponding to a vacuum of 50kPa, and held there for 30 seconds after which the vacuum was broken.

Post-infiltration the seedlings were planted in sterile soil (supplemented with silica sand and vermiculite [8:1:1]) and placed in a clear plastic bag to progressively harden off over 4 – 5 days. Plants were then grown under prevailing greenhouse conditions as stated before. Unless stated otherwise, leaves three to five from eight-week old plants were harvested at midday, rapidly flash frozen and stored at -80°C until sample processing.

Total sPP activity measurements

Total leaf protein extracts were obtained from TRV2-psPP and TRV2-empty vector infected plants by vortexing homogenised tissue with pre-cooled extraction buffer (50mM Tris-HCl (pH 7.5), 5mM DTT, 2mM EDTA, 0.1% (v/v) Triton X100). The total protein extract was centrifuged (13000g for 20min, 4°C) and the supernatant was recovered. Activity assays were performed as previously described (Schulze et al., 2004).

Chloroplast enrichment

Leaf material was harvested from plants which had been destarched by darkening over a 48h period. Leaves were submerged in 100ml ice cold homogenization buffer (0.45M sorbitol, 20mM Tricine-KOH (pH 8.4), 10mM EDTA, 10mM NaHCO₃ and 0.1% (w/v) bovine serum albumin) for 5min before being homogenized by three 1 second bursts in a polytron blender. The material was filtered through two layers of cheese cloth and centrifuged at 300g for 5min at 4°C. The plastid pellet was resuspended in 2ml resuspension buffer (0.3M sorbitol, 20mM Tricine-KOH (pH 7.6), 5mM MgCl₂, 2.5mM EDTA). The mixture was overlaid onto a Percoll discontinuous step gradient of 80 and 40% (v/v) and centrifuged for 6min at 13000g, 4°C. Plastids were collected from the resulting interphase and resuspended in three volumes of resuspension buffer. After centrifugation at 3000g for 2min, 4°C the pellet was resuspended in 500µl protein extraction buffer described above and sonicated for three 1 second bursts separated by 10 second incubations on ice. Marker enzyme assays were performed to determine the degree of plastid enrichment. For this purpose non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ADP-glucose pyrophosphorylase (AGPase), UDP-glucose pyrophosphorylase (UGPase), and

phosphoenolpyruvate carboxylase (PEP carboxylase) activities were determined for plastidial and cytosolic enrichments, respectively. GAPDH was assayed according to Fernie et al. (2001), AGPase according to Sweetlove et al. (1996), PEP carboxylase according to Merlo et al. (1993) and UGPase according to Sowokinos (1976).

In-gel sPP activity and denaturing protein gel analysis

sPPase in-gel assays were performed by running either total or plastid enriched protein extracts on a non-denaturing 10% (v/v) polyacrylamide gel at 4°C. The polyacrylamide gel was subsequently incubated in 20ml pyrophosphatase assay buffer (as described above) for 1h at room temperature. It was then washed for 5min in distilled water before a further 5min incubation in 1% (w/v) ammonium molybdate, 5% (w/v) FeSO₄ and 0.5M H₂SO₄. Colour development was stopped by washing the gel for 5 min in excess water. Total protein was extracted from leaf disks as described above. Protein was denatured according to Laemmli et al. (1970) and run on a 10% (v/v) denaturing gel which was subsequently stained with Coomassie Blue. Immunoblot analysis was performed by running 30 µg of total protein on a 10% (v/v) SDS-PAGE gel. Following separation of proteins, the samples were blotted onto PVDF membrane using a semi-dry method (Sambrook et al. 1989) and immunoblots were performed using previously described antibodies (Rojas-Beltrán et al. 1999).

PGM and AGPase assays

Protein aliquots, extracted as described above, were assayed for phosphoglucomutase (PGM) and ADP glucose pyrophosphorylase (AGPase) activity. In gel assays for PGM were performed by loading 30µg of total protein onto a 10% (v/v) native polyacrylamide gel containing 4mg glycogen (*Mytilus edulis* type VII, Sigma Co.). The gel was stained according to Vallejos (1983). AGPase was measured by adding 20µl protein extract to 20mM Hepes-KOH (pH 7.1), 2mM MgCl₂, 0.5mM NAD⁺, 2mM 3-PGA, 1.5mM ADP-glucose, 1U glucose-6-phosphate dehydrogenase (*Leuconostoc*) and 1U PGM (from rabbit muscle). The addition of 10µl of 20mM PP_i was used to start the reaction and the activity was followed spectrophotometrically at 340nm (Sweetlove et al., 1996).

Total protein concentrations were measured according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

Drought stress treatment

TRV2 control and TRV2-psPP plants were subjected to a mild drought stress by drenching the root system with 10% (w/v) polyethyleneglycol 6000 (PEG-6000) for 12h and subjected

to phenotypical and biochemical evaluation. Well-watered plants served as a control for each transformant.

Stomatal conductance and carbon assimilation

Stomatal conductance was measured on a EGM-4 Environmental Gas Monitor (PP Systems, USA) Readings were taken directly prior to harvesting at midday on the fourth leaf of each plant with the flow rate ($50\text{ml}\cdot\text{min}^{-1}$) and temperature (25°C) kept constant. Carbon assimilation rates were estimated using an infrared gas analyzer (Ciras-1, PP system, UK). Each measurement was made on the third fully expanded leaf from psPP and control plants. CO_2 concentrations were modulated by mixing external CO_2 -free air with pure CO_2 to obtain concentrations of 0, 380, and $1000\ \mu\text{mol}\cdot\text{mol}^{-1}$. The measurements were performed at flow rate of $350\ \text{ml}\cdot\text{min}^{-1}$, at $25\ ^\circ\text{C}$, using a PAR of $1400\ \mu\text{mol}\ \text{m}^{-2}\ \text{s}^{-1}$ inside leaf chamber.

PP_i determination

PP_i was extracted from leaf tissue by a TCA/ether method (Jelitto et al., 1992). Briefly, homogenized tissue (50mg) was extracted with $300\ \mu\text{l}$ 16% (w/v) TCA/ether and incubated for 3h, 4°C . After adding $250\ \mu\text{l}$ of 5mM EGTA, 16% (w/v) TCA/H₂O the samples were centrifuged at $14000g$, 4°C . The bottom phase was transferred to a new tube, washed four times with water saturated ether, and the supernatant adjusted to pH6.0 using HCl. PP_i was determined using the colorimetric P_iPer pyrophosphate cycling assay kit (Invitrogen, USA) according to the manufacturers' specifications. PP_i levels were deduced from P_i by using a sample blank with or without sPPase for each individual sample, and total P_i calculated by comparison of fluorescence at 595nm with a linear authentic P_i standard curve.

Soluble sugar and starch measurements

Soluble sugars were extracted from leaf material by incubating 50mg homogenized tissue in 1ml 80% (v/v) ethanol at 80°C for 1h. Samples were then cooled on ice for 5min and centrifuged at $16000g$ for 10min, 4°C . The supernatant was used for the enzymatic determination of glucose, fructose and sucrose according to Müller-Röber et al. (1992). Starch was extracted from the pellet with the addition of $400\ \mu\text{l}$ 0.2M KOH and incubated at 95°C for 1h. Samples were neutralized with $7\ \mu\text{l}$ glacial acetic acid, the starch digested to glucose and quantified as previously described (Müller-Röber et al., 1992).

Primary metabolite determination

Primary metabolites levels were extracted and derivatized as previously described (Roessner et al., 2001). Samples were injected splitless into a GCT Premier™ benchtop

orthogonal acceleration time-of-flight (oa-TOF) MS (Waters, USA) and run according to specifications of Erban et al. (2007). The GC was mounted with a 5PDM VF-5 ms, 0.25 μ m film thickness, 30m \times 0.25mm fused silica capillary column (Varian, Darmstadt, Germany), which had an integrated 10m guard column. The injector temperature was 230 $^{\circ}$ C and helium carrier gas used at a constant flow of 1 mL.min⁻¹. Purge time and flow reduction was 1min. The temperature programming comprised of an initial 1min isothermal period at 70 $^{\circ}$ C and a 7.7 $^{\circ}$ C.min⁻¹ ramp to 310 $^{\circ}$ C. TOF-detection was performed using a MassLynx mass spectrometry system (Waters, USA). Mass spectral recording was set to 20 scans.s⁻¹. Transfer line and ion source temperatures were set to 250 $^{\circ}$ C. The monitored mass range was m/z 70–600. Data integration and quantification was processed using TagFinder software (Luedemann et al., 2008).

HPLC analysis of carotenoids and xanthophylls

Carotenoid and xanthophyll extraction and determination was based on a method from Taylor et al. (2006). Homogenised leaf discs (64 mm²), taken from the middle of the fourth leaf of TRV2 and TRV2-psPP plants, were incubated for 5min with 100 μ l methanol containing β -apo-caroten-8-al as internal standard. After adding 100 μ l 50mM Tris-HCl (pH 8.0) and 1M NaCl the mixture was incubated for another 5min. The carotenoids and xanthophylls were partitioned from this solution by addition of 400 μ l chloroform and centrifugation at 3000g for 5min, 4 $^{\circ}$ C. This was done twice and the lower chloroform phases were pooled and dried under vacuum. Samples were immediately resuspended in an ethyl acetate:methanol (1:4) mixture with 0.1% (w/v) butylated hydroxytoluene (BHT). The samples were run exactly according to specification from Taylor et al. (2006), and normalized as a response equivalent to 53.5ng β -apocaroten-8-al injected.

Phytohormone profiling

Phytohormones were extracted according to Edlund et al. (1995). In brief, 500 μ l of 0.05M Na-phosphate buffer (pH 7.0) was added in a 10:1 ratio to homogenized leaf tissue, and incubated for 1h in the dark, continuous shaking, 4 $^{\circ}$ C. After extraction, the pH was adjusted to 2.6 using HCl, the sample enriched with ~35mg Amberlite XAD-7 (Serva, Heidelberg, Germany) and further incubated for 1h in the dark, continuous shaking at 4 $^{\circ}$ C. After centrifugation, the XAD-7 was washed twice with 500 μ l 1% (v/v) acetic acid before elution with 500 μ l dichloromethane for 30min and elution repeated once more. The combined dichloromethane fractions were reduced under vacuum till dryness. Derivatisation of the sample (modified from Schmelz et al., 2003) was achieved by adding 50 μ l 2.0M trimethylsilyl diazomethane in hexane (Sigma Co.) and 10 μ l methanol, and incubating at RT for 30min.

Excess TMS diazomethane was destroyed by adding 50 μ l 1% acetic acid. *n*-Alkane time standards was added to each sample prior to reducing the sample to dryness under vacuum. Samples were resuspended in 50 μ l heptane and injected splitless into a GCT Premier™ benchtop orthogonal acceleration time-of-flight (oa-TOF) MS (Waters, USA). Running conditions was exactly as previously described (Edlund et al., 1995), and phytohormone identification and quantification was done by means of linear calibration curves for authentic standards and the mass spectrums adjusted accordingly.

Statistical analysis

Unless otherwise specified, statistical analyses were performed using *Students t*-test embedded in the Microsoft Excel software (Microsoft, Seattle). Only the return of a p-value < 0.05 was designated significant. Linear correlations were performed in the Pearson linear regression algorithm embedded in the XLSTAT software (Microsoft, Seattle).

3.3 Results

VIGS to repression of plastidial sPPase activity

The Arabidopsis *AtPPa6* cDNA sequence (Schulze et al., 2004) was used to identify a *Solanum lycopersicum* EST (CLET20N17) that might encode for a plastidial localized sPP protein. CLET20N17 shares 78.2% identity to the plastidial *AtPPa6* cDNA while it is only between 39.2% and 44.5% identical to the cytosolic targeted *AtPPa1-5* Arabidopsis sequences. This sequence (designated *SlpsPP*) was ligated into the multiple cloning site of the deconstructed TRV2 vector, previously reported for the VIGS platform in *Nicotiana benthamiana* (Liu et al., 2002a). Subsequently, *N. benthamiana* seedlings were infiltrated with an *Agrobacterium tumefaciens* mixture of both TRV1 and TRV2 (control) or both TRV1 and TRV2-*psPP* (silencing) plasmids. In order to monitor transfection efficiency and growth conditions, the TRV2-PDS vector (containing a cDNA encoding phytoene desaturase; Liu et al., 2002b) was used in parallel as an internal control (data not shown).

When assessing the degree of down-regulation of the native sPP protein, total activity was measured in crude protein extracts from leaves. TRV2-*psPP* silenced plants showed a significant reduction in total sPP activity when compared to the TRV2 controls (Figure 2 A, B and C). To examine whether the repression was specific to the plastidial isoform, a fractional enrichment study was performed. Protein extracts from the plastid enriched fraction were not significantly contaminated by the cytoplasm, as demonstrated by the negligible cytosolic marker enzymes (UDP-glucose pyrophosphorylase and PEP carboxylase) activities (Supplemental Table I). The crude extract and plastid enriched fractions were separated by non-denaturing SDS PAGE and in-gel assayed for sPP activity. A single band of similar size could be observed in both the crude and plastid enriched TRV2 control extracts (Figure 2A). The activity band was significantly reduced in crude extracts from the TRV2-*psPP* leaves (Figure 2B). In addition, determination of total pyrophosphate content from leaves of TRV2-*psPP* silenced and TRV2 control plants indicated a ~60% increase in cellular PP_i content in the *psPP* silenced plants compared to the TRV2 control plants (Figure 2D). To determine whether this increase was indeed caused by a reduction in the plastidial isoform an immunoblot for the soluble pyrophosphatases was performed which showed a complete reduction in the plastidial isoform where the cytosolic was still detectable in the *psPP* line of plants (Figure 2E).

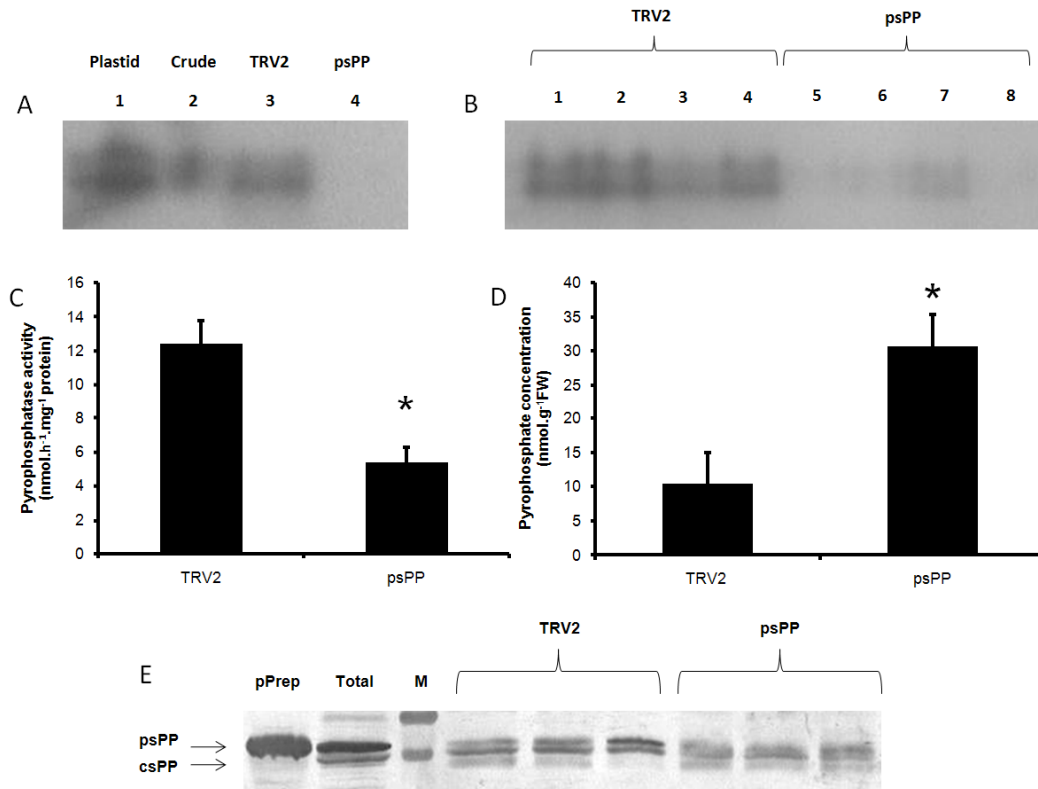


Figure 2 In gel activity assays of plastidial TRV2 control preparations (A, lane 1) and crude TRV2 control extracts (A, lane 2) in *N. benthamiana* leaves. Crude protein activity assays for TRV2 control (A, lane 3; B, lane 1-4) and TRV2-psPP silenced plants (A, lane 4; B, lane 5-8). Total leaf pyrophosphatase activity of crude protein extracts from TRV2 control and TRV2 psPP silenced plants (C). Pyrophosphate (PP_i) content in *N.benthamiana* leaves of TRV2 control and TRV2 psPP silenced leaves (D). (E) Immunoblot for soluble pyrophosphatases in isolated plastids (pPrep), total leaf extract (Total), and three TRV2 control as well as three psPP plants. Arrows to the left denote the plastidial and cytosolic sPP isoforms. Values are presented as mean ± SE of five individual plants per line and values with an * were determined by *Students t*-test to be significantly different ($P < 0.05$) from the TRV2 control.

