Increasing Heat Tolerance in Pollen by Modifying its Carbohydrate Metabolism

by

Zongbin Wu

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

Carleton University

Ottawa, Ontario

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-93534-7

Our file Notre référence
ISBN: 978-0-494-93534-7

Abstract

Anther/pollen development is especially susceptible to high temperatures in many commercially important crops. Although carbohydrate metabolism is essential for normal pollen development and appears to be significantly affected by high temperature stress, there are only very rare examples of attempts to modify carbohydrate metabolism in pollen. In this study, transgenic Arabidopsis plants overexpressing 6 *Arabidopsis thaliana* genes: *sugar transport protein 6, cell wall invertase 2, sucrose synthase 1, hexokinase 1, phosphofructokinase 3* or *ADP-glucose pyrophosphorylase small subunit 1* under the control of a strong pollen-specific promoter were generated and studied under high temperature stress. The number and percentage of healthy looking siliques, as well as seed production of the transgenic plants were compared to untransformed plants and to heat tolerant transgenic plants overexpressing fructokinase. Most candidate genes did not appear to improve the heat tolerance of the transgenic plants, although lines overexpressing sucrose synthase or cell wall invertase may be worth further investigation. This work has made contributions towards the study of the effect of heat stress on the expression of carbohydrate genes in pollen, identified candidate genes which may contribute to improving pollen heat tolerance, and provided a foundation for future investigations in this important field of research.

Acknowledgements

Firstly, I would like to express my deepest gratitude to Dr. Laurian Robert for his excellent supervision, advice, guidance and patience throughout this research. He provided me encouragement and support in various ways, helping my growth from a student to a passionate researcher. I am very grateful to him more than he knows. Many thanks go in particular to my other co-supervisor Dr. Ashkan Golshani and to my thesis advisor Dr. Christiane Charest for their excellent advice and crucial contribution, guiding my research for the past two and half years. Special thank goes to Dr. David Granot for his advice and providing LeFRK transgenic seeds. I was extraordinarily fortunate in having Madeleine Lévesque-Lemay, John Chan, Frances Tran, Nazila Nazemof and Mohsin Zaidi as my colleagues. I would also like to thank them for their help and best suggestions. I would also like to acknowledge Renlin Xu for his advice and help on quantitative RT-PCR, Dr. Shea Miller for teaching me how to use a fluorescence microscope and Kathie Upton for helping me with my growth cabinets and plants. Finally, I would like to thank my close friends and family for their support and encouragement, especially my wife Lili Hao. She was always there to support me and cheer me up through the good times and bad.

Table of Contents

2. Materials and Methods

List of Tables

List of Figures:

List of Abbreviations:

ADP-G:	ADP-glucose
AGPase:	ADP-glucose pyrophosphorylase
AtAPS1:	*Arabidopsis thaliana* ADP-glucose pyrophosphorylase small subunit 1
AtcwINV2:	*Arabidopsis thaliana* cell wall invertase 2
AtHXK1:	*Arabidopsis thaliana* hexokinase 1
AtPFK3:	*Arabidopsis thaliana* phosphofructokinase 3
AtSTP6:	*Arabidopsis thaliana* sugar transport protein 6
AtSUS1:	*Arabidopsis thaliana* sucrose synthase 1
BCP:	Bicellular pollen
CC:	Companion cells
cINV:	cytosolic invertase
cwINV:	cell wall invertase
DNA:	Deoxyribonucleic acid
F1,6BP:	Fructose 1,6-biphosphate
F6P:	Fructose 6-phosphate
FRK:	Fructokinase
G1P:	Glucose 1-phosphate
G6P:	Glucose 6-phosphate
GFP:	Green fluorescent protein
HXK:	Hexokinase
HTS:	High temperature stress
MPG:	Mature pollen grain
PCR:	Polymerase chain reaction
PFK:	Phosphofructokinase
PMC:	Pollen mother cells
PPi:	Pyrophosphate
RNA:	Ribonucleic acid
SE:	Sieve elements
STP:	Sugar transport protein
Suc-P:	Sucrose 6-phosphate
SUS:	Sucrose synthase
SUT:	Sucrose transporter
TCP:	Tricellular pollen
TPT:	Triose phosphate/phosphate translocator (TPT)
Triose-P:	Triose-phosphate
UDP-G:	UDP-glucose
UNM:	Uninucleate microspore
vINV:	Vacuolar invertase

1. Introduction

1.1 Statement of Research Problem

High temperature stress (HTS) occurs as temperature rises above an optimum for a period of time which is detrimental to a plant's physiological activities and development. It can impair vegetative growth, tiller production, and reproduction, thus markedly reducing fruit set and seed yield (Prasad *et al.*, 2011). It is one of the main abiotic stresses limiting plant biomass production and productivity. Significant temperature-stress-induced yield losses happen across the world. For example, in the United States during August 2000, HTS led to approximately US$4.2 billion in agricultural losses (Sakata *et al.*, 2010). Global climate change, with predicted 1.5-5.8°C increases in temperatures by 2100 (Fischer *et al.* 2005; Houghton *et al.*, 2001), should make HTS an increasingly serious problem to agricultural production. Crop yields are predicted to decrease by approximately 10% for every one-degree increase in temperature (USDA release no. 0501.09, 2009). Global temperature rising will not stop in 2100, especially if high carbon dioxide emissions continue, and may lead to a temperature increase of another 10-12°C by 2300 according to the IPCC (Intergovernmental Panel on Climatic Change) (Sherwood & Huber, 2010). Developing nations that already suffer from heat stress-related crop failures are predicted to be especially susceptible to climate change, particularly those located in sub-Saharan Africa (Mittler, 2006). Therefore, overcoming the adverse effect of HTS on crop production is vital for food security in the future.