Effect of reduced psPP activity on primary metabolism

In order to evaluate the effect of reduced psPP activity on leaf metabolism the physiology and intermediates of central metabolism were further investigated. The most striking visible phenotype of plants infiltrated with the TRV2-psPP construct was a mottled appearance of the leaves compared with the TRV2 control (Figure 3) suggesting that chlorophyll content and photosynthetic performance might be significantly affected in these plants. To further investigate this, photosynthetic leaf pigments were analyzed by HPLC and number of chlorophylls, carotenoids and xanthophylls were identified (Table 1). This analysis indicated that β -carotene, chlorophyll a (chl *a*) and violaxanthin content of psPP silenced plants were

reduced between 30 and 50% compared to the TRV2 controls (Table 1). In contrast, chlorophyll b (chl *b*) and lutein remained invariable from the control, whilst zeaxanthin content was increased three fold compared to the TRV2 control (Table 1).

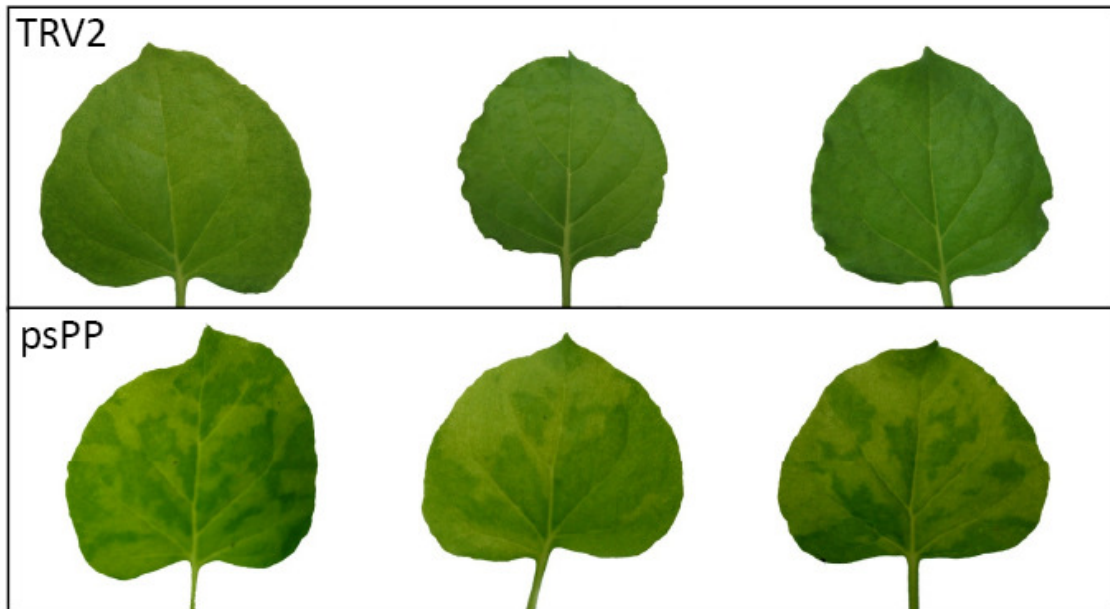


Figure 3 Phenotypic assessment of VIGS infiltrated TRV2 control plants (upper pane) compared to TRV2-psPP silenced leaves (lower pane). The psPP silenced plants showed a mottled phenotype three weeks post infection.

Table 1 Pigment levels of VIGS infiltrated TRV2 control and TRV2-psPP silenced leaf discs grown under greenhouse conditions. Values are presented as mean \pm SE of three individual plants per line and bold values were determined using *Students t*-test to be significantly different ($P < 0.05$) from the TRV2 control. Values are normalized to the internal standard β -apocaroten-8-al (response.g⁻¹ FW).

<i>Pigment</i>	<i>TRV2 control</i>	<i>TRV2-psPP silenced</i>
<i>(response.g⁻¹FW)</i>		
β -carotene	29.7 \pm 0.8	20.9 \pm 2.0
Chlorophyll a	504.4 \pm 5.3	377.9 \pm 30.9
Chlorophyll b	134.8 \pm 1.8	129.4 \pm 12.9
Lutein	20.4 \pm 0.3	17.4 \pm 2.3
Neoxanthin	2.3 \pm 0.2	2.6 \pm 0.1
Violaxanthin	6.2 \pm 1.0	3.2 \pm 1.1
Zeaxanthin	3.6 \pm 0.0	10.9 \pm 1.5

To further determine the consequence(s) of reduced sPP activity, leaf starch content of TRV2-psPP silenced plants and TRV2 controls was measured under prevailing greenhouse conditions. The leaves of the psPP silenced plants showed a significant reduction in starch levels (Table 2) while the soluble sugars (sucrose, glucose and fructose) did not change in comparison with the TRV2 control (Table 2). The carbon assimilation rate of psPP silenced leaves compared to TRV2 controls was tested to determine whether the reductions in chl-a would affect the rate of photosynthesis (Figure 4a). According to A/ci curve analysis it was found that indeed, in the psPP silenced plants, the rate of carbon assimilation was less than half that observed in the controls. The amount of Rubisco is known to affect carboxylation rates in photosynthesising leaves (Farquhar et al. 1980). Rubisco is easily identified in leaf extracts as one of the most abundant proteins and so to determine whether it was being influenced by silencing sPP protein extracts were made from leaf discs and separated on a denaturing acrylamide gel before being stained with Coomassie brilliant blue (Figure 4b). While there was no obvious difference in any other protein, both subunits of Rubisco are greatly reduced in the leaves of the psPP silenced plants.

Table 2 Effect of down-regulation of plastidial soluble pyrophosphatase (psPPase) activity on soluble sugar and starch levels of *N.benthamiana* leaves of TRV2 control and TRV2-psPP silenced plants under well watered and drought induced conditions. Values are presented as mean \pm SE of five individual plants per line and bold values were determined using *Students t*-test to be significantly different ($P < 0.05$) from the respective TRV2 control.

<i>Metabolite</i>	<i>TRV2</i>	<i>TRV2-psPP</i>	<i>TRV2</i>	<i>TRV2-psPP</i>
$(\mu\text{mol.g}^{-1}\text{FW})$	<i>watered</i>	<i>watered</i>	<i>drought stressed</i>	<i>drought stressed</i>
Starch	5.5 \pm 1.0	3.0 \pm 0.1	7.5 \pm 1.0	3.0 \pm 1.0
Glucose	0.4 \pm 0.1	0.5 \pm 0.1	0.6 \pm 0.2	0.4 \pm 0.1
Fructose	0.8 \pm 0.1	1.1 \pm 0.2	1.1 \pm 0.1	0.7 \pm 0.1
Sucrose	0.9 \pm 0.1	0.8 \pm 0.1	0.9 \pm 0.1	0.8 \pm 0.1

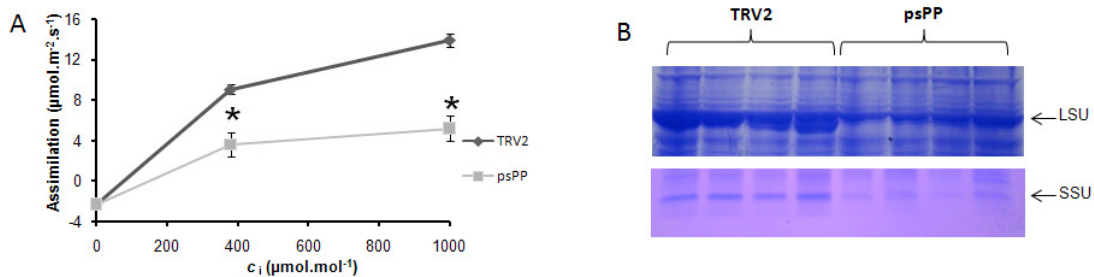


Figure 4 Photosynthetic carbon assimilation of psPP when supplied with different concentrations of CO₂ (n=4) (A). Total protein extracts from leaf disks of control and psPP silenced plants, where the large and small subunits (LSU) of Rubisco are indicated (B). Values are presented as means \pm SE and the values marked with an * were determined by *Students t*-test to be significantly different ($P < 0.05$) from the TRV2 control.

Although the activity of phosphoglucomutase (PGM) remained unchanged from the TRV2 control (data not shown), determinations of ADP-glucose pyrophosphorylase (AGPase) indicated a 40% increase in the total activity (Figure 5A). To establish that the observed decrease in starch was due to increased PP_i levels and not to an indirect effect resulting from a reduction in photosynthetic pigments, transfected plants were dark-adapted for three days to allow complete starch degradation in the leaves. Subsequently, the leaf discs were transferred onto sucrose, kept in the dark, and starch accumulation measured at defined intervals (Figure 5B). Starch levels increased significantly in the TRV2 control plants, while psPP silenced plants could not accumulate starch under these conditions (Figure 5B).

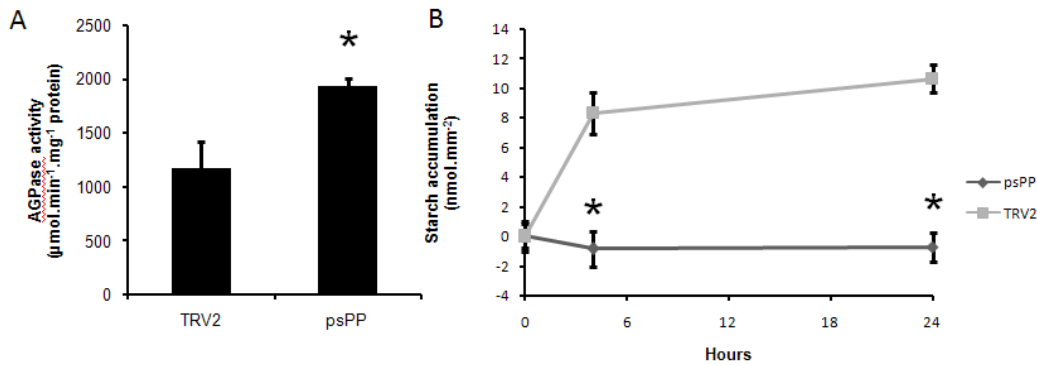


Figure 5 Characterization of enzyme activity of AGPase in TRV2 control and TRV2-psPP silenced plants (n=4) (A). Starch accumulation rates on sucrose media of de-starched leaf discs of TRV2 control and TRV2-psPP silenced plants during a darkened 24h period (n=7) (B). Values are presented as means \pm SE and the values marked with an * were determined by *Students t*-test to be significantly different ($P < 0.05$) from the TRV2 control.

Influence of drought stress in leaves with reduced plastidial sPP activity

In order to evaluate the vitality of plants with reduced plastidial sPP activity, a short term mild drought stress was induced, and phenotypically and biochemically evaluated. Stomatal conductance was similar between the TRV2 control and TRV2-psPP plants under well watered conditions (Figure 6A). Treatment with 10% (w/v) PEG-6000, however, reduced stomatal conductance by approximately 55% in the control and 30% in the TRV2-psPP silenced plants (Figure 6A), suggesting that the transpiration rate was significantly greater under the mild drought stress in the psPP silenced plants than the control. Because of this observation, ABA levels were measured. This showed an opposite pattern with respect to the drought-stressed TRV2 control and TRV2-psPP silenced plants. ABA was increased 3-fold in the drought stressed control plants, while in TRV2-psPP plants ABA levels were invariable between treatments or from the TRV2 unstressed control (Figure 6B). As a consequence, TRV2-psPP stressed plants showed an accelerated wilting phenotype compared to the control (Figure 6C). In addition, PP_i concentrations in the psPP silenced plants remained significantly increased in comparison with the TRV2 controls under both well watered and PEG-6000 treated plants (Figure 6D). Similarly, although soluble sugar levels were indistinguishable between the plants, there was still significantly less starch in the TRV2-psPP plants than the controls under drought stress conditions (Table 2). Other phytohormones measured indicated, with the exception of an unknown mass spectral tag (MST) (designated MST8350_24105), no significant differences between control and silenced plants from the same treatment (Supplemental Table 2).

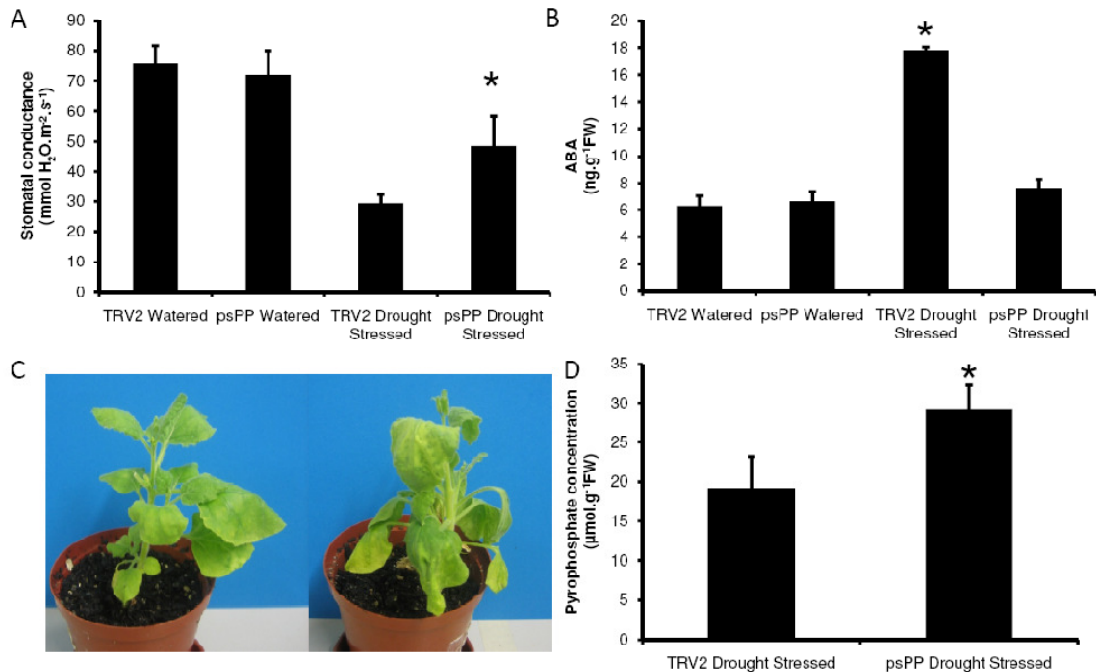


Figure 6 Stomatal conductance measured for well watered and 12h drought stressed treatment in TRV2 control and TRV2-psPP silenced plants (A). Abscisic acid (ABA) levels measured in the leaves under similar conditions for TRV2 control and TRV2-psPP silenced plants (B). Phenotypical assessment of wilting response of TRV2 control (left pane) compared to the TRV2-psPP silenced plants (right pane) (C). Effect of drought stress treatment on PP_i levels in TRV2 control and TRV2-psPP plants (D). Values are presented as mean \pm SE of five individual plants per line and values with an * were determined by *Students t*-test to be significantly different ($P < 0.05$) from the respective TRV2 control.

In order to evaluate other changes in metabolism, a GC TOF MS based metabolite profile of the TRV2 control and TRV2-psPP silenced plants under the different treatments was compiled (Figure 7). The non-redundant metabolites identified (119) indicated that, in TRV2-psPP silenced plants under well-watered conditions, citrate levels significantly decreased, whilst glyceraldehyde 3-phosphate, malate, quinate, myo-inositol, tryptophan, histidine, serine, ferulate, coniferylalcohol, 3-caffeoyl quinate, 4-caffeoyl quinate, 5-caffeoyl quinate, 1-pyrroline-2-carboxylate, MST_1431.3_TOF and MST_1856.23_TOF (a MST similar in fragmentation pattern to pinitol) levels significantly increased compared to the respective TRV2 control (Figure 7). Interestingly, the majority of these metabolite changes were also reflected when exposing the TRV2 control to drought-stressed conditions. TRV2-psPP drought-stressed plants, on the other hand, revealed few further changes compared to the TRV2 stressed control (Supplemental Table III). The exception to this was a significant increase in 2-oxoglutarate and 3,5-dimethoxy-4-hydroxycinnamate (Figure 7), and decreased homocysteine levels in the TRV2-psPP stressed leaves (Supplemental Table III).

Furthermore, significant positive correlations between PP_i levels and ferulate, coniferylalcohol, malate, quinate, MST_1856.23_TOF, histidine, glyceraldehyde 3-phosphate and 5-caffeoyl quinate ($R^2 > 0.83$), and significant negative correlations between PP_i levels and chlorophyll a and β -carotene ($R^2 > 0.81$) content were observed (Supplemental Table 4).