1.2 Abiotic Stresses and Plant Reproduction

Abiotic stresses, such as heat stress, cold stress, and drought stress can have negative effects on the reproductive process, thus leading to poor seed set (Saini & Westgate, 2000; Thakura *et al.*, 2010; Zinn *et al.*, 2010).

Sexual plant reproduction is a process of generating offsprings. The male gametophyte is the mature pollen grain produced in the anthers which are made up of four microsporangia (or pollen sacs). Each microsporangium initially contains diploid pollen mother cells (PMC) lined with a single tissue layer called the tapetum which provides nutrition to the developing pollen grains. These PMC undergo microsporogenesis and microgametogenesis and in the Brassicaceae produce a mature tricellular pollen grain which contains one vegetative cell and two generative cells. The mature pollen grain is eventually released and transferred to the stigma, the receptive portion of the pistil. After proper adhesion, a compatible pollen grain will germinate and the pollen tube will grow through the style to the ovule where the female gametes are located and double fertilization occurs (Raven *et al.*, 2005).

Abiotic stress can affect various phases throughout the entire reproductive process from gamete formation to fertilization and seed maturation (Lardon & Triboi-Blondel, 1994). Drought stress at any time during the reproductive phase can lead to a reduction of crop yield, but it was shown to have a greater negative effect on two reproductive phases, PMC meiosis and anthesis (Saini & Westgate, 2000). For example, sorghum was found to be highly sensitive to drought stress at the male meiotic stage reducing seed set due to

pollen sterility (Craufurd *et al.*, 1993). Drought stress was also shown to cause pollen sterility and the failure of pollination in rice, as well as zygotic abortion in maize (Saini & Westgate, 2000). Cold temperature can also cause structural and functional abnormalities in reproductive organs resulting in the failure of double fertilization and premature seed abortion in rice (Takeoka *et al.*, 1992). Another study demonstrated that temperatures below 15°C during the reproductive phase lead to flower abscission and reduced seeds set in chickpea (Berger *et al.*, 2006). Furthermore, cold temperature was found to not only disrupt microsporogenesis and microgametogenesis in rice leading to male sterility, but also impaired ovule development and viability, resulting in the failure of fertilization (Thakura *et al.*, 2010). In wheat, heat stress can also cause male reproductive structural abnormalities, such as tapetum degradation during the period of microspore meiosis, leading to male sterility (Sakata *et al.*, 2000). Heat stress was shown to not only stimulate early flowering before enough biomass had sufficiently accumulated for proper seed development in Arabidopsis (Tonsor *et al.*, 2008), but also shorten the period of time the stigma is receptive to pollen thus decreasing the chance of pollination (Zinn *et al.*, 2010). Another study also demonstrated that heat stress could cause poor anther dehiscence leading to a reduction of released pollen in tomato (Sato *et al.*, 2002).

Abiotic stresses frequently affect carbohydrate metabolism during plant reproduction. Drought stress was shown to inhibit starch accumulation thus reducing carbohydrate availability during pollen development and leading to developmental failure (Franchi *et al.*, 1996). Saini and Westgate (2000) also reported that starch failed to build up properly

in developing pollen of both wheat and rice during drought stress. A study in rice showed that cold stress repressed the sugar supply to the tapetum and pollen grains by inhibiting the transcription of the tapetum-specific cell wall invertase gene *OsINV4* during pollen development, leading to pollen sterility and reduced grain set (Oliver *et al.*, 2005). Sakata *et al.* (2010) proposed that the early phase of anther development is especially susceptible to high temperatures in wheat, barley, and various other commercially important crops. Heat stress was also found to impair plant reproduction by causing disturbances in carbohydrate metabolism in the anther and the developing pollen. Pressman *et al.* (2006) and Firon *et al.* (2006) demonstrated that heat stress caused a significant reduction in starch and sucrose levels of the developing pollen of tomato resulting in reduced numbers of viable pollen grains. Therefore, carbohydrate metabolism during pollen development and germination is often affected by abiotic stresses.

1.3 Carbohydrate Metabolism During Pollen Development and Germination

Sugars, the primary product of photosynthesis in plants, are the initial building blocks of most organic matter found in nature. In higher plants, CO_2 fixation occurs predominantly in mesophyll cells of mature leaves. These are net exporters of sugars and are known as 'carbon sources'. Heterotrophic cells in roots, reproductive structures, storage and developing organs rely on a supply of sugars for their nutrition, these are known as 'carbon sinks' (net importers) (Williams *et al.*, 2000). All carbon sink tissues receive an adequate supply of carbohydrate for growth and development from carbon

sources via sugar transporters (STP). In many plants, the transported sugar is mainly sucrose. Developing pollen grains can be considered as strong sinks that require carbohydrate import from the apoplast during maturation, germination and tube growth (Williams *et al.*, 2000).