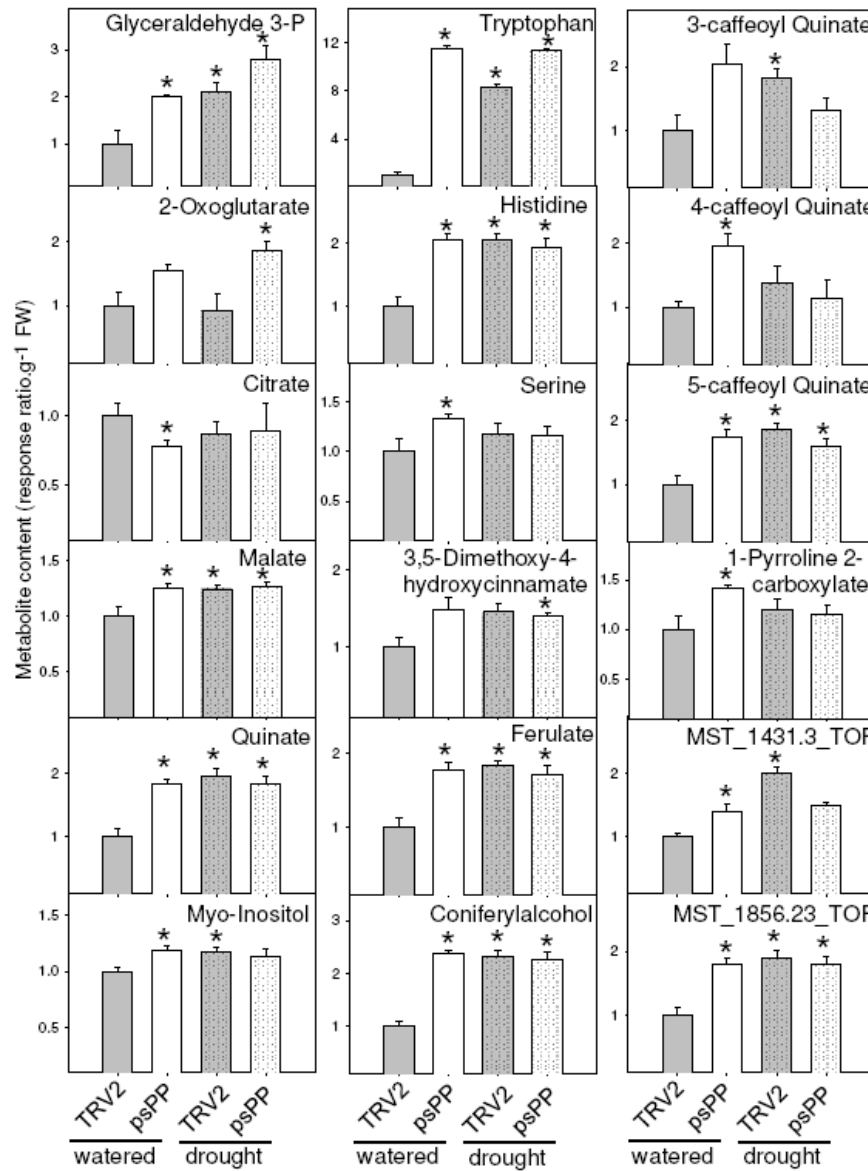


Figure 7 Relative metabolite content in *N.benthamiana* leaves of TRV2 control and TRV2-psPP plants. Metabolites levels were determined as described in Materials and Methods. Data are normalized with respect to the mean response calculated for the TRV2 unstressed control (to allow statistical assessment in the same way). Values are presented as mean \pm SE of five individual plants per genotype/treatment and an asterisk * were determined using *Students t*-test to be significantly different ($P < 0.05$) from the unstressed TRV2 control.

3.4 Discussion

The gene coding for the psPP has been identified (Schulze et al., 2004), however, we have been unable to identify insertion mutants of it in *Arabidopsis*. We decided, therefore, to use VIGS to study its role in *N. benthamiana* leaf metabolism. The cDNA sequence used for VIGS infiltration showed much greater similarity to genes known to code for plastidial sPP isoforms than cytosolic ones. Analysis of total sPP activity in leaf material of eight week old plants revealed that VIGS repressed sPP activity by approximately 60% in the TRV2-psPP plants compared to the TRV2 control demonstrating that the majority of the sPP activity is repressed by the VIGS construct (Figure 2B, C). It has been shown that the plastidial sPP accounts for the majority (approximately 80%) of the total sPP activity in higher plants (Gross and ap Rees 1986, Weiner et al. 1987). In-gel assays confirmed that the plastidial enriched fraction had a reduction in intensity staining (Figure 2A, B). Specificity of the targeting was also tested by immunoblot analysis (Figure 2E) where it is shown that the plastidial isoform is abolished while the cytosolic one is still detectable. Taken together, these results strongly suggested that the reduction in sPP activity observed here comes from the specific repression of the plastidial isoform.

The decrease in the sPP activity led to an approximately three fold increase in PP_i levels in the TRV2-psPP silenced plants compared with the TRV2 control. Increased PP_i concentrations in turn affect many biosynthetic pathways which generate PP_i , and so we examined several pathways spanning different compartments to evaluate this further. Leaves of the psPP silenced plants were characterized by a mottled appearance of light green islands along the leaf adaxial lamina and suggested that leaf isoprenoid biosynthesis could be severely compromised. Isoprenoids play important roles as photosynthetic pigments (chlorophylls, carotenoids), electron carriers (quinones), radical scavengers (tocopherols), membrane components (sterols) and growth and defence regulators (abscisic acid (ABA), gibberellins, brassinosteroids, cytokinins, monoterpenes, sesquiterpenes, and diterpenes). Originally thought to be exclusively synthesized in the cytosol (in a acetate/mevalonate (MVA) dependent manner), terpenoid biosynthesis in plants has also been recognized to follow a plastidial 1-deoxy-D-xylulose-5-phosphate (DOXP) (also known as methylerythritol phosphate (MEP)) dependent route (Lichtenthaler, 1999). The metabolic advantage of this ensures that certain constituents such as thiamine, mono- and diterpenes, chlorophyll, carotenoids and tocopherols are synthesized in the same compartment where they are utilized. Isoterpenoids affected in psPP silenced plants were largely restricted to those which play a predominant role in plastidial metabolism. Carotenoids, such as β -carotene, are synthesized in a two step PP_i -dependent manner (Hirschberg, 2001) catalyzed

by plastidial phytoene synthase, (PSY; Seo and Koshiba, 2002) (Figure 1). Carotenoids are involved in light harvesting and photoprotection of photosystem II (PSII) where they channel away excess quanta of energy from reaction centre II (RCII) to quenching complexes (Nayak et al. 2002). Transgenic overexpression of PSY in wheat kernels, tomato fruit and rapeseed leads to increased β -carotene levels (Shewmaker et al., 1999; Fraser et al., 2002; Paine et al., 2005). However, down-stream metabolites, such as tocopherols and chlorophylls were either significantly reduced (Shewmaker et al., 1999; Busch et al., 2002) or unchanged (Fraser et al., 2002). In transgenic tomato plants, the constitutive expression of PSY additionally leads to reduced gibberellin levels resulting in dwarfism (Fray et al., 1995). In psPP silenced plants several interesting observations concerning these metabolites were made. For instance, chlorophyll-a, but not chlorophyll- b amounts were significantly decreased compared to the TRV2 controls (Table 1). Similarly the β -Carotene, but not lutein, content was significantly affected by reductions in cellular PP_i amounts (Table 1). β -carotene is the precursor for the xanthophylls zeaxanthin and violaxanthin and sunflowers deficient in ζ -carotene desaturase [ZDS], an enzyme in the carotenoid biosynthetic pathway, results in a concomitant decrease in β -carotene, violaxanthin and chlorophyll content (Conti et al. 2004). In this study it was found that psPP silenced plants led to a reduction in violoxanthin but not zeaxanthin (Table 1). Our data illustrate that the increase in a common up-stream metabolite, namely PP_i , had a marked and synchronized effect on the levels of all three pigment classes. However, not all the pigments in these three classes are reduced, indicating complex regulatory steps within each pathway that cannot be explained solely by the perturbation in PP_i amounts.

In contrast, when evaluating the levels of the phytohormones (ABA, cytokinins and gibberellins) where the initial biosynthetic steps occur in the plastid, no significant differences between the TRV2 control and TRV2-psPP silenced plants could be observed under normal conditions (Figure 6B, Supplemental Table 2). However, when grown under mild drought stress ABA concentrations were significantly decreased in the psPP silenced plants compared to the controls (Figure 6B). ABA may be synthesized either through a 9-cis-violaxanthine (C40 indirect carotenoid) pathway (mainly found in higher plants) (Zeevaart and Creelman, 1988; for review Schwartz et al., 2003) or a farnesyl diphosphate (C15 precursor) pathway (Oritani and Kiyota, 2003). ABA-deficient or insensitive plant mutants identified to date (Zeevaart and Creelman, 1988; Parry and Horgan, 1991; Willmer and Fricker, 1996; Pugliesi et al., 1994; Koornneef et al., 1982; Koornneef et al., 1984; Rock et al., 1992) indicate that most of them are deficient in steps involved in the xanthophyll cycle (Marin et al., 1996; Koornneef et al., 1998) suggesting that this route might be more

prevalent in ABA biosynthesis. Furthermore, North et al. (2007) isolated an ABA deficient mutant, *aba4*, which lacks neoxanthin, accumulates violaxanthin and synthesizes sufficient ABA to give a normal phenotype under control conditions. Under drought conditions ABA is, however, up-regulated and triggers stomatal closure to limit water loss (Mittelheuser and van Steveninck, 1969). Upon dehydration conditions, *aba4* was severely limited in ABA accumulation (North et al., 2007) suggesting that the major source of stress-induced ABA in *aba4* is derived from neoxanthin. In the current study, psPP repressed plants were characterized by reduced violaxanthin, normal neoxanthin and increased zeaxanthin content under prevailing greenhouse conditions (Table 1). ABA was also unchanged in the psPP repressed plants under normal conditions and synthesis could not be induced when the plants were challenged with mild water stress. This suggested that stress-induced ABA synthesis in TRV2-psPP plants are probably derived from violoxanthin. However, similar to observations from North et al. (2007), it cannot be ruled out that under water stress conditions, the substrates might become limiting due to compensatory mechanisms that might exist in the photoprotective capacities associated with the light harvesting complex (Snyder et al., 2006; Dall'Osto et al., 2007). In accordance with the ABA levels, no difference in transpiration rate between unstressed TRV2-psPP and TRV2 control plants could be found under normal conditions. The rate decreased in both genotypes under drought stress; however, the decrease was far less severe in the TRV2-psPP plants than in the TRV2 controls and led to the TRV2-psPP plants wilting faster than the respective TRV2 control (Figure 6C).

In contrast to metabolites that are only evident when exposed to drought-stressed conditions, the TRV2-psPP plants exhibited a drought mock response under well-watered conditions. These included the increases in metabolite levels of several phenylpropanoids or precursors (quinic acid, coniferylalcohol, ferulate, 3-caffeoyl quinate, 4-caffeoyl quinate and 5-caffeoyl quinate) which may be involved in lignification processes associated with drought stress (Lee et al., 2007). Protection against drought stress can also be facilitated by the induction of osmoprotective compounds such as the amino acids proline and glutamate, sugar or sugar polyols and/or inorganic ions (Mahajan and Tuteja, 2005, and references therein). Increases in 1-pyrroline-2-carboxylate (precursor to proline), myo-inositol and a metabolite similar to pinitol were also observed in this dataset (Figure 7). When TRV2 control plants were drought induced, the majority of these metabolites accumulated in a similar manner to the TRV2-psPP unstressed plant, and also were not significantly different from the TRV2-psPP stressed metabolite levels (Figure 7). This suggests that PP_i metabolism might be indirectly involved in mediating drought stress responses in

N.benthamiana leaves. Interestingly, also malate levels showed similar patterns to those observed for the osmoprotective responses. Whilst the exact mechanism is still unknown, modulation of malate metabolism in transgenic tomato leaves have been shown to induce opposite photosynthetic responses (Nunes-Nesi et al., 2005; 2007), with antisense fumarase plants impaired in regulating stomatal aperture (Nunes-Nesi et al., 2007). Cumulatively these results suggest that malate might play a profound role in mediating photosynthetic performance, and that these responses are also integrated with prevailing PP_i levels.

One other pathway that might be affected by increased amounts of plastidial PP_i is that of starch synthesis, which acts as a source of carbon assimilates that can be used during dark phases to supply sugars to glycolysis. ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed reaction in the starch biosynthetic pathway (Figure 1). This enzyme uses glucose-1-phosphate and ATP to produce ADP-glucose and PP_i . The increase in PP_i in TRV2-psPP silenced plants would be expected to make the AGPase reaction less thermodynamically favourable in the forward direction as previously reported for purified plant AGPases (Amir and Cherry, 1972). In addition, sodium fluoride inhibition of soluble phosphatases leads to reduced starch levels in potato tuber discs (Viola and Davies, 1991). Chemical complementation by either glucose 1-phosphate or ADP-glucose demonstrate that only ADP-glucose could restore starch synthesis, suggesting that reduced synthesis of ADP-glucose from glucose 1-phosphate limits starch accumulation. This observation agrees well with the significant reduction in starch levels found in TRV2-psPP silenced plants compared to the TRV2 controls (Table 2). Photosynthesis and amount of Rubisco was reduced in the psPP plants (Figure 4) which could explain the reduction observed in starch. It has been shown that reduction in the level of Rubisco, in tobacco leaves, to 60% found in wild type leaves has only a mild effect on the rate of photosynthesis (Quick et al. 1991a). When Rubisco is decreased to between 20 – 50% the wild type value there is a proportional effect on photosynthetic carbon assimilation. This reduced carbon assimilation did not, however, alter the volume of starch turnover during a day night cycle (Quick et al. 1991b). In order to determine whether the decreased Rubisco and rate of carbon assimilation observed in the psPP line was the cause of the reduced starch content, starch accumulation was measured. The reduced rates of starch accumulation in leafdisks supplemented with sucrose (Figure 5b) suggest that these reduced starch levels are caused by PP_i induced thermodynamic inhibition of the AGPase enzyme. Curiously, an increase in plastidial AGPase maximal activity was observed (Figure 5A). AGPase plays an important role in leaf starch accumulation, and its activity is regulated on several levels. Regulation by P_i and 3-phosphoglyceric acid (Ballicora et al., 2004) are important allosteric controls while more

recently redox regulation of AGPase by trehalose 6-phosphate has also been proposed (Geigenberger et al., 2005; Kolbe et al., 2005). Increases in AGPase activity observed here could either illustrate the increase in reversibility of the AGPase activity under thermodynamically unfavourable PP_i levels and/or the overriding effect of altered PP_i metabolism on allosteric and redox regulators on its endogenous activity.

Metabolite profiling further revealed little changes in respect to non-plastidial metabolism. The levels of the soluble sugars, several amino acids and the majority of organic acids remained largely unaltered (Supplemental Table 3). Those organic and amino acids that did respond to the perturbation could not be characterized to be restricted to a specific cellular compartment (Farré et al., 2000). It remains unclear whether PP_i is transported across the plastid membrane, and whether this could affect metabolism in different compartments. Lunn and Douce (1993) described a transporter from isolated chloroplast preparations that is able to import PP_i over the chloroplast membrane, however, neither a PP_i export mechanism nor the corresponding gene have been isolated to date. Similarly, in developing maize embryos a L-malate/ PP_i transporter has been demonstrated (Lara-Núñez and Rodríguez-Sotres 2004) but gene identification still remains elusive. Data from silenced psPP plants suggests little linkage between cytosolic and plastidial metabolism due to transport of PP_i .

3.5 Conclusion

Since the plastidial pyrophosphatase gene was isolated in 2004 (Schulze et al., 2004), its exact metabolic role has, until now, not been elucidated. The data presented here assigns a function to the psPP gene while at the same time shedding light on pyrophosphate metabolism as a whole. It was shown that silencing psPP leads to an increase in cellular PP_i content which in turn led to a range of metabolic perturbations mostly restricted to reactions occurring in the plastid. Starch was reduced in the psPP silenced plants suggesting that the increased PP_i levels inhibit starch biosynthesis. Chlorophyll and carotenoids were also reduced in the psPP silenced plants. These are both products of PP_i generating pathways which are subjected to thermodynamic inhibition. The continuation of the carotenoid biosynthetic pathway leads to the production of xanthophylls which, with the exception of zeaxanthin, were also reduced. The major ABA biosynthetic pathway uses xanthophylls as a substrate and under well watered conditions there was no change in ABA levels. The plants ability to respond to drought stress by producing ABA was, however, severely impaired in the psPP plants leading to reduced drought tolerance. In contrast to the changes we demonstrated in plastidial biosynthetic pathways, metabolite profiling of the psPP silenced plants revealed little or no changes in metabolites associated with other compartments.

There has been debate surrounding the level of compartmentation of PP_i between the cytosolic and plastidial pools (Farré et al., 2000; 2006). Conjecture based on import studies performed in spinach leaf and wheat endosperm plastids (Lunn and Douce 1993; Lara-Núñez and Rodríguez-Sotres, 2004), as well as biochemical studies (Farré et al., 2000), have suggested the presence of PP_i transporters. The data in this study suggests that, at least in *N.benthamiana* leaves, there is very little to no communication between the two pools and that metabolic changes caused by increases in the plastidial PP_i pool were limited to that compartment. In this paper we demonstrated that psPP was necessary for the normal functioning of plastidial metabolism and that reduction of its activity led to altered plastidial metabolite levels and reduced ability to respond to stress.

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3.8 Supplementary material

Supplemental Table I Marker enzyme activities determined in plastid enrichments for TRV2 control leaves. NADP dependant glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ADP-glucose pyrophosphorylase (AGPase), UDP-glucose pyrophosphorylase (UGPase), and phosphoenolpyruvate carboxylase (PEP carboxylase) represents the markers for the plastidial and cytosolic fractions, respectively. Values are presented as mean \pm SE of two activity assays performed on each fraction.

	Crude extract	Plastid Purification 1	Plastid Purification 2
UGPase ($\mu\text{mol G-1-P produced.mg protein-1.min-1}$)	2.3 \pm 0.3	0.1 \pm 0.1	0.2 \pm 0.2
PEP carboxylase ($\mu\text{mol oxaloacetate produced.mg protein-1.min-1}$)	39.3 \pm 3.6	0.1 \pm 0.0	0.3 \pm 0.3
AGPase ($\mu\text{mol NADPH produced.mg protein-1.min-1}$)	3.8 \pm 0.1	3.0 \pm 0.5	2.7 \pm 0.6
GAPDH ($\mu\text{mol NADPH produced.mg protein-1.min-1}$)	3.4 \pm 0.2	3.2 \pm 0.7	3.9 \pm 0.2

Supplemental Table II List of phytohormones in *N.benthamiana* leaves of TRV2 control and TRV2-psPP plants. Levels were determined as described in Materials and Methods. Absolute values were determined by linear calibration of authentic standards, and ratios were determined for putative identified phytohormones to a relevant phytohormone corresponding to the same class. Values are presented as mean \pm SE of five individual plants per genotype/treatment and values in bold were determined using *Students t*-test to be significantly different ($P < 0.05$) from the respective TRV2 control.

Metabolite (amount)	TRV2 well-watered	psPP well-watered	TRV2 drought stressed	psPP drought stressed
IBA (nmol)	7.58 \pm 2.83	7.39 \pm 2.79	4.70 \pm 1.80	7.11 \pm 3.07
IAA (nmol)	0.38 \pm 0.09	0.47 \pm 0.13	0.43 \pm 0.03	1.14 \pm 0.27
GA25 (nmol)	10.07 \pm 0.61	10.47 \pm 0.76	7.32 \pm 1.56	8.94 \pm 0.80
GA3 (nmol)	0.08 \pm 0.01	0.05 \pm 0.01	0.52 \pm 0.25	0.99 \pm 0.31
GA9 (nmol)	2.24 \pm 0.54	1.90 \pm 0.24	1.93 \pm 0.49	2.37 \pm 0.42
JA (nmol)	7.10 \pm 1.55	11.22 \pm 2.44	7.47 \pm 0.86	6.94 \pm 0.69
SA (nmol)	4.25 \pm 0.49	8.01 \pm 0.81	5.16 \pm 0.65	4.31 \pm 0.66
Zeatin (nmol)	1.95 \pm 0.62	1.90 \pm 0.32	2.79 \pm 0.90	3.51 \pm 0.86
Cytokinin (nmol)	5.86 \pm 0.30	6.40 \pm 0.60	5.41 \pm 0.42	5.32 \pm 0.58
Zeatin riboside (nmol)	3.76 \pm 0.30	4.14 \pm 0.41	4.21 \pm 0.97	3.80 \pm 0.62
Zeatin ribosamide (nmol)	3.99 \pm 0.27	4.28 \pm 0.34	3.60 \pm 0.21	4.03 \pm 0.53
MST_9500_TOF_258.07 (μ mol)	2.59 \pm 0.16	2.33 \pm 0.17	1.95 \pm 0.05	2.06 \pm 0.24
ABA (nmol)	1.76 \pm 0.16	2.04 \pm 0.15	5.00 \pm 0.74	1.83 \pm 0.15
GA1/GA25	0.20 \pm 0.07	0.21 \pm 0.11	0.49 \pm 0.13	0.25 \pm 0.06
GA5/GA25	1.75 \pm 1.16	1.59 \pm 0.43	1.19 \pm 0.40	1.27 \pm 0.32
GA3/GA25	0.55 \pm 0.27	0.26 \pm 0.15	0.37 \pm 0.18	0.42 \pm 0.18
GA8 TMS1/GA25	3.43 \pm 2.42	0.55 \pm 0.50	2.58 \pm 1.79	2.82 \pm 1.99
GA8 TMS2/GA25	3.98 \pm 1.73	4.97 \pm 3.21	8.02 \pm 3.13	4.71 \pm 2.67
GA77/GA25	0.09 \pm 0.03	0.20 \pm 0.11	0.94 \pm 0.71	0.09 \pm 0.03
JA-leu-ME /JA	6.88 \pm 0.64	7.32 \pm 0.95	7.02 \pm 0.68	6.47 \pm 0.51
I-Ile-ME/IAA	0.95 \pm 0.22	1.35 \pm 0.40	1.72 \pm 0.43	0.47 \pm 0.17

Supplemental Table III Full list of relative metabolite content in *N.benthamiana* leaves of TRV2 control and TRV2-psPP plants. Metabolites levels were determined as described in Materials and Methods. Data are normalized with respect to mean response calculated for the TRV2 unstressed control (to allow statistical assessment in the same way). Values are presented as mean \pm SE of five individual plants per genotype/treatment and values in bold were determined using *Students t*-test to be significantly different ($P < 0.05$) from the respective TRV2 control.

Metabolite	TRV2 well-watered	psPP well-watered	TRV2 drought stressed	psPP drought stressed
1-Amino-cyclopropanecarboxylate	1.00 \pm 0.10	1.18 \pm 0.04	0.96 \pm 0.12	1.10 \pm 0.08
1-Pyrroline-2-carboxylate	1.00 \pm 0.14	1.41 \pm 0.04	1.20 \pm 0.10	1.16 \pm 0.09
2-Hydroxy cinnamate	1.00 \pm 0.16	0.99 \pm 0.20	1.19 \pm 0.21	0.97 \pm 0.12
2-Hydroxyglutarate	1.00 \pm 0.12	1.00 \pm 0.07	1.23 \pm 0.05	1.10 \pm 0.10
2-Methyl-citrate	1.00 \pm 0.05	0.91 \pm 0.02	0.99 \pm 0.11	0.99 \pm 0.14
2-Oxoglutarate	1.00 \pm 0.21	1.54 \pm 0.11	0.92 \pm 0.26	1.87 \pm 0.14
2-Oxoisocaproate	1.00 \pm 0.12	1.31 \pm 0.09	1.28 \pm 0.22	0.80 \pm 0.16
3,5-Dimethoxy-4-hydroxycinnamate	1.00 \pm 0.13	1.48 \pm 0.16	1.45 \pm 0.12	1.40 \pm 0.04
3-caffeoyl Quinate	1.00 \pm 0.24	2.04 \pm 0.32	1.83 \pm 0.14	1.30 \pm 0.22
3-cyano-Alanine	1.00 \pm 0.05	0.90 \pm 0.25	0.91 \pm 0.14	0.57 \pm 0.20
4-Amino butyrate	1.00 \pm 0.13	1.39 \pm 0.11	1.05 \pm 0.35	1.18 \pm 0.08
4-caffeoyl Quinate	1.00 \pm 0.10	1.95 \pm 0.20	1.37 \pm 0.28	1.15 \pm 0.29
5-caffeoyl Quinate	1.00 \pm 0.13	1.75 \pm 0.12	1.86 \pm 0.11	1.60 \pm 0.12
Aconitate	1.00 \pm 0.19	0.90 \pm 0.28	0.86 \pm 0.21	0.69 \pm 0.22
Amino-oxy-acetate	1.00 \pm 0.35	0.62 \pm 0.19	0.87 \pm 0.20	0.66 \pm 0.29
Caffeate	1.00 \pm 0.12	0.94 \pm 0.09	0.81 \pm 0.04	0.82 \pm 0.07
Citrate	1.00 \pm 0.09	0.77 \pm 0.05	0.87 \pm 0.09	0.90 \pm 0.19
Dehydroascorbate	1.00 \pm 0.17	1.02 \pm 0.22	1.06 \pm 0.18	1.08 \pm 0.17

Ferulate	1.00 ± 0.13	1.78 ± 0.10	1.82 ± 0.08	1.70 ± 0.12
Fumarate	1.00 ± 0.13	1.08 ± 0.04	1.02 ± 0.06	1.05 ± 0.05
Galactonate	1.00 ± 0.17	0.99 ± 0.14	0.92 ± 0.12	0.99 ± 0.09
Galactonic acid-1,4-lactone	1.00 ± 0.08	0.94 ± 0.12	1.05 ± 0.12	0.82 ± 0.12
Galacturonate	1.00 ± 0.19	0.99 ± 0.21	1.17 ± 0.29	0.90 ± 0.18
Gluconate	1.00 ± 0.17	0.99 ± 0.14	0.92 ± 0.12	0.99 ± 0.09
Gluconic acid-6-phosphate	1.00 ± 0.22	0.89 ± 0.08	0.68 ± 0.07	1.12 ± 0.27
Glucuronate	1.00 ± 0.19	0.99 ± 0.21	1.17 ± 0.29	0.90 ± 0.18
Glucuronate-e-lactone	1.00 ± 0.20	0.79 ± 0.18	0.82 ± 0.23	0.82 ± 0.28
Glutamate	1.00 ± 0.31	0.82 ± 0.24	0.83 ± 0.11	0.72 ± 0.34
Glutarate	1.00 ± 0.11	0.79 ± 0.10	0.96 ± 0.12	0.84 ± 0.04
Isocitrate	1.00 ± 0.17	0.99 ± 0.14	0.92 ± 0.12	0.99 ± 0.09
Lactate	1.00 ± 0.14	0.80 ± 0.08	0.75 ± 0.03	0.87 ± 0.08
Malate	1.00 ± 0.09	1.25 ± 0.05	1.24 ± 0.04	1.26 ± 0.04
Maleate	1.00 ± 0.11	0.96 ± 0.13	1.04 ± 0.03	0.97 ± 0.13
n-Propyl-gallate	1.00 ± 0.21	1.01 ± 0.13	1.46 ± 0.12	2.26 ± 0.52
Pantothenate	1.00 ± 0.19	1.11 ± 0.25	1.29 ± 0.31	1.04 ± 0.23
Phosphoric acid	1.00 ± 0.1	2.42 ± 0.1	1.56 ± 0.14	1.81 ± 0.22
Quinate	1.00 ± 0.12	1.82 ± 0.09	1.95 ± 0.13	1.82 ± 0.12
Shikimate	1.00 ± 0.41	1.27 ± 0.24	0.95 ± 0.28	1.09 ± 0.13
Succinate	1.00 ± 0.03	1.10 ± 0.09	1.46 ± 0.15	1.11 ± 0.10
Threonate-1,4-lactone	1.00 ± 0.16	1.14 ± 0.12	1.13 ± 0.12	0.99 ± 0.11
4-Hydroxy-proline	1.00 ± 0.10	0.98 ± 0.18	0.90 ± 0.09	0.67 ± 0.25
Arginine	1.00 ± 0.24	1.01 ± 0.19	1.48 ± 0.21	0.78 ± 0.14
Aspartate	1.00 ± 0.01	1.00 ± 0.01	0.99 ± 0.01	1.02 ± 0.01
beta-Alanine	1.00 ± 0.17	0.65 ± 0.09	0.96 ± 0.25	0.83 ± 0.15
beta-Homoserine	1.00 ± 0.16	0.72 ± 0.12	0.91 ± 0.05	0.70 ± 0.10

Glycine	1.00 ± 0.33	0.63 ± 0.11	0.93 ± 0.15	0.85 ± 0.26
Histidine	1.00 ± 0.15	2.04 ± 0.11	2.05 ± 0.10	1.93 ± 0.15
Homocysteine	1.00 ± 0.07	0.98 ± 0.05	1.08 ± 0.05	1.07 ± 0.04
Homoserine	1.00 ± 0.06	1.00 ± 0.04	1.05 ± 0.05	1.07 ± 0.04
Lysine	1.00 ± 0.11	0.84 ± 0.15	1.00 ± 0.17	0.82 ± 0.22
Methionine	1.00 ± 0.12	0.89 ± 0.02	0.95 ± 0.13	1.03 ± 0.09
Ornithine	1.00 ± 0.20	0.85 ± 0.20	0.95 ± 0.07	1.44 ± 0.24
Phenylalanine	1.00 ± 0.14	1.14 ± 0.11	0.91 ± 0.07	0.90 ± 0.11
Serine	1.00 ± 0.13	1.34 ± 0.05	1.18 ± 0.11	1.16 ± 0.09
Spermidine	1.00 ± 0.15	0.78 ± 0.13	0.91 ± 0.13	0.79 ± 0.20
Threonine	1.00 ± 0.13	0.79 ± 0.09	0.76 ± 0.03	0.86 ± 0.06
Tryptophan	1.00 ± 0.26	8.28 ± 0.30	11.50 ± 0.25	11.40 ± 0.04
Tyrosine	1.00 ± 0.10	0.93 ± 0.11	0.78 ± 0.12	1.03 ± 0.22
1,3-Dihydroxyacetone	1.00 ± 0.18	0.79 ± 0.13	1.14 ± 0.20	0.59 ± 0.15
3-O-methyl glucose	1.00 ± 0.20	0.79 ± 0.18	0.82 ± 0.23	0.82 ± 0.28
Cellobiose	1.00 ± 0.10	0.94 ± 0.07	0.91 ± 0.12	0.85 ± 0.11
Dihydroxyacetone phosphate	1.00 ± 0.05	0.91 ± 0.02	0.99 ± 0.11	0.99 ± 0.14
Fructose	1.00 ± 0.08	0.94 ± 0.12	1.05 ± 0.12	0.82 ± 0.12
Galactitol	1.00 ± 0.17	0.99 ± 0.14	0.92 ± 0.12	0.99 ± 0.09
Galactose	1.00 ± 0.17	0.99 ± 0.14	0.92 ± 0.12	0.99 ± 0.09
Galactose-6-phosphate	1.00 ± 0.22	0.89 ± 0.08	0.68 ± 0.07	1.12 ± 0.27
Gentiobiose	1.00 ± 0.10	0.94 ± 0.07	0.91 ± 0.12	0.85 ± 0.11
Glucopyranose	1.00 ± 0.13	0.91 ± 0.07	0.87 ± 0.06	0.80 ± 0.07
Glucose	1.00 ± 0.08	0.94 ± 0.12	1.05 ± 0.12	0.82 ± 0.12
Glucose-6-phosphate	1.00 ± 0.22	0.89 ± 0.08	0.68 ± 0.07	1.12 ± 0.27
Glycerate-3-phosphate	1.00 ± 0.08	0.94 ± 0.12	1.05 ± 0.12	0.82 ± 0.12
Glyceraldehyde-3-	1.00 ± 0.29	2.00 ± 0.03	2.11 ± 0.19	2.79 ± 0.30

phosphate					
Maltose	1.00 ± 0.10	0.94 ± 0.07	0.91 ± 0.12	0.85 ± 0.11	
Mannitol	1.00 ± 0.17	0.99 ± 0.14	0.92 ± 0.12	0.99 ± 0.09	
Mannose-1-phosphate	1.00 ± 0.13	0.85 ± 0.08	0.83 ± 0.08	0.99 ± 0.11	
Mannose-6-phosphate	1.00 ± 0.22	0.89 ± 0.08	0.68 ± 0.07	1.12 ± 0.27	
myo-Inositol	1.00 ± 0.04	1.19 ± 0.03	1.18 ± 0.04	1.14 ± 0.07	
Ribose	1.00 ± 0.09	0.94 ± 0.04	0.95 ± 0.04	0.98 ± 0.04	
Ribose-5-phosphate	1.00 ± 0.13	0.85 ± 0.08	0.83 ± 0.08	0.99 ± 0.11	
Ribulose-5-phosphate	1.00 ± 0.07	0.84 ± 0.07	0.87 ± 0.11	1.07 ± 0.09	
Sorbose	1.00 ± 0.19	0.99 ± 0.21	1.17 ± 0.29	0.90 ± 0.18	
Sucrose	1.00 ± 0.10	0.94 ± 0.07	0.91 ± 0.12	0.85 ± 0.11	
Threose	1.00 ± 0.31	0.59 ± 0.14	0.90 ± 0.06	0.67 ± 0.20	
Trehalose	1.00 ± 0.10	0.94 ± 0.07	0.91 ± 0.12	0.85 ± 0.11	
Xylitol	1.00 ± 0.12	1.44 ± 0.11	1.84 ± 0.14	1.34 ± 0.14	
Xylulose	1.00 ± 0.13	0.90 ± 0.14	1.26 ± 0.07	0.97 ± 0.12	
Norleucine	1.00 ± 0.04	1.13 ± 0.10	1.47 ± 0.18	1.35 ± 0.15	
Calystegine B2	1.00 ± 0.34	0.88 ± 0.12	1.09 ± 0.14	0.92 ± 0.17	
Coniferylalcohol	1.00 ± 0.10	2.38 ± 0.07	2.32 ± 0.10	2.25 ± 0.16	
Glycerate	1.00 ± 0.31	0.59 ± 0.14	0.90 ± 0.06	0.67 ± 0.20	
Glycerol	1.00 ± 0.31	0.59 ± 0.14	0.90 ± 0.06	0.67 ± 0.20	
Glycerol-3-phosphate	1.00 ± 0.07	0.84 ± 0.07	0.87 ± 0.11	1.07 ± 0.09	
Glycerophosphoglycerol	1.00 ± 0.11	0.87 ± 0.08	0.88 ± 0.11	0.77 ± 0.14	
Guanine	1.00 ± 0.15	1.72 ± 0.19	1.65 ± 0.13	1.53 ± 0.05	
Inosine	1.00 ± 0.08	1.03 ± 0.05	1.01 ± 0.05	1.04 ± 0.05	
Nicotinamide	1.00 ± 0.16	0.92 ± 0.11	0.90 ± 0.18	1.11 ± 0.10	
Nicotine	1.00 ± 0.11	0.82 ± 0.12	0.68 ± 0.16	1.06 ± 0.11	
N-methyl-calystegine B2	1.00 ± 0.45	0.95 ± 0.21	1.27 ± 0.18	0.87 ± 0.17	