Carbohydrate metabolism is essential for normal pollen development. Developing pollen grains receive their carbohydrate supply from the tapetum layer and the surrounding locular fluid. The transported sucrose is released from the sieve elements of the phloem into the anther wall layers and the tapetum apoplast, probably via a sucrose transporter (Williams *et al.*, 2000). Pollen germination and pollen tube growth is also greatly dependent on the availability of sugars since the main metabolic activity during this process is the biosynthesis of polymers that will form the elongating cell wall. While the volume of the protoplast does not change significantly during pollen tube elongation since it moves forward, the cell wall forms an immobile tube that continuously expands at the apex. Given that a single pollen tube has to achieve a total length often many times its diameter within a short time, the supply of cell wall precursors needs to be uninterrupted and extremely rapid. To support this high level of carbohydrate synthesis pollen tubes use both stored reserves that can include sucrose and starch, as well as external sources of necessary substrates from the pistil. Efficient sugar metabolism is therefore crucial for the success of the fertilization process (Raven *et al.*, 2005).

Thus, during normal development, pollen grains accumulate sucrose during their maturation. In order to be metabolized in the pollen grain, sucrose must be cleaved by

invertase (INV) or sucrose synthase (SUS), the only two sucrose-cleaving enzymes identified in plants. INV cleaves sucrose into the monomer hexoses, glucose and fructose, while SUS cleaves sucrose in the presence of UDP to yield fructose and UDP-glucose (UDP-G) (Granot, 2008). Therefore, at the onset of pollen germination, stored sucrose is rapidly hydrolyzed by vacuolar invertases (vINV) into glucose and fructose, whereas the imported sucrose is irreversibly cleaved into glucose and fructose by an extra-cellular cell wall invertase (cwINV) that is ionically bound to the cell wall. All glucose and fructose liberated from both pathways are then phosphorylated by hexokinase (HXK) or fructokinase (FRK) to produce glucose 6-phosphate (G6P) or fructose 6-phosphate (F6P), respectively. G6P is then used for metabolic processes such as glycolysis and respiration (Karni & Aloni, 2002) (Figure 1).

Pollen grains also accumulate starch that serves as the energy source for subsequent pollen germination and pollen tube growth (Dorion *et al.*, 1996). Starch is the major storage polysaccharide in plants and is accumulated as granules in many different organs such as pollen grains. The regulatory and rate-limiting step of starch biosynthesis is the synthesis of the glucosyl donor ADP-glucose (ADP-G) by ADP-glucose pyrophosphorylase (AGPase). Therefore, as demonstrated in many different plant species, AGPase is a major enzyme controlling starch synthesis (Denyer *et al.*, 1995; Neuhaus & Stitt, 1990; Tiessen *et al.*, 2002). Whenever sugars are needed, starch can be degraded into glucose 1-phosphate (G1P), glucose or maltose. Among those three resultants, only glucose and maltose are exported to the cytoplasm. The breakdown of maltose in the

cytoplasm yields glucose monomers that, together with the transported glucose, must be phosphorylated to produce G6P, which is then used for metabolic processes such as glycolysis and respiration (Karni & Aloni, 2002). G6P can be isomerized into fructose 6-phosphate (F6P) or converted to G1P, which is attached to UDP to form UDP-G. In the cytoplasm UDP-G and F6P are combined to form sucrose 6-phosphate (Suc-P), which is dephosphorylated to produce sucrose. This sucrose can then be stored in vacuoles or exported out of the photosynthetic (source) tissues to non-photosynthetic (sink) tissues, where it serves as initial substrate for all organic metabolic pathways (Granot, 2008) (Figure 1).

Glycolysis is the main pathway of carbohydrate degradation and it is a central metabolic pathway that is present in all organisms. It provides substrates to fuel energy production and anabolic processes of living cells. Glycolysis is also of importance for adaptations to stress conditions such as nutrient limitations, cold, drought, or oxygen deficiency (Churchill *et al.*, 1994; Plaxton, 1996; Shenton & Grant, 2003). The regulation of the glycolytic process is essential for all cell types. Key regulatory enzymes in plant glycolysis include pyruvate kinase and phosphofructokinase (PFK) (Mustroph *et al.*, 2007).

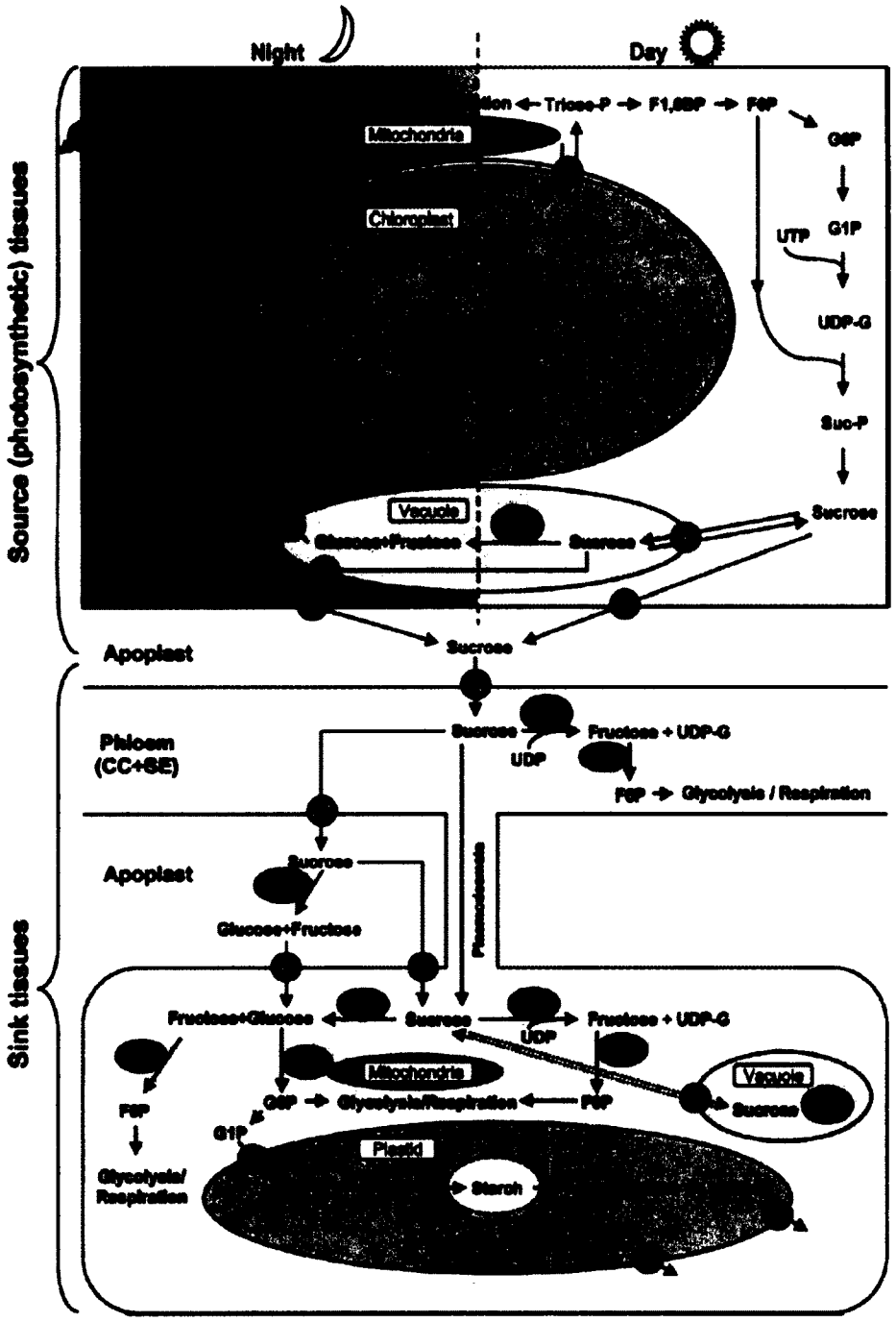

Figure 1. Schematic presentation of sugar metabolism and transport in source tissues during the day and at night, and in sink tissues. Triose-phosphate (Triose-P), triose phosphate/phosphate translocator (TPT), fructose 1,6-biphosphate (F1,6BP), cytosolic invertase (cINV), sucrose transporter (SUT), companion cells (CC), sieve elements (SE), hexokinase (HXK), fructokinase (FRK), cytosolic invertase (cINV), vacuolar invertase (vINV), sucrose synthase (SUS), cell wall invertase (cwINV). (Granot, 2008, copyright © Reprinted with permission of http://www.sciencedirect.com)

1.4 Effect of HTS on Anther Carbohydrate Metabolism

High temperature stress (HTS) causes many physiological and biochemical changes at both the cellular and whole plant levels that significantly reduce crop yields and those effects are often observed at the reproductive stages (Wahid *et al.*, 2012). The optimum temperature of reproductive processes and grain filling (15°C) is much lower than vegetative growth and development (20°C) for wheat, peanut and sorghum (Prasad *et al.*, 2011).

HTS impairs pollen development, germination and fertilization by causing disturbances in carbohydrate metabolism in the anther and the developing pollen. Several studies (e.g. Firon *et al.*, 2006; Pressman *et al.*, 2002; Pressman *et al.*, 2006) showed that constant exposure of tomato plants to mild high temperatures (~29°C) lead to a significant reduction in the starch and sucrose levels of immature pollen grains. The reduced carbohydrate condition then causes a noticeable decrease in the number of pollen grains produced and a marked decline in the capacity of the viable pollen to germinate. This confirmed that starch and sucrose accumulation which rely on sugar supplied via the anther locular fluid are important to the maturing pollen grains and play a critical role in pollen germination. Under normal growth conditions, the level of starch increases in the pollen grains and will reach a maximum 3 days before anthesis. From this stage until anthesis, starch will be rapidly degraded into glucose, which will be used for pollen germination (Pressman *et al.*, 2002). Another study showed that starch and sucrose levels at 1 and 2 days before anthesis were significantly higher in pepper pollen under HTS than

at normal temperature, suggesting that HTS represses the activity of invertase (INV) and reduces carbohydrate metabolism in the developing pollen grains (Aloni *et al.*, 2001). Firon *et al.* (2006) compared heat sensitive and heat tolerant tomato cultivars under heat stress conditions. They observed that the number and quality of pollen grains, the number of fruits and seeds per fruit, as well as the starch concentration in the developing pollen grains at 3 days before anthesis were less affected by HTS in the heat tolerant tomato cultivars, suggesting that keeping up the level of carbohydrates in the developing pollen grains under HTS can overcome the adverse effect of HTS.