Quercetin	1.00 ± 0.08	0.84 ± 0.05	0.79 ± 0.08	1.29 ± 0.28
Sinapaldehyde	1.00 ± 0.12	0.96 ± 0.15	1.18 ± 0.20	1.01 ± 0.11
Taxifolin	1.00 ± 0.17	0.79 ± 0.15	0.70 ± 0.20	1.15 ± 0.35
Uracil	1.00 ± 0.13	1.08 ± 0.04	1.02 ± 0.06	1.05 ± 0.05
MST_1272.27_TOF	1.00 ± 0.11	0.96 ± 0.13	1.04 ± 0.03	0.97 ± 0.13
MST_1403.63_TOF	1.00 ± 0.12	1.00 ± 0.07	1.23 ± 0.05	1.10 ± 0.10
MST_1426.15_TOF	1.00 ± 0.11	0.79 ± 0.10	0.96 ± 0.12	0.84 ± 0.04
MST_1426.93_TOF	1.00 ± 0.09	1.40 ± 0.16	1.72 ± 0.09	1.30 ± 0.09
MST_1431.3_TOF	1.00 ± 0.06	1.40 ± 0.11	1.99 ± 0.11	1.48 ± 0.05
MST_1453.47_TOF	1.00 ± 0.05	0.90 ± 0.25	0.91 ± 0.14	0.57 ± 0.20
MST_1760.93_TOF	1.00 ± 0.11	0.79 ± 0.16	0.89 ± 0.17	0.86 ± 0.11
MST_1765.77_TOF	1.00 ± 0.08	0.94 ± 0.12	1.05 ± 0.12	0.82 ± 0.12
MST_1850.6_TOF	1.00 ± 0.06	1.17 ± 0.21	0.99 ± 0.03	1.69 ± 0.18
MST_1856.23_TOF (cyclitol)	1.00 ± 0.11	1.80 ± 0.08	1.89 ± 0.13	1.80 ± 0.12
MST_1929.03_TOF	1.00 ± 0.06	1.17 ± 0.21	0.99 ± 0.03	1.69 ± 0.18
MST_2002.87_TOF	1.00 ± 0.08	0.94 ± 0.12	1.05 ± 0.12	0.82 ± 0.12
MST_2242.35_TOF	1.00 ± 0.11	0.87 ± 0.08	0.88 ± 0.11	0.77 ± 0.12
MST_2843.55_TOF	1.00 ± 0.23	1.00 ± 0.17	1.09 ± 0.20	0.88 ± 0.19
MST_2907.17_TOF	1.00 ± 0.10	0.94 ± 0.07	0.91 ± 0.12	0.85 ± 0.11
MST_3126.16_TOF	1.00 ± 0.09	0.88 ± 0.10	0.89 ± 0.12	0.92 ± 0.12
MST_3144.5_TOF	1.00 ± 0.13	2.88 ± 0.16	2.82 ± 0.16	2.23 ± 0.20

Supplemental Table IV Linear correlation matrix of PP_i levels and selected metabolites. Correlations were performed with the linear Pearson regression algorithm embedded in the XLSTAT software, and represented of five individual plants of each genotype in each treatment.

Chapter 4

Virus Induced Multiple Gene Silencing to Study Metabolic Pathways

4 Virus Induced Multiple Gene Silencing to Study Metabolic Pathways

Gavin M George, Rolene Bauer, Jens Kossmann, James R Lloyd

4.1 Introduction

Post-transcriptional gene silencing (PTGS) has been described in plants, animals, and fungi (Vaucheret et al. 1998, Fire et al. 1998, Romano and Macino, 1992). Virus-induced gene silencing (VIGS) is a form of PTGS and has the ability to specifically down-regulate targeted endogenous genes (Kumagai et al. 1995). Sequence specific silencing is achieved through the action of DICER, which cleaves dsRNA into 21 – 25 nt fragments, and the RNA-induced silencing complex (RISC), which incorporates these fragments to guide the cleavage of homologous mRNA (Xie et al. 2005; Martinez et al. 2002). VIGS offers an attractive alternative to stable forms of PTGS, such as antisense silencing (Sheehy et al. 1988) or RNAi silencing (Smith et al. 2000), through the speed at which it can be performed. Whereas antisense and RNAi both require the creation and selection of transgenic lines VIGS can reproducibly elicit silencing, in excess of 90% repression of enzyme activity in Solanaceous species, within two weeks allowing for the rapid analysis of gene function (Orzaez et al. 2006). Given the advantages of VIGS, we wanted to determine whether this silencing system could be used effectively for the study of biological pathways as opposed to single genes.

Starch studies are often complicated by the fact that the synthesis and degradation of this highly structured polymer involves the combined action of a great number of enzymes (Smith et al. 1996; Kossmann and Lloyd, 2000; Zeeman et al. 2007). The highly complex nature of this biological pathway along with the fact that it has been relatively well characterised makes it an excellent target for this study. Most transitory leaf starch studies have been performed using *Arabidopsis* as model system, mainly due to the relative ease of genetic modification of this species. While silencing of single genes can be highly informative with regard to determining a gene function often the analysis of attenuated genes in a mutant background or simultaneous silencing of multiple genes has been necessary to produce a phenotype when a high level of enzyme isoform redundancy exists (Lloyd et al. 1999; Edwards et al. 1999; Dumez et al. 2006).

In this paper we aimed to both optimise a VIGS system for silencing of single and multiple genes in the starch metabolic pathway and furthermore to demonstrate that the silenced plant lines can be used to elucidate complex enzyme interactions within the metabolism of starch. We chose a number of target genes involved in starch metabolism (Figure 1) where

silenced or mutant plants have been previously characterised in potato or *Arabidopsis*. These include an α -glucan, water dikinase (GWD), a disproportionating enzyme (DPE1), and a transglucosidase (DPE2) (Lorberth et al. 1998; Takaha et al. 1993; Critchley et al. 2001; Lloyd et al. 2004). In *Arabidopsis thaliana* mutations of the GWD enzyme results in an accumulation of starch up to seven times that which occurs in wild type leaves (Yu et al. 2001), while in potato antisense repression of the enzyme also leads to a starch excess phenotype in the leaves (Lorberth et al. 1998). The DPE1 enzyme catalyses the transfer of maltosyl units from one maltooligosaccharide (MOS) to another and through this process releases glucose. In *Arabidopsis* it is the only enzyme which can metabolise maltotriose so, when knocked out results both in a slower rate of starch degradation and a buildup of maltotriose and higher order MOS in the leaves at night (Critchley et al. 2001).

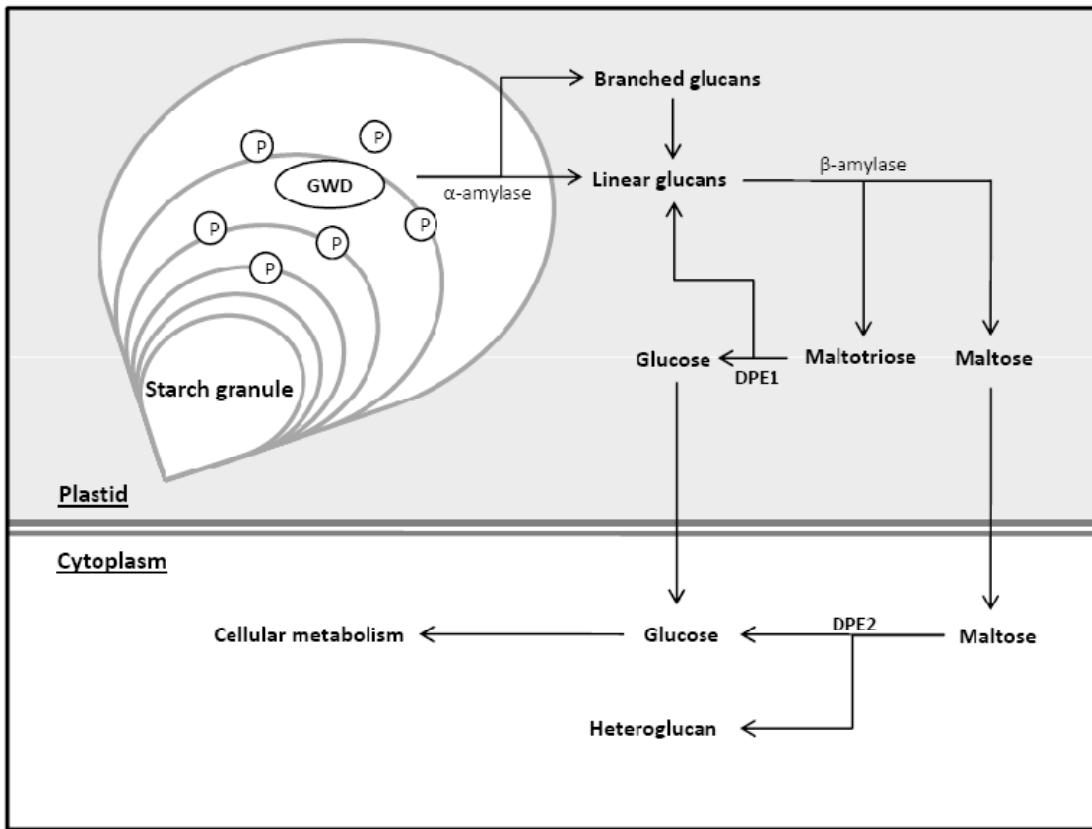


Figure 1 A schematic representation of the starch degradative pathway. GWD catalyses the phosphorylation of glucose molecules in amylopectin, partially solubilizing the surface of the granule. The α - and β -amylases act on the partially solubilised starch to produce maltose and maltotriose which can either be exported to the cytoplasm or acted on by DPE1 to produce glucose. Maltose exported to the cytoplasm can be broken down into glucose by DPE2.

The transglucosidase, DPE2, transfers a glucosyl residue from maltose to polymers such as glycogen liberating a glucose molecule. *Arabidopsis* knockout mutants of DPE2 (Chia et al. 2004), and transgenic potato plants lacking it (Lloyd et al. 2004), accumulate large amounts of maltose and also show a starch excess phenotype.

We demonstrate that using VIGS we can effectively achieve silencing of one, and two genes along with marked reduction of a third gene simultaneously and in so doing reveal novel phenotypes in the leaves of *N. benthamiana*. In this way we aimed to show that VIGS can be used effectively to study complex biological pathways such as that seen in starch metabolism.

4.2 Methods

Plant material and growth conditions

Nicotiana benthamiana seeds were surface-sterilized by successive 70% (v/v) ethanol (3 x 1 min), 1% (v/v) sodium hypochlorite (1 x 3 min), and sterile water washes (3 x 30 sec). Seeds were germinated on 0.4% (w/v) agar (Plant Gel, Highveld Biotechnology, South Africa), 4.32g.l⁻¹ Murashige and Skoog basal salt media, 15% (w/v) sucrose. The plantlets were grown at 24°C under fluorescent light in a 16h/8h day night cycle. Seedlings at the four leaf stage were transferred to 1L tissue culture bottles (Sigma) containing growth media described above, Chapter 3.2, and acclimatized for ten days before being subjected to VIGS infiltration.

VIGS plasmid construction, transfection and plant generation

Construct design and principles of deconstructed vectors of tobacco rattle virus (pTRV1, pTRV2, and pTRV2-PDS) for virus induced gene silencing (VIGS) were previously described (Liu et al., 2002). The silencing constructs were produced using 380-bp fragments of the *DPE1*, *DPE2*, and *GWD* genes, each chosen based on the theoretical production of 20 or more siRNAs (siRNA Finder, Ambion: http://www.ambion.com/techlib/misc/siRNA_finder.html). Each fragment was amplified by PCR using the following primer sets in Table 1 before gel purification performed as per the QIAquick Spin Handbook (Qiagen Gel Extraction Kit, <http://www.qiagen.com>).

Table 1 Primer sets used for the PCR amplification of gene fragments which were cloned into TRV2 for use in this study. The forward and reverse primer sequence as well as the included restriction sites and gene fragment product length are indicated.

Targeted gene	Primer sequences	Restriction sites on primer	Product length
<i>DPE1</i>	5'- GCACGCGTCTCGAGAACTTTTCGC-3' 5'- GCCTCGAGAAGGCGTCTGGAGGA-3'	<i>MluI</i> <i>XhoI</i>	380 bp
<i>DPE2</i>	5'- GCAGGCCTGCTTCAGAATCCCAT-3' 5'- GCTCTAGACAGGGACCTTTCACC-3'	<i>StuI</i> <i>XbaI</i>	380 bp
<i>DPE2</i>	5'-CGCGCCCGGGGCTTCAGAATCCCATACTAT-3' 5'-CGCGCCCGGGCAGGGACCTTTCACCAT-3'	<i>SmaI</i> <i>SmaI</i>	380 bp
<i>GWD</i>	5'-GCACGCGTTTTCTGGTTTCTTCA-3' 5'-GCGAGCTCAAATCCACAAAGGGAC-3'	<i>MluI</i> <i>SacI</i>	380 bp

These primers contained restriction sites which would be used for cloning into the pTRV2 vector. The *GWD* fragment was cut with the *MluI* and *SacI* restriction enzymes and then ligated into pTRV2 using the same sites to create pTRV2-GWD. *DPE1* was ligated into pTRV2 and pTRV2-GWD to create pTRV2-DPE1 and pTRV2-GWD-DPE1 respectively using the *MluI* and *XhoI* enzymes. *DPE2* was excised from the pGEM-T Easy vector (Promega) using *EcoRI* and ligated into pTRV2, pTRV2-DPE1, pTRV2-GWD, pTRV2-GWD-DPE1 to create pTRV2-DPE2, pTRV2-DPE2-DPE1, pTRV2-DPE2-GWD, and pTRV2-DPE2-GWD-DPE1 respectively. In order to create the triple construct pTRV-GWD-DPE1-DPE2, new *DPE2* primers, Table 1, were used to amplify the fragment which was subsequently cloned into the *SmaI* site of TRV2-GWD-DPE1. Each of these eight silencing constructs as well as the empty TRV2 control vector were transformed into *A. tumefaciens* (strain GV2260) by the freeze-thaw method (Höfgen and Willmitzer 1988). Transformed *A. tumefaciens* were grown and resuspended in infiltration media as described in Ryu et al. (2004). Seedlings (ca. 4 – 5 days post germination) were placed inside a 60 ml syringe containing 20 ml of the *A. tumefaciens* suspension; In cases where only a single TRV2 line of *A. tumefaciens* was used the mixture was 1:1 TRV1 to TRV2 however if several lines of TRV2 were co-infiltrated the ratios were adjusted to 1:1:1 TRV1 to TRV2-X to TRV-Y. With the leaves of the plant submerged the nib of the syringe was stoppered and the air volume was doubled to create a half atmospheric vacuum pressure for 30 seconds after which the vacuum was broken.

Seedlings were then planted in soil (supplemented with silica sand and vermiculite [8:1:1]) and placed in a clear plastic bag to harden off over 5 days. Plants were then grown under prevailing greenhouse conditions and analyzed after three weeks.

Protein extracts

Total protein was extracted from leaf discs or pre-ground material by grinding in protein extraction buffer (50mM Tris-HCl, pH 7.5, 2mM EDTA, 0.002% (v/v) Triton X-100). The supernatant was recovered after centrifugation at 13000g for 20 min at 4°C and the protein concentration measured as by Bradford (1976).

Protein analysis

Measurement of the DPE1 enzyme activity was measured in the direction of glucose production and performed according to Zeeman et al. (1998a). 50µl of total protein was assayed in 50mM MOPS, pH 6.8, containing 60mM maltotriose. The reactions was stopped after 0 and 20 min by heating for 5 min at 95°C and glucose production was measured according to Müller-Röber et al. (1992). Immunoblot analysis was performed by running 30 µg of total protein on a 10% (v/v) SDS-PAGE gel. Following separation of proteins, the samples were blotted onto PVDF membrane using a semi-dry method (Sambrook et al. 1989) and immunoblots were performed using previously described antibodies (Lloyd et al. 2004 and Lorberth et al. 1998) which recognise either DPE2 or GWD respectively.

Starch analysis

Total leaf starch was extracted as described in Tomlinson et al. (1997) and quantified based on the method described in Muller-Rober et al. (1992). Starch and sugars were extracted from 50mg of ground leaf material by adding 1ml 80% (v/v) ethanol and heating at 80°C for 1 hour. The samples were centrifuged at 13000g, for 10 min and the pellet resuspended in 0.4ml 0.2M KOH and heated for 1 hour at 95°C. The samples were allowed to cool before being neutralised with 7µl glacial acetic acid. Starch in the samples was then determined by digesting 10µl in 10µl 50mM NaAC, pH 5.6, containing 10 U/ml amyloglucosidase for 2 hours at 37°C. The liberated glucose units were measured enzymatically through the addition of Hexokinase and Glucose 6-phosphase dehydrogenase as described previously (Muller-Rober et al. 1992).