A number of studies addressed the effects of HTS on specific anther/pollen enzymes involved in carbohydrate metabolism. For example, a study in wheat demonstrated that heat stress had a negative effect on the activity of ADP-glucose pyrophosphorylase during pollen starch accumulation and suggested that an alteration in carbohydrate metabolism may be involved in pollen abortion (Dorion *et al.*, 1996). Jain *et al.* (2007) pointed out that under high temperature conditions, the anther cell wall invertase (cwINV) in sorghum doesn't function properly thus hindering sucrose hydrolysis and leading to alterations in carbohydrate metabolism and starch deficiency. Another study demonstrated that the activity of INV, SUS and AGPase in heterozygous canola genic male sterile lines was greatly increased under HTS when compared to homozygous sterile lines. This resulted in significantly increased sugar accumulation in developing pollen grains of heterozygous canola lines leading to high numbers of pollen grains with enhanced pollen viability (Hua *et al.*, 2012). A comparison between heat tolerant and heat

sensitive tomato lines under HTS revealed that the heat tolerant lines had a higher level of transcript of a cwINV gene, *Lin7,* leading to higher sucrose level in the young fruit (Li *et al.*, 2012). The data suggested that higher activity of cwINV could improve heat tolerance in plants.

Evidently, increasing or maintaining carbohydrate metabolism as well as starch and sugar levels in developing and mature pollen may improve pollen survival and germination under HTS. Accordingly, promoting sugar metabolism throughout pollen development may help to enhance heat tolerance.

1.5 The Model Organism: *Arabidopsis thaliana*

In this project, *Arabidopsis thaliana* is used as a model organism. Arabidopsis is a small flowering plant native to Asia, Europe and Northwestern Africa. It is an angiosperm, a dicot from the mustard family (Brassicaceae). It is an ideal and popular organism for understanding the molecular biology of many plant traits such as pollen development (Meinke & Sussex, 1979).

These are some of its advantages as a model organism:

1) Since Arabidopsis is a member of the Brassicaceae, it shares recent common ancestry with a large number of species of significant economic importance, including a diverse range of vegetable and oil producing crops, the majority of which are Brassica species such as *Brassica napus.* A number of publications have addressed the level of conserved synteny between regions of the Arabidopsis and *B. napus* genomes (e.g. Cavell

et al., 1998; Scheffler *et al.*, 1997).

2) Arabidopsis has a relatively short life cycle. It only takes 5-6 weeks to complete its entire life cycle (from germination to mature seeds). Its short generation time facilitates rapid genetic studies.

3) Arabidopsis is also a small plant usually growing to 20–25 cm tall. The size of Arabidopsis is advantageous for high-throughput screening. It can be easily grown in a relatively small space and each individual plant can produce several thousand seeds, facilitating genetic studies.

4) Arabidopsis was the first plant to have its genome fully sequenced (The Arabidopsis Genome Initiative, 2000). It has one of the smallest genomes in the plant kingdom with about 157 million base pairs of DNA distributed within 5 chromosomes (Bennett *et al.*, 2003). Its genome sequence, along with a broad range of genetic and molecular information, is maintained by The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/).

5) Transgenic Arabidopsis plants can be made easily using *Agrobacterium tumefaciens* to introduce foreign genes into the plant genome. The current protocol, termed "floral-dip", involves simply dipping inflorescences into a solution of the *Agrobacterium* strain containing the binary vector with the genetic construct of interest. This method avoids the need for tissue culture or plant regeneration (Clough & Bent, 1998).

6) The developing Arabidopsis anther is well characterized at the ultrastructural

level and twelve separate stages of development have been described in depth (Owen & Makaroff, 1995).

7) *Arabidopsis* has served as a model to study the impact of abiotic stresses on plant reproduction. For example, a study showed that Arabidopsis plants lacking the heat shock protein HSP101, which is required for high temperature survival, not only had reduced total dry biomass, number of inflorescences and fruit production, but also displayed pre-mature germination and bolting (Tonsor *et al.*, 2008). Elevated temperature-induced early flowering was also reported by Balasubramanian *et al.* (2006). Both heat stress and cold stress were demonstrated to cause low ovule number and ovule abortion in Arabidopsis plants (Whittle *et al.*, 2009). Another study pointed out that cold stress also substantially reduced pollen tube growth and seed production in Arabidopsis (Lee & Lee, 2003).

The above criteria lead to the selection of *Arabidopsis thaliana* as a genetic model organism for this project.

1.6 Six Candidate Genes

1.6.1 *AtSTP6* (*Arabidopsis thaliana Sugar Transport Protein 6*, At3G05960)

In higher plants, sink tissues receive their supply of carbohydrate for growth and development from carbon sources. Pollen grains are strong sinks that require carbohydrate import from the apoplast during maturation, germination and tube growth (Williams *et al.*, 2000). The long-distance distribution of sugars at the sub-cellular level

requires several transport steps across membranes.

The transport of sugars across membrane barriers is largely mediated by transport proteins which catalyze either passive (but selective) diffusion or energy-dependent active transport thereby allowing accumulation of sugar substrates (Büttner, 2007). Therefore, sugar transport proteins play a pivotal role in the membrane transport of sugars and their cell-to-cell and long-distance distribution throughout the plant (Williams *et al.*, 2000). Higher plants possess two distinct families of sugar transporters: the disaccharide transporters that primarily catalyze sucrose transport and the monosaccharide transporters that mediate the transport of a variable range of monosaccharides. AtSTP6 is a plasma membrane monosaccharide transporter. All known STPs are plasma membrane proteins and catalyze the uptake of hexoses from the apoplastic space into the cell (Scholz-Starke *et al.*, 2003).

In Arabidopsis, 13 *STP* genes have been identified to date. Among all AtSTPs, only AtSTP2, AtSTP4, AtSTP6, AtSTP9 and AtSTP11 were found to be expressed during pollen development (Bennett *et al.*, 2003; Clough & Bent, 1998; Firon *et al.*, 2006; Jain *et al.*, 2007; The Arabidopsis Genome Initiative, 2000). AtSTP2 is expressed during early phases of pollen maturation (Firon *et al.*, 2006) and AtSTP9 is expressed only after pollen germination in the pollen tube (Jain *et al.*, 2007). AtSTP4 plays a role in pollen germination and pollen tube growth (Clough & Bent, 1998), while AtSTP11 was characterized as a pollen tube-specific monosaccharide transporter (The Arabidopsis Genome Initiative, 2000). Since *AtSTP6* is most highly expressed in tricellular pollen

(Büttner, 2007; Scholz-Starke *et al.*, 2003) (Table 1), increasing its expression earlier in development might improve the transport of monosaccharides at these critical stages and it was therefore chosen as a candidate gene for this study.

1.6.2 *AtcwINV2* (*Arabidopsis thaliana cell wall Invertase 2*, AT3G52600)

As mentioned above, pollen grains can be considered as a sink tissue and accumulate sucrose during their maturation. The transported sucrose must be cleaved into glucose and fructose by an extra-cellular cell wall invertase (cwINV) in order to be metabolized in sink tissues. In addition to cwINV, other invertases also exist such as vacuolar invertase (vINV) and cytosolic invertase (cINV) isoforms. INV activity is generally due mainly to cwINV and vINV, whereas cINV activity is comparatively very low. Therefore, sucrose degradation by vINV and cwINV predominate during sink tissue initiation and expansion. The activity of cwINV will determine whether a cell is provided with apoplastic sucrose or hexoses (Sherson *et al.*, 2003). Claeyssen & Rivoal (2007) demonstrated that high cwINV activity correlated with elevated expression levels of MSTs (monosaccharide transporters). Since pollen development relies on sucrose import from the apoplast and its hydrolysis by cwINV, cwINV plays a critical role in pollen development (Claeyssen & Rivoal, 2007).

In Arabidopsis, 6 *cwINV* genes have been identified. All six genes show distinct levels and spatial patterns of expression. Remarkably, five of the six *AtcwINV* genes are expressed in developing Arabidopsis seeds, and of these, four appear to be expressed

Table 1. Microarray expression data at different pollen developmental stages.

Gene	UNM	BCP	TCP	MPG
PSP	971	1884	3563	4178
AtAPS1	892	817	0	0
AtPFK3	470	407	1579	576
AtHXK1	818	1353	1629	868
AtcwINV2	0	158	2718	3809
AtSUS1	305	245	218	0
AtSTP6	107	836	1425	297

UNM - uninucleate microspore, BCP - bicellular pollen, TCP - tricellular pollen and MPG - mature pollen grain, from Honys and Twell (2004).

more strongly in the cell division stages. These observations are consistent with the idea that cwINVs have important roles in seed development (Sherson *et al.*, 2003). AtcwINV1 has been reported to be expressed in stems, leaves and roots, whereas AtcwINV2 expression was anther-specific. Additionally, high expression of AtcwINV2 was detected in the late stages of pollen development, in the tricellular pollen and mature pollen (Honys & Twell, 2004; Tymowska-Lalanne & Kreis, 1998) (Table 1). Therefore, given the important role of cwINV to pollen development and the fact that AtcwINV2 is the only anther-specific cwINV, increasing its expression may improve pollen sink strength and it was thus selected as a candidate gene for this study.