Maltooligosaccharide analysis

Maltooligosaccharides (MOS) were measured based on Critchley et al. (2001) by grinding 20mg of leaf material in 200µl 0.7M perchloric acid. The samples were incubated at 4°C for 30 min and centrifuged at 3890g for 3 min at 4°C. 50µl of the supernatant was removed and to this 30µl 2M KOH, 0.4M MES pH 5.6, 0.4M KCl was added. The potassium perchlorate precipitate was removed by centrifugation at 3890g for 10 min at 4°C and 50µl of the

supernatant was added to 66µl sugar removal buffer (0.1M MES pH 5.6, 0.75 U β-fructosidase, 0.1 glucose oxidase, 65 U catalase) and incubated for 2 hours at 32°C followed by a further incubation for 40 min at 70°C. The malto-oligosaccharide content was determined by adding 50µl to an equal volume of assay buffer (50mM sodium acetate (pH 5.6) containing 0.25 U maltase and 2 U amyloglucosidase), incubated for 2 hours at 37°C. Glucose units were determined as described in starch analysis above.

Starch granule purifications

Starch granules were purified using a technique adapted from Zeeman et al. (1998a, 2002). 100mg of leaf material was partially homogenised in 50mM MOPS (pH 7.5), 5mM EDTA and centrifuged at 5000g for 10 min. The supernatant was removed and the starch pellet washed by resuspending in the same buffer and centrifuging a further twice. Two more washes were performed with the same buffer containing 0.5% SDS followed by three washes in acetone at -20°C without centrifugation but rather allowing the starch to settle out of solution. The starch was then dried for 1 hour under vacuum.

Statistical analysis

Statistical analyses were performed using either the *Students t*-test embedded in the Microsoft Excel software (Microsoft, Seattle) or ANOVA (Statistica 8, StatSoft Inc.). Data analysed by ANOVA was further processed using the Fisher's LSD Test, embedded in the StatSoft software.

4.3 Results and Discussion

Here we describe a system for simultaneous silencing multiple genes in *N. benthamiana*. The TRV vectors used in this study were designed and described by Liu et al. (2002) and there are several reviews on the topic (Brigneti et al. 2004, Burch-Smith et al. 2004). Many agroinfiltration techniques have been described (Ryu et al. 2004 and Burch-Smith et al. 2004) but we established an efficient system for high throughput vacuum infiltration, on plants 4 – 6 weeks old. Following infiltration the plants were hardened off and allowed to grow for a further four weeks before analysis. It was found that the third fully expanded leaf, which developed post infiltration, consistently displayed silencing across the entire lamina as validated by targeting of the phytoene desaturase gene, which results in photobleaching in *N. benthamiana* (Figure 2).



Figure 2 Photobleaching resulting from the silencing of the PDS gene. The TRV2 (above) and TRV2-PDS (below) were used to validate the vacuum infiltration technique. The third expanded leaves, which consistently showed near complete silencing across the leaf lamina, are marked with an arrow.

Targeting of the PDS in *N. benthamiana* using vacuum infiltration consistently led to complete photobleaching in the third expanded leaf. Leaves which existed at the time of infiltration, those below the fourth leaf, display the 'patchy' silencing which has been previously described (Liu and Page, 2008). Using vacuum infiltration all the leaves on the seedlings are exposed to the *Agrobacterium* which allows for efficient transfer of the virus to the plant when compared to localized infiltration techniques. This efficient transfer could

result in a higher inoculation ‘dose’ of the virus which in turn leads to the efficient silencing seen. Having established an efficient infiltration technique and size of targeting sequences the system could be tested for its ability to co-silence multiple genes.

Co-Infiltration of TRV Targeting Different Genes Does Not Lead To Multiple Gene Silencing

In an attempt to silence multiple genes *N. benthamiana* seedlings were vacuum infiltrated with mixtures of Agrobacterium containing TRV constructs targeting three different genes, namely to the genes encoding DPE1, DPE2, and GWD. The plants were infiltrated with TRV1 and either TRV2-DPE1, TRV2-DPE2, or TRV2-GWD alone or with mixtures of these VIGS constructs to determine whether multiple silencing would occur. A complete list of the constructs used in this paper is shown in Figure 3.

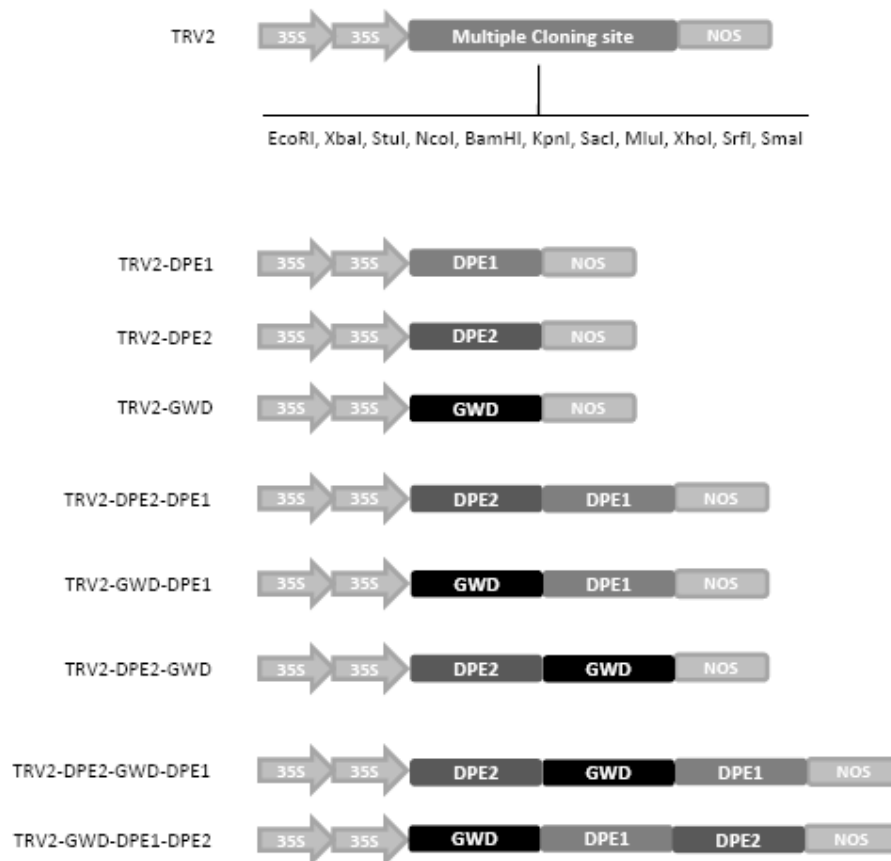


Figure 3 TRV2 vectors constructed for use in this study. The gene fragments contained between the dual 35S promoters and the NOS terminator sites are all 380 bp in length

and were ligated into the pre-existing sites in the TRV2 vector. These constructs were transformed into *Agrobacterium* and co-infiltrated into *Nicotiana benthamiana* plants along with TRV1 using a vacuum infiltration system.

The results of these experiments are shown in Figure 4 and Table 2 where immunoblots and enzyme activities were measured to determine the extent of the silencing that occurred. Plant extracts were tested for the presence of the DPE2 enzyme in each of the plant lines.

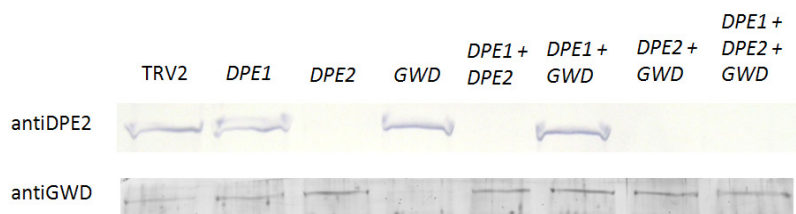


Figure 4 Immunoblots were produced by probing total protein extracts, from the lines of plants where distinct viruses were co-infiltrated into the seedlings, with the antiDPE2 (above) and antiGWD (below) antibodies. DPE2 was undetectable in all the lines targeted whereas GWD was only undetectable where it alone was targeted.

Table 2 Activity of DPE1 in mixed infiltration plant lines. Values are presented as means \pm SE (n=5) where the annotations show lines with no significant difference ($P > 0.05$ by Anova, Fisher's LSD test).

Plant Line	DPE1 Activity ($\mu\text{mole glucose units}\cdot\text{h}^{-1}\cdot\mu\text{g}^{-1}$ protein)
TRV2	2.4 \pm 0.3 ^a
DPE1	0.5 \pm 0.2 ^{b/c}
DPE2	1.9 \pm 0.4 ^{a/b}
GWD	2.9 \pm 0.2 ^a
DPE1 + DPE2	2.4 \pm 0.3 ^a
DPE1 + GWD	0.1 \pm 0.4 ^{b/c}
DPE2 + GWD	2.8 \pm 0.9 ^a
DPE1 + DPE2 + GWD	2.2 \pm 0.5 ^a

Using this technique the DPE2 protein was undetectable in lines where it was targeted whether it was infiltrated alone or co-infiltrated with TRV targeting either of other two genes. Similarly, the presence of GWD was also determined using immunoblots. In contrast to DPE2, GWD was only reduced in the line where it alone was the target. These plant lines showed a similar pattern of silencing for DPE1 (Table 2) where it only showed a significant reduction in activity when silenced alone or when GWD was the other target. Never, in each of the plant lines where single or multiple genes were targeted, was more than one gene silenced. These data, taken together, show a hierarchical pattern of silencing where DPE2 is preferentially silenced over DPE1 and GWD, DPE1 over GWD while GWD is never silenced in the presence of either of the others. It has been shown in the leaves of potato plants where *DPE2* was silenced that there was an increase in DPE1 activity (Lloyd et al. 2004). While no significant increases in DPE1 activity were observed when DPE2 was reduced (Table 2) it does not exclude the possibility that some level of transcriptional control over this gene exists. It is possible that upregulation of DPE1 when it is targeted along with DPE2 by mixed infiltration could allow the plant to overcome the silencing effect of the virus. With GWD catalysing one of the earliest steps in starch breakdown, it is conceivable then that similar transcriptional control over this enzyme also exists. A more likely explanation is that silencing is related to steady state mRNA levels of a particular gene. It has been shown in tomato that co-suppression of a gene is avoided if the endogenous mRNA transcript level is below a certain threshold (Han et al. 2004). It is therefore possible that in VIGS systems the steady state mRNA expression level could be a determining factor in which gene is preferentially silenced. While transcriptional control and endogenous mRNA levels may be factors in determining which gene is silenced, more research into the nature of gene targeting motifs in plants is required before this selective process will be fully understood.

TRV2 gene fragment size is a determining factor in which gene will be silenced

Chimeric viral constructs were then created each targeting two genes, TRV2-DPE2-DPE1, TRV2-GWD-DPE1, and TRV2-DPE2-GWD. To determine whether the size of the insert in the virus was a determining factor on the level of silencing these constructs were each infiltrated into plants along with a second TRV2 targeting only one gene. In Figure 5 and in Table 3, it can be seen again that each of the genes targeted individually resulted in silencing. When the plants were infiltrated with combinations of the chimeric and single gene targeting viruses again only one gene was ever affected. In contrast to the mixing of single gene targeting TRV constructs, here it was always the gene targeted by a single fragment containing construct that was preferentially silenced.

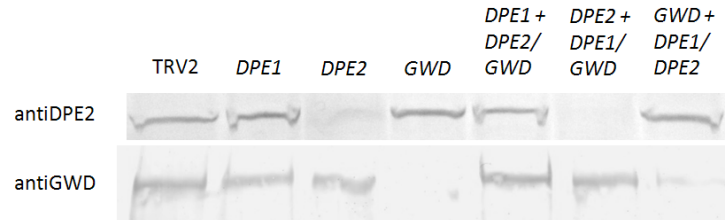


Figure 5 Immunoblots probed with antiDPE2 (above) and antiGWD (below) antibodies were produced using plant extracts infiltrated using single gene targeting TRV as well as mixtures of double gene targeting and single gene targeting viruses. The DPE2 and GWD were both silenced when targeted with the single gene fragment containing virus whether it be alone or mixed with a two gene fragment containing version of the virus.

Table 3 Activity of DPE1 in plant lines infiltrated with chimeric and single gene targeting TRV simultaneously. Values are presented as means \pm SE (n=5) where the annotations show lines with no significant difference between them ($P > 0.05$ by Anova, Fisher's LSD test).

Plant Line	DPE1 Activity (p mole glucose units.h ⁻¹ . μ g ⁻¹ protein)
TRV2	2.4 \pm 0.3 ^a
<i>DPE2/GWD + DPE1</i>	0.1 \pm 0.5 ^b
<i>GWD/DPE1 + DPE2</i>	2.5 \pm 0.4 ^a
<i>DPE2/DPE1 + GWD</i>	1.8 \pm 0.5 ^a

The ability of the smaller viral construct to overcome the strict preferential silencing observed earlier could point to two distinct determining factors for silencing. In the first case, due to the identical size of the inserts, the primary determinant of which gene is silenced is most likely the one that is least transcriptionally upregulated when two or more are targeted together or based on steady state level of mRNA (Han et al. 2004). The RNA-dependent RNA polymerases (RdRPs) are responsible for the amplification of silencing signal through siRNA primed creation of dsRNA from targeted mRNA which can be utilised by DICER, and mutants of it result in suppression of silencing (Mourrain et al. 2000; Dalmay et al. 2000). The exponential increase in siRNAs generated through this process of amplification could be impaired for one target gene when two genes are targeted by distinct viruses. This impaired amplification could be due to saturation of the silencing machinery by the effectively targeted

gene fragment and its corresponding mRNA. This selection of which gene is effectively targeted could be the steady state concentration of the mRNA for either gene. In the second instance the preferential targeting of the single gene construct was likely based on which version of the virus was more viable in the plant. It has been shown that viruses, including TRV, containing foreign sequences often have impaired replication or can be modified at a higher rate through recombination (Guo et al. 1998; Mueller and Wimmer, 1998; German-Retana et al. 2000; McFarlane, 1999). Furthermore, it has been shown that identical but differentially labelled viruses can not infect the same cell (Deitrich and Maiss, 2003). It is highly possible that two similar viruses, one containing a larger insert, will spread through the plant at different rates based on these factors and that the version containing the smaller insert will be more able to out-compete the other. In this way co-infection by distinct viruses could lead to selection based on insert sequence size which is observed in my experiments.

Chimeric constructs elicit simultaneous gene silencing

To determine the ability of the chimeric constructs to elicit silencing eight lines of plants were generated by infiltrating them with *Agrobacterium* containing either TRV2, TRV2-DPE1, TRV2-DPE2, TRV2-GWD, TRV2-DPE2-DPE1, TRV2-GWD-DPE1, TRV2-DPE2-GWD, or TRV2-DPE2-GWD-DPE1. The eight lines were then analyzed for DPE1 activity and it was found that in each of the lines containing a DPE1 fragment the activity of the enzyme was significantly reduced compared to the control as can be seen in Table 4.

Table 4 Activity of DPE1 in plant lines infiltrated with chimeric multiple gene targeting TRV. Values are presented as means \pm SE (n=5) where the annotations denote lines with no significant difference ($P > 0.05$ by Anova, Fisher's LSD test).

Plant Line	DPE1 Activity pmole glucose units.h ⁻¹ . μg^{-1} protein
TRV2	2.1 \pm 0.4 ^a
DPE1	0.3 \pm 0.3 ^b
DPE2	3.3 \pm 0.4 ^c
GWD	2.2 \pm 0.2 ^a
DPE1/DPE2	0.1 \pm 0.3 ^b
DPE1/GWD	0.2 \pm 0.1 ^b
DPE2/GWD	3.6 \pm 0.3 ^c
DPE2/GWD/DPE1	0.5 \pm 0.1 ^b

The lines were then analyzed for the presence of DPE2 and GWD using immunoblots, Figure 6a, where again, the protein was not detected in the lines where they were targeted with the exception of GWD in the TRV2-DPE2-GWD-DPE1 line.

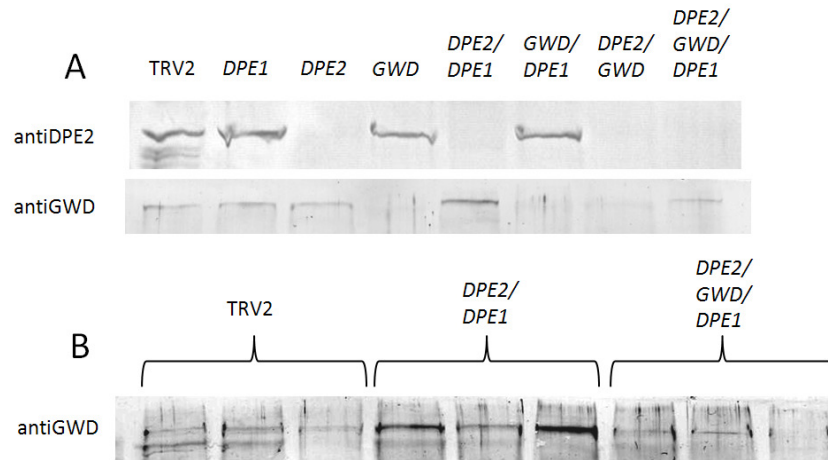


Figure 6 Immunoblots of protein extracts from plant lines infiltrated with chimeric TRV constructs targeting the DPE1, DPE2, and GWD alone and in different combinations. (A) shows the immunoblots using the antiDPE2 (above) and antiGWD (below) antibodies. DPE2 is silenced in each line where targeted while GWD is silenced in all lines where targeted with the exception of TRV2-DPE2-GWD-DPE1. (B) Immunoblots of three control, TRV2-DPE2-DPE1 and TRV2-DPE2-GWD-DPE1 plants from each line using the antiGWD antibody showing the consistent increase in the former and only partial reduction in the latter of GWD.

Lines where DPE1 and DPE2 are silenced together consistently showed an increase in the amount of GWD. Figure 6b shows three pTRV2 control, DPE2-DPE1, and DPE2-GWD-DPE1 plants where it can be seen that the signal obtained for the GWD protein in the double silenced line is stronger than in the control, and is only slightly reduced in the triple gene silenced line. There is a parallel between this result and those seen in the mixed infiltration of single gene targeting viruses above. That is that both DPE1 and DPE2 are consistently more strongly reduced than GWD. In addition, GWD is being upregulated when DPE1 and DPE2 are simultaneously targeted (Figure 6b). Taking this into account there are two explanations for why GWD is still detectable in the TRV2-DPE2-GWD-DPE1 line of plants. Either GWD is being upregulated in response to the loss of DPE1 and DPE2 to the point where it is detectable or its position within this viral construct (see Figure 3) is preventing it from effectively targeting the endogenous gene. To determine whether the positional effect

of the gene fragments within the viral constructs is a factor in the level of silencing a rearranged version of the virus was constructed and tested.

Position of gene fragment within the triple construct has little effect on level of silencing

In order to determine whether the position of the GWD gene fragment within the triple gene targeting, TRV2-DPE2-GWD-DPE1, construct was in some way preventing silencing a new rearranged version of the virus was produced. The TRV2-GWD-DPE1-DPE2 contained each gene fragment in a different position compared to the previous triple gene targeting construct and also placed GWD in the first position in the TRV multiple cloning site, previously occupied by the always strongly repressed DPE2 targeting fragment. The new construct was analyzed for the presence of the targeted genes as can be seen in Figure 7 using the TRV2 as a control. It can be seen that in all lines targeting the DPE2 it was again completely silenced, and this was also true for the newly constructed TRV2-GWD-DPE1-DPE2.

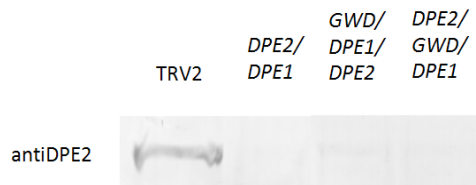


Figure 7 Immunoblots probed with the antiDPE2 antibody on protein extracts from the TRV2, TRV2-DPE2-DPE1, TRV2-GWD-DPE1-DPE2, and TRV2-DPE2-GWD-DPE1 lines of plants. DPE2 was undetectable in all the lines where it was targeted.

The lines were then tested for DPE1 activity and again each was found to be repressed compared to the control lines as can be seen in Table 5. While DPE1 activity appeared to be the least repressed in the TRV2-DPE2-GWD-DPE1 line of plants it was statistically indistinguishable from TRV-DPE1, TRV2-DPE2-DPE1 and the new TRV2-GWD-DPE1-DPE2 line.

Table 5 Activity of DPE1 in plant lines infiltrated with chimeric multiple gene targeting TRV. Values are presented as means \pm SE (n=5) where the annotations show lines with no significant difference ($P > 0.05$ by Anova, Fisher's LSD test).

Plant Line	DPE1 Activity pmole glucose units.h ⁻¹ . μg^{-1} protein
TRV2	2.3 \pm 0.2 ^a
<i>DPE2/DPE1</i>	0.6 \pm 0.1 ^b
<i>GWD/DPE1/DPE2</i>	0.7 \pm 0.1 ^b
<i>DPE2/GWD/DPE1</i>	1.1 \pm 0.2 ^b

In order to clearly show the consistent incomplete (reduced but still detectable) repression of the GWD in the triple silencing constructs, Figure 8, three plant extracts of both the TRV2-GWD-DPE1-DPE2 and TRV2-DPE2-GWD-DPE1 were compared to TRV2 controls. Again it can be seen that the previously constructed triple silencing construct only causes incomplete repression of the GWD gene. This is mirrored in the new, TRV2-GWD-DPE1-DPE2, construct where again the repression of the gene was incomplete. From this data it can be seen that because GWD is inefficiently silenced in both the TRV2-DPE2-GWD-DPE1 and the TRV2-GWD-DPE1-DPE2 lines and so the lack of silencing is not determined by the position of the fragment within the TRV constructs.



Figure 8 Immunoblots showing the presence of GWD in the TRV2 control, TRV2-DPE2-GWD-DPE1, TRV2-GWD-DPE1-DPE2 lines of plants. Protein extracts were probed with the antiGWD antibody.

Analysis of chimeric constructs for their effect on starch metabolism

Each of these lines was then analyzed for starch content at the end of the day to determine whether any starch excess (SEX) phenotype existed in the plants after silencing. This SEX phenotype has been demonstrated in Arabidopsis and tomato mutants for GWD, as well as in the leaves of antisense GWD silenced potato plants (Yu et al. 2001; Nashilevitz et al.

2009; Lorberth et al. 1998). It has also been demonstrated in the leaves of DPE2 silenced potato and Arabidopsis, as well as DPE1 Arabidopsis mutants, however, the SEX phenotype was far less severe than in the GWD mutants (Lloyd et al. 2004; Chia et al. 2004; Lu and Sharkey, 2004; Critchley et al. 2001). In Figure 9a it can be seen that indeed the starch content is altered in many of the lines. The lines where *DPE1* and *-2* were targeted individually there was no observable change in the starch content, most likely due to the fact that in potato plants where DPE2 is silenced the SEX phenotype only develops after 10 weeks and only a slight increase in starch is expected in DPE1 silenced leaves (Lloyd et al. 2004, Critchley et al. 2001). The TRV2-GWD line shows a large increase in the amount of starch present in the leaves.

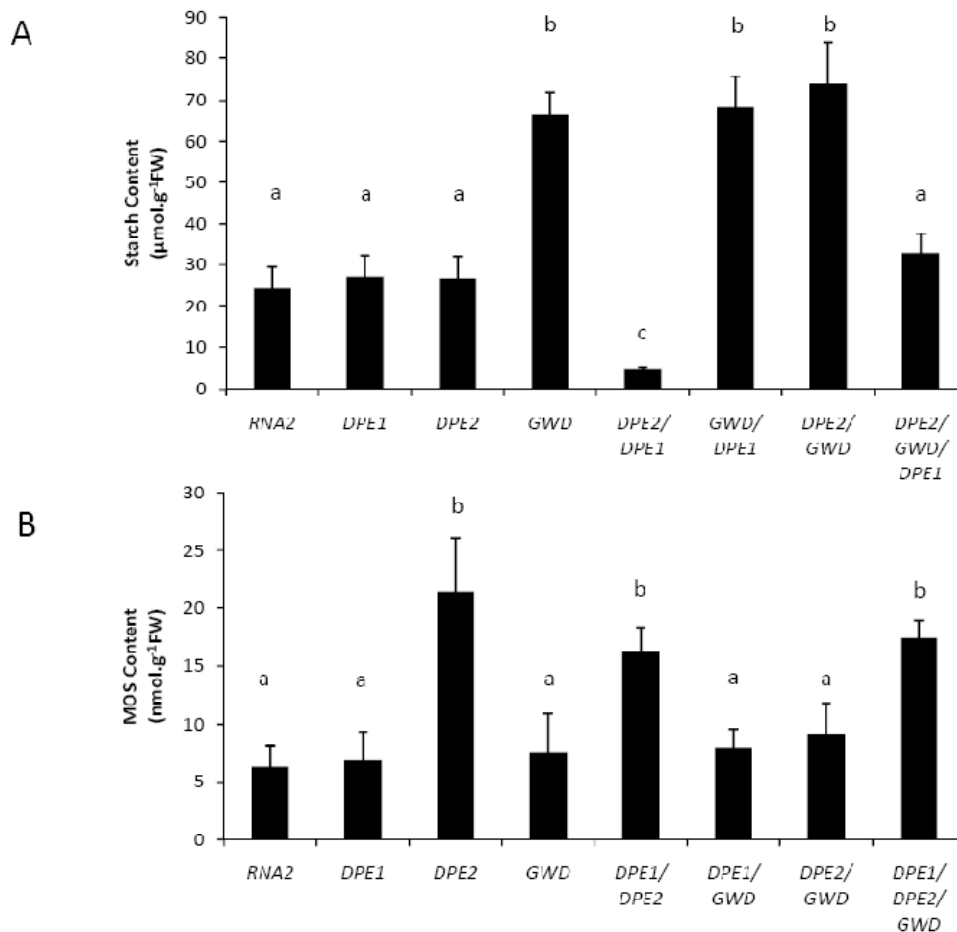


Figure 9 The (A) starch and (B) maltooligosaccharide content of the leaves of plants infiltrated with chimeric TRV constructs. Values are presented as means \pm SE (n=5). ANOVA (one way, $P > 0.05$) was performed on the data which was subsequently grouped using the post hoc Fisher's LSD test where letters a, b, and c denote groups within which there was no significant difference.

This phenotype is observed in all the lines where GWD is targeted with the sole exception of TRV2-DPE2-GWD-DPE1. Interestingly, in the line where DPE1 and DPE2 are co-silenced there is in fact a large reduction in the level of leaf starch over the control plants. Along with this large reduction in starch levels, these plants also exhibited a chlorotic leaf phenotype which was also observed in the TRV2-DPE2-GWD-DPE1 line.

In the plastid, linear glucans are broken down by β -amylase to maltose and maltotriose, Figure 1 (Scheidig et al. 2002; Lao et al. 1999; Fulton et al. 2008). The maltose can then be exported to the cytoplasm through the MEX1 transporter to be converted to glucose by DPE2 (Niittylä et al. 2004). DPE1 also produces glucose from maltotriose which is subsequently exported to the cytoplasm (Takaha et al. 1993). Large increases in total MOS content were seen in the DPE2 repressed line as well as the DPE2-DPE1 and the DPE2-GWD-DPE1 silenced lines. It has been suggested that MOS acts as a primer for granule bound starch synthase (GBSS) or could modify its affinity for amylose which it synthesizes (Denyer et al. 1996, 1999). It was also shown in *C. reinhardtii* that D-enzyme mutants possessed an impaired ability to synthesize amylopectin (Colleoni et al. 1999). A possibility for the decrease in starch seen in DPE2-DPE1 line could be that the increased MOS is in some way interfering with this priming of the starch granule either through loss of ability to transfer oligosaccharides directly to amylopectin or through alterations in the MOS population interfering with the normal activity of the starch synthesizing enzymes. To investigate whether this was the case the starch morphology in the TRV2-DPE1-DPE2 line was characterized through imaging by scanning electron microscopy (SEM, Figure 10).

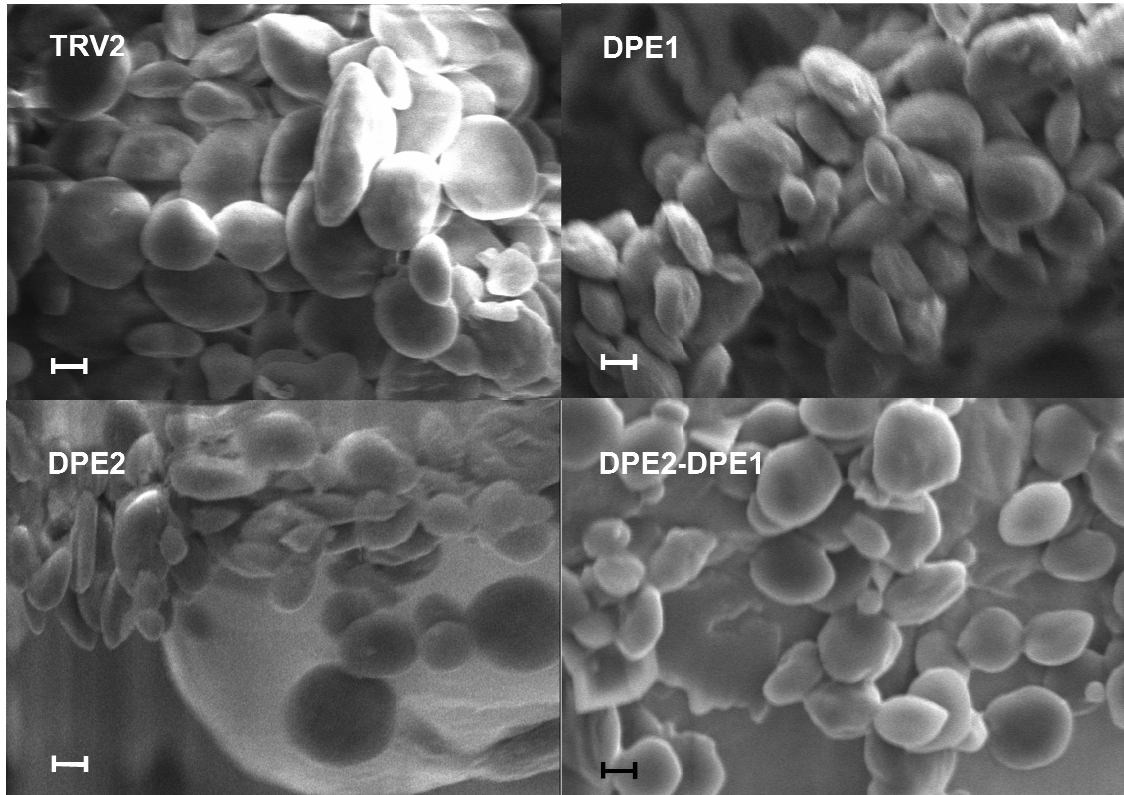


Figure 10 Scanning electron micrographs of starch granules isolated from TRV2 control, TRV2-DPE1, TRV2-DPE2, and TRV2-DPE2-DPE1 plants.

The decreased starch in this line is due either to a net reduction in starch synthesis or a rapid breakdown of any starch which is synthesized. Starch synthesis could be inhibited either by a reduction in the number of granule nucleation events, which would lead to fewer normal sized granules, or through impaired growth of starch granules which would produce many smaller starch granules. The SEM visualization showed no obvious alteration to the morphology of the starch compared to the controls. Similar reductions in starch content, to less than 5%, have been observed in the *C. reinhardtii* mutant STA11 which has been shown to carry a lesion in the *DPE1* gene (Wattebled et al. 2003). The mutant resulted in an increase in the MOS content of the algae and it was hypothesized that it was this increase in MOS that was inhibiting starch production. These results, however, were not mirrored in *Arabidopsis* where both the DPE1 and DPE2 mutants resulted in increased starch levels (Critchley et al. 2001; Chia et al. 2004). It is possible that the simultaneous silencing of both DPE1 and DPE2, which both result in increased MOS, are creating an intraplasmidial population of linear MOS which is more comparable to that found in the STA11 mutant. If this is true and starch synthesis is indeed being inhibited by the increased MOS found in the TRV2-DPE2-DPE1 line then it is possible that they are interfering with the action of the one

or more of the many enzymes which produce linear MOS such as the limit dextrinase (LDA) and isoamylase enzymes (ISA) (Nakamura, 1996; Delatte et al. 2006; Wattedled et al. 2005). Mutations in an Arabidopsis chloroplastic ISA led to an approximate 90% reduction of starch with a concomitant increase in water soluble polysaccharides known as phytoglycogen (Zeeman et al. 1998b). Water soluble polysaccharides were measured in the TRV2-DPE2-DPE1 line of plants however no significant difference was observed compared to control plants (Data not shown). An accumulation of phytoglycogen could indicate that loss of DPE1 and -2 were interfering with the structure of starch leading to a less ordered and so soluble polymer being formed. The lack of observable phytoglycogen thus shows that the pool of stored carbohydrates is reduced.

As mentioned above, the TRV2-DPE2-DPE1 line of plants displayed a chlorotic leaf phenotype as did the TRV2-DPE2-GWD-DPE1 and TRV2-GWD-DPE1-DPE2 lines (Figure 11). Based on the immunoblot analysis of DPE2 and the activity of the DPE1 enzymes in these plants there is little difference in the strength of the silencing in these lines compared to TRV2-DPE2-DPE1 and the controls. This chlorotic phenotype has been observed in *Arabidopsis mex1/dpe1* plants which contain a mutation in the genes for the chloroplastic maltose export protein and DPE1 (Niittylä et al. 2004). The maltose transported through MEX1 into the cytoplasm is converted directly to glucose by DPE2. While it is unlikely that the loss of the DPE2 activity is directly related to this chlorotic phenotype, the fact that *mex1/dpe1* mutants also show it indicates that it is rooted in the increases in the levels of MOS in the plastid.

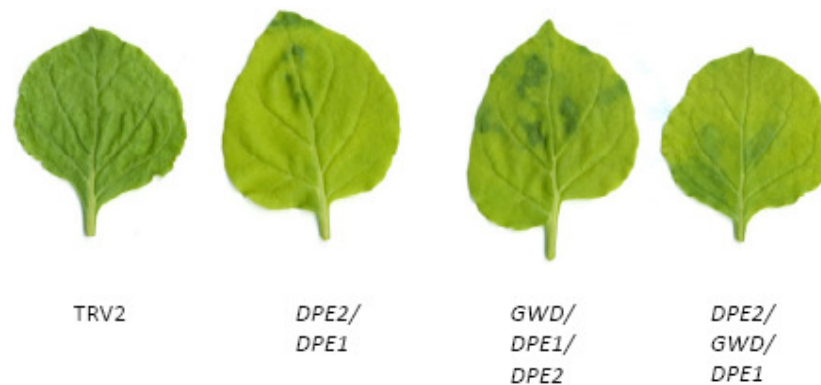


Figure 11 Leaves from TRV2 control, TRV2-DPE2-DPE1, TRV2-DPE2-GWD-DPE1, and TRV2-GWD-DPE1-DPE2 plant lines showing the chlorotic phenotype in all but the control plants and where pPGM is also targeted.