1.6.3 *AtSUS1* (*Arabidopsis thaliana Sucrose Synthase 1*, AT5G20830)

Sucrose synthase (SUS) is a key enzyme involved in sucrose metabolism. Sucrose, the transported sugar in many plants, must be cleaved by either invertase or sucrose synthase in order to undergo further metabolism in sink tissues. Invertase and sucrose synthase are the only two sucrose-cleaving enzymes identified in plants (Granot, 2008). While INV is active in both source and sink tissues, SUS is thought to act mainly in sink tissues. SUS is located in the cytosol and it is also found within the mitochondrion where it possibly fulfills a non-catalytic function in signalling (Claeyssen & Rivoal, 2007). INV cleaves sucrose into the monomer hexoses, glucose and fructose, while SUS catalyses the reversible conversion of sucrose and UDP to UDP-G (UDP-glucose) and fructose (Granot, 2008). Several studies established that SUS is a significant sucrose-cleaving

enzyme feeding sucrose into metabolic pathways in sink tissues (Chengappa *et al.*, 1999; D'Aoust *et al.*, 1999; Ruan *et al.*, 2003; Tang *et al.*, 1999). Further supporting this theory, overexpression of SUS in tobacco plants led to significant increases in plant height, suggesting a role for this enzyme in determining sink strength (Coleman *et al.*, 2006). SUS activity has been studied in various plants and has been shown to play a major role in energy metabolism by controlling the mobilization of sucrose into various pathways important for the metabolic, structural, and storage functions of the plant cell (Baud *et al.*, 2004). Its activity also has been found to mediate transitions through the prominent stages of sink organ development (Claeyssen & Rivoal, 2007).

There are 6 sucrose synthase genes in Arabidopsis and they were all shown to express weakly in seeds (Baud *et al.*, 2004). AtSUS1 is the only Arabidopsis sucrose synthase with activity in the anther (Honys & Twell, 2004) and it exhibited relatively low expression at all stages of pollen development and was not detected in the mature pollen grain (Table 1). *AtSUS1* could be a good candidate for increased pollen expression and it was therefore chosen as a candidate gene for this study.

1.6.4 *AtHXK1* (*Arabidopsis thaliana Hexokinase 1*, AT4G29130)

Pollen grains accumulate sucrose during their maturation. At the onset of germination, the stored sucrose, together with imported sucrose will be hydrolysed by invertase. In plants, all liberated glucose and fructose must be phosphorylated by

hexokinase (HXK) or fructokinase (FRK) to produce G6P or F6P before undergoing further metabolism (Karni & Aloni, 2002). HXK can phosphorylate glucose and fructose, with a high affinity for glucose and a low affinity for fructose, whereas FRK phosphorylates only fructose with a high affinity (Granot, 2008). Therefore, hexokinase is pivotal enzyme for the initial step of sugar utilization in sink tissues.

Hexose + ATP -> hexose-6-phosphate + ADP

HXK isozymes have been identified in various plant species and have been classified into two major groups; plastidic isozymes (Type A) located in the plastid stroma and those containing a membrane-anchor domain (Type B), which are located mainly adjacent to the mitochondria (Olsson *et al.*, 2003). In Arabidopsis, AtHXK1 was the first hexokinase to be isolated from a plant and it is a Type B hexokinase (Damari-Weissler *et al.*, 2007). AtHXK1 is expressed widely, but displays the highest level of expression of the AtHXKs in the stamen and pollen grain (Claeyssen & Rivoal, 2007). AtHXK1 was found to be expressed at the highest level in the late stage of pollen development, in the tricellular pollen (Honys & Twell, 2004) (Table 1). Since higher levels of AtHXK1 may improve sugar utilization throughout pollen development, germination and tube growth it was also selected as a candidate gene.

1.6.5 *AtPFK3* (*Arabidopsis thaliana PhosphoFructoKinase 3*, AT4G26270)

Carbohydrates are the principal energy source for most living organisms, and the main pathway of carbohydrate degradation is through glycolysis. Glycolysis provides

substrates to fuel energy production and anabolic processes of living cells. Glycolysis is also of importance for adaptations to stress conditions such as nutrient limitations, cold, drought or oxygen deficiency (Churchill *et al.*, 1994; Plaxton, 1996; Shenton & Grant, 2003). The regulation of the glycolytic process is essential for all cell types. Phosphofructokinase (PFK) is a key regulatory enzyme of glycolysis (Mustroph *et al.*, 2007).

Phosphofructokinase catalyzes the conversion of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate (Figure 2).

fructose 6-Phosphate + ATP → fructose 1,6-bisPhosphate + ADP

The reaction between F6P and ATP to give F1,6BP is the first unique step in glycolysis and is a non-equilibrium reaction, therefore, regulation of phosphofructokinase could determine the rate of glycolysis (Turner & Turner, 1975). Three different types of phosphofructokinases are known that differ with respect to the phosphoryl donor. ATP-dependent PFK uses ATP as a phosphoryl donor and is widely distributed in plants and a number of prokaryotes. It is a key enzyme of glycolysis. The second type of phosphofructokinase is a pyrophosphate-dependent phosphofructokinase that uses pyrophosphate (PPi) instead of ATP as a phosphoryl donor (pyrophosphate fructose-6-phosphate-phosphotransferase, PFP). PFP shares sequence similarity with the ATP-dependent PFK. However, phosphorylation of F6P catalyzed by PFK is virtually irreversible in vivo, while PFP reacts near equilibrium and catalyzes the reaction in both directions. PFP was reported to be found in plants, various protists and some prokaryotes.