Starch was then measured in the new triple construct lines. Again a large reduction in the starch levels was observed in the TRV2-DPE2-DPE1 lines, Figure 12a, which contained less than 5% of that observed in controls. The starch in the TRV2-GWD-DPE1-DPE2 line also showed a partial recovery of the starch levels while in the TRV2-DPE2-GWD-DPE1 starch was recovered to wild type levels. The total MOS was then measured in the lines and significant increases were seen in the TRV2-DPE2-DPE1, TRV2-DPE2-GWD-DPE1, and TRV2-GWD-DPE1-DPE2 lines.

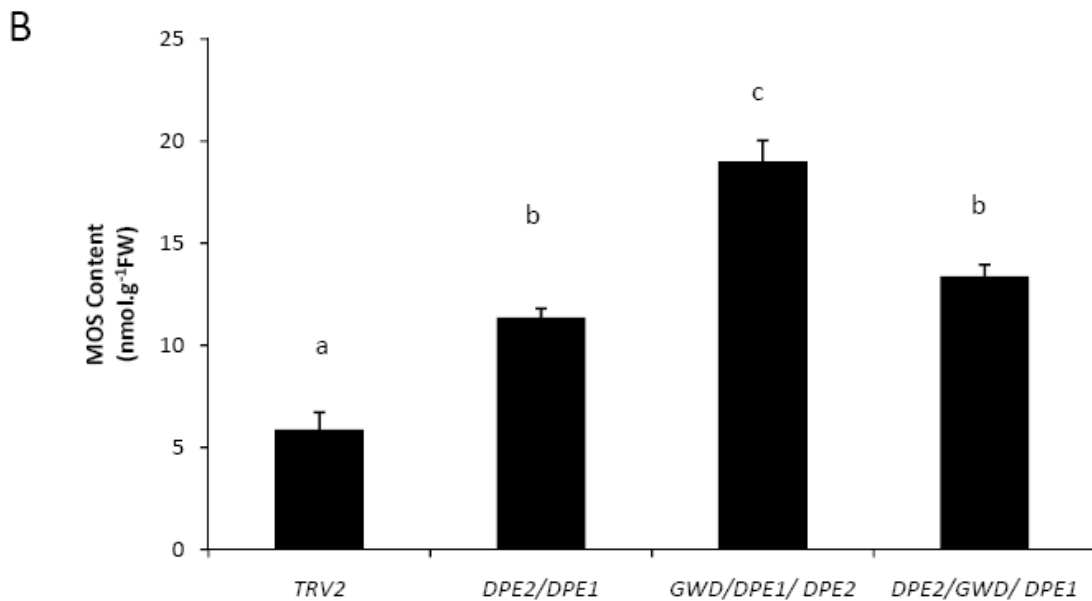
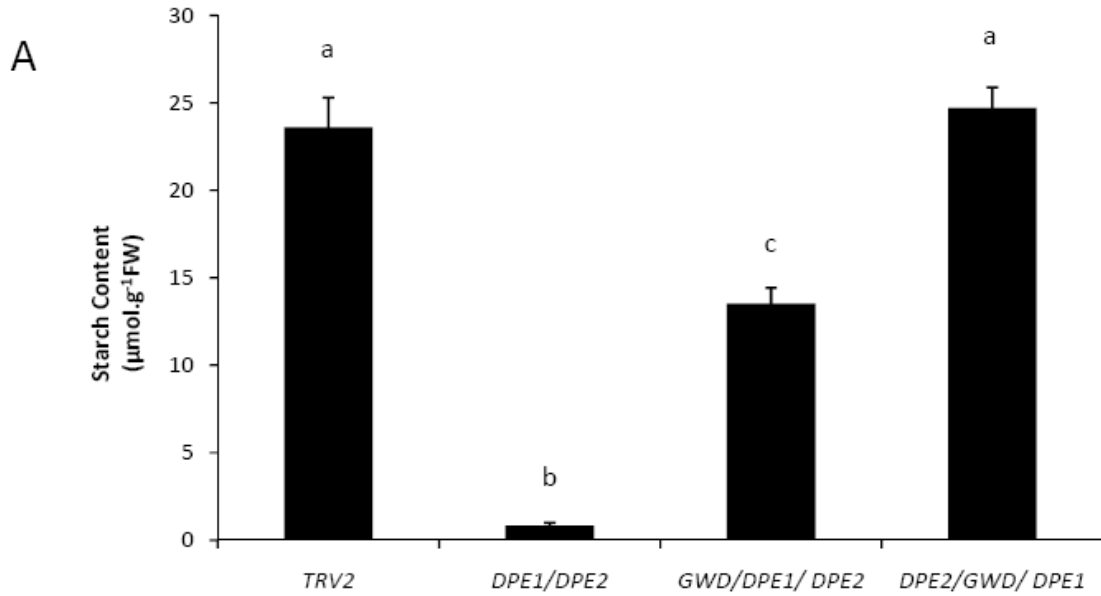


Figure 12 The (A) starch and (B) maltooligosaccharide content of the leaves of plants infiltrated with TRV2, TRV-DPE2-DPE1, TRV2-GWD-DPE1-DPE2, TRV2-DPE2-GWD-DPE1 viral constructs. Values are presented as means \pm SE (n=5). ANOVA (one way, $P>0.05$) was performed on the data which was subsequently grouped using the post hoc Fisher's LSD test where letters a, b, and c denote groups within which there was no significant difference

Considering the only partial reduction of GWD in the TRV2-GWD-DPE1-DPE2 and TRV2-DPE2-GWD-DPE1 lines the recovery of starch is difficult to explain. However, the excess of MOS in the TRV-DPE2-DPE1 line is indicative of flux through starch, even though the total amount is greatly reduced compared to the controls. It is possible that the reduction in GWD causes starch degradation to proceed more slowly in these lines and in so doing affect an accumulation of starch over time. Both triple gene targeting lines still maintain a high level of MOS by comparison to the TRV2 controls. This taken together with the maintenance of the chlorotic phenotype observed in these lines show that DPE1 and DPE2 are still strongly repressed and that while co-reduction of GWD can recover the starch to wild type values it does not recover the chlorotic phenotype.

4.4 Conclusion

Here we present data that not only one, but two genes can be silenced with a third reduced simultaneously through the use of chimeric TRV constructs. A summary of these results can be seen in Table 6 below. Under these experimental conditions we have determined that the position of the gene fragments within the TRV virus is not a major determinant of silencing strength, however it is important to consider whether there may be any gene interactions when undertaking similar experiments. We speculate whether the build-up of MOS leads to an induction of the gene for GWD and thus compromising the strength of silencing. To prevent the build-up of MOS we have co-silenced DPE1, DPE2 and plastidial phosphoglucomutase (pPGM). We have produced preliminary data showing that three genes *DPE1*, *DPE2*, and a pPGM can be strongly silenced simultaneously using a chimeric construct. Furthermore, co-silencing of pPGM with DPE1 and DPE2 leads to a recovery of the chlorotic phenotype.

Table 6 Summary of the data obtained in this study where dashes represent lack of silencing and one or two plus signs indicate mild and strong silencing respectively.

TRV Construct	Silencing Strength of Genes Targeted		
	<i>DPE1</i>	<i>DPE2</i>	<i>GWD</i>
TRV2	-	-	-
<i>DPE1</i>	++	-	-
<i>DPE2</i>	-	++	-
<i>GWD</i>	-	-	++
<i>DPE1 + DPE2</i>	-	++	-
<i>DPE1 + GWD</i>	++	-	-
<i>DPE2 + GWD</i>	-	++	-
<i>DPE1 + DPE2 + GWD</i>	-	++	-
<i>DPE2/GWD + DPE1</i>	++	-	-
<i>GWD/DPE1 + DPE2</i>	-	++	-
<i>DPE2/DPE1 + GWD</i>	-	-	++
<i>DPE1/DPE2</i>	++	++	-
<i>DPE1/GWD</i>	++	-	++
<i>DPE2/GWD</i>	-	++	++
<i>GWD/DPE1/DPE2</i>	++	++	+
<i>DPE2/GWD/DPE1</i>	++	++	+

Leaf starch metabolic studies have been predominantly performed in *Arabidopsis* and much valuable knowledge has come from silencing or mutating multiple genes simultaneously (Dumez et al. 2006). Here we propose that studies of this kind can be performed in *N. benthamiana* using VIGS to rapidly and effectively perform loss of function studies of multiple genes simultaneously. Based on the data presented in this paper, we propose that this system could be used to rapidly study complex biochemical pathways.

4.5 References

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Chapter 5

General Discussion

5.1 Virus Induced Gene Silencing for Metabolic Studies

VIGS is a powerful technique for the rapid study of plant metabolism. Since the discovery of RNA-induced gene silencing and the subsequent developments in antisense, RNAi, and virus-induced gene silencing techniques they have been used in a broad range of forward genetics studies (Fire et al. 1998; Napoli et al. 1990; Waterhouse et al. 1998; Smith et al. 2000; Baulcombe, 1999). VIGS was initially validated in plants with the silencing of the gene, phytoene desaturase, which is involved in carotenoid biosynthesis and results in rapid photobleaching of leaves where it is repressed (Baulcombe, 1999). That study was performed in *Nicotiana benthamiana* using a modified potato virus X vector but since then more efficient vectors, such as a modified tobacco rattle virus (TRV) (Liu et al. 2002a, 2002b). This virus had the advantage of having reduced symptoms of infection making phenotypic results easier to interpret (Liu et al. 2002a). It also proved to be effective in a broader range of species including tobacco, potato, Arabidopsis, and tomato thus expanding the array of possible studies into flower development, leaf, fruit and even root metabolism (Liu et al. 2002a, Brigneti et al. 2004; Burch-Smith et al. 2006; Lui et al. 2002b; Liu et al. 2004; Fu et al. 2005; Valentine et al. 2004).

While VIGS is becoming more widespread as a technique for studies on metabolism, several practical limitations still exist. Among these are the widespread reports the 'patchy silencing' associated with VIGS. This manifests as a mosaic of silenced and non-silenced plant material (Liu and Page, 2008). Widespread use of VIGS as a technique has also been limited by insufficient knowledge about the optimal size of cDNA inserts into the tobacco rattle virus, and the level of silencing that can be expected from any given insert.

In this study I have addressed many of these questions and also added a novel use for VIGS into the field of research. In terms of the optimal size of the insert required for efficient silencing, several reports have shown that anywhere between 30 and 1500 bp sequences of cDNA insert into the TRV can elicit silencing in *N. benthamiana* (Burch-Smith et al. 2004; Thomas et al. 2001). This being said, more recent and practical studies have shown that 200 bp size inserts to be the lowest recommended by the authors for genes expressed at low levels while often researchers recommend 500 bp as the optimal length for efficient silencing (Liu and Page, 2008; Burch-Smith et al. 2004). For the purpose of this study (Chapter 4) I decided to use inserts of 380 bp to avoid incomplete silencing due to lack of targeting sequences. This insert size proved to be efficient in silencing all three of the genes targeted.

While optimising VIGS for my study I often encountered the 'patchy silencing' of leaf material described above. I showed that, similarly to studies performed in Arabidopsis, optimal coverage of silencing is based strongly on the age of the seedlings when Agrobacterium infiltration is performed (Burch-Smith et al. 2006). The method I developed for infiltration of seedlings involved the growth of the plants in tissue culture until ready for infiltration, which avoids unnecessary exposure to pathogens which might elicit a PTGS response (see Methods in Chapters 3 and 4 above). The seedlings were then infiltrated under a mild vacuum where it could be seen that whole leaves were efficiently exposed to the Agrobacterium, and in this way ensuring a higher initial 'dose' compared to other methods (Burch-Smith et al. 2004; Ryu et al. 2004). After infiltration the plants were hardened off and allowed to grow for four weeks in which time new leaves developed that were consistently silenced across the entire leaf lamina (Chapter 4).

It has been suggested in the literature that VIGS could be used for the silencing of gene families by targeting conserved sequences within the genes (Lu et al. 2003). I show here, in Chapter 4, that VIGS can also be used for the specific and simultaneous down-regulation of one, two and three genes of diverse homology by cloning multiple cDNA fragments into TRV. Using this system we targeted a number of genes involved in starch metabolism and produced a number of expected phenotypes as well as some which have not yet been reported. Having validated this system of multiple gene silencing it is now possible to apply the technology to any number of biological pathways to study the complex interactions between the enzymes within them.

In Chapter 3 I profiled plants where the plastidial isoform of the inorganic pyrophosphatase gene was targeted. Pyrophosphate is efficiently metabolised by the psPP into inorganic phosphate which can be exported from the plastid so maintaining the intraplastidial PP_i concentration at a very low level (Rojas-Beltrán et al., 1999; Farré et al., 2001; Geigenberger et al., 1998). It is believed that this low level of PP_i is thermodynamically required to allow for specific anabolic reactions to take place such as starch and carotenoid biosynthesis (Stitt, 1998; Sonnewald, 2001; López-Marqués et al., 2004). Upon profiling of the plants I found that starch and carotenoid synthesis was impaired, and with this so was the plants ability to respond to drought stress through limitations of the precursors to abscisic acid. Many studies have been performed where mutagenised Arabidopsis and maize have been screened for reduced starch, chlorophyll, ability to respond to drought stress and ABA insensitivity and to date none have identified a lesion in the psPP (Yu et al. 2000; Sulmona et al. 2006; Hable et al. 1998; Finkelstein, 1994). Due to the fact that none of these screens have ever identified the psPP as the cause of the phenotypes observed it is likely that loss of its activity is

deleterious. This demonstrates another major advantage for VIGS in that it can be used for the study of genes that when silenced would normally prove lethal. While this use for VIGS has been suggested, this is the first time to my knowledge that it has actually been applied to the study of a novel gene.

In this study as a whole I have shown that VIGS can be used for studies into complex metabolic pathways. I have optimised VIGS to allow for near complete silencing of genes across fully expanded leaves in eight to ten week old *N. benthamiana* leaves which has allowed for the study of starch, chlorophyll, and carotenoid metabolism, as well as the plants ability to respond to drought stress. These techniques can now be applied to a broad range of studies where either gene function is masked by a redundant enzyme, to complicated biological pathways, or even to genes where mutation may be deleterious and so difficult to study by traditional means.

5.2 References

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Apendix

List of Abbreviations

ABA	Abscisic acid
Ac	<i>Activator</i>
ADP	Adenosine diphosphate
ae	Amylose extender
AGO	Argonaute
AGPase	ADP-glucose pyrophosphorylase
AMP	Adenosine monophosphate
AMY	A-amylases
ATP	Adenosine triphosphate
BAM	B-amylase
BE	Branching enzyme
BHT	Butylated hydroxytoluene
CaMV	Cauliflower mosaic virus
DCL	Dicer-like
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DPE1	Disproportionating enzyme; 4-alpha-glucanotransferase
DPE2	Disproportionating enzyme 2
dsRNA	Double stranded RNA
EGM	Environmental gas monitor
EMS	Ethyl methane sulphonate
G1P	Glucose 1-phosphate

G6P	Glucose 6-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBSS	Granule bound starch synthase
GFP	Green fluorescent protein
GWD	α -glucan, water dikinase
ISA	Isoamylases
LAPS	Low amylopectin starch
LDA	Limit dextrinases
LSU	Large subunit of agpase
MDs	Maltodextrins
MEX	Maltose excess
MOS	Malto-oligosaccharides
mRNA	Messenger RNA
<i>Mu</i>	<i>Mutator</i>
PANT	Plastidial adenine nucleotide transporters
PAZ	PIWI/Argonaute/Zwille domain
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PEP	Phosphoenolpyruvate
PFP	Pyrophosphate:fructose 6-phosphate phosphotransferase
PGM	Phosphoglucomutase
Pi	Inorganic phosphate
PP_i	Pyrophosphate
psPP	Plastidial soluble pyrophosphatase
PTGS	Post-transcriptional gene silencing
PVX	Potato virus X

PWD	Phosphoglucan, water dikinase
<i>RDR</i>	<i>Rna-dependent polymerase6</i>
RdRP	RNA-dependant RNA polymerase
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
SBE	Starch branching enzymes
<i>SDE</i>	<i>Silencing defective1</i>
SEX	Starch excess
<i>SGS</i>	<i>Suppressor of gene silencing</i>
siRNAs	Small interfering RNAs
sPP	Soluble pyrophosphatase
SS	Starch synthase
ssRNA	Single stranded RNA
SSU	Small subunit of AGPase
tasiRNAs	Trans-acting siRNAs
TILLING	Targetting induced local lesions in genomes
TRV	Tobacco rattle virus
TUSC	Trait Utility System for Corn
UGPase	UDP-glucose pyrophosphorylase
VIGS	Virus induces gene silencing
WGS	Water soluble glucans
ZFN	Zinc-finger nucleases