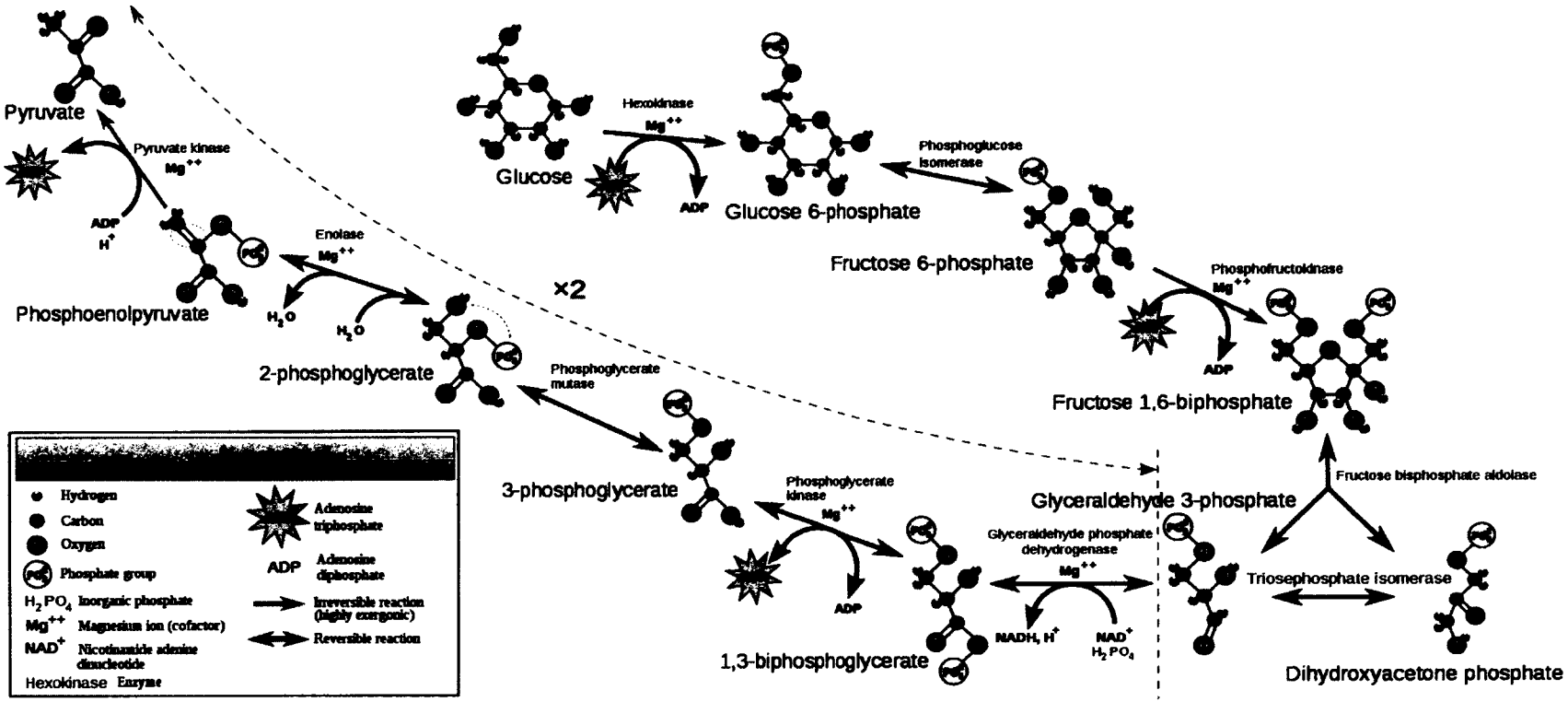

Figure 2. The pathway of glycolysis. (Copyright © GNU Free Documentation License) (http://commons.wikimedia.org/wiki/File:Glycolysis.svg)

The last phosphofructokinase is ADP-dependent. However, ADP-dependent phosphofructokinases have been reported only in certain groups of archaebacteria. In plants, only the first two types of PFK are present (Mustroph *et al.*, 2007). According to the phosphoryl donor, AtPFK3 is an ATP-dependent phosphofructokinase.

Based on bioinformatic and phylogenetic analyses, there are seven sequences that encode ATP-dependent phosphofructokinases (AtPFK1-7) in Arabidopsis and they are all expressed in floral tissues (Mustroph *et al.*, 2007). According to microarray data (Honys & Twell, 2004), *AtPFK2* didn't display any expression during pollen development, while the other *AtPFK*s showed relatively low expression in the pollen with the exception of *AtPFK3* late in development (Table 1). The different AtPFK isoforms have different functions in Arabidopsis during development and adaptation towards environmental changes. AtPFK1 and AtPFK3 were shown to be induced following heat stress (Mustroph *et al.*, 2007). Given that *AtPFK3* has the highest level expression in pollen of all AtPFKs and that it has been shown to be induced by HTS, it seemed to be a good candidate for pollen overexpression.

1.6.6 *AtAPS1* (*Arabidopsis thaliana ADP-glucose Pyrophosphorylase Small subunit 1*, AT5G48300)

Starch is the major storage polysaccharide in plants and is accumulated as granules in many different organs including pollen grains where it is used as a carbon and energy source for pollen germination and tube growth. The regulatory and rate-limiting

step of starch biosynthesis is the synthesis of the glucosyl donor, ADP-glucose, by ADP-glucose pyrophosphorylase (AGPase). AGPase generates the sugar nucleotide ADP-glucose and inorganic pyrophosphate (PPi) from glucose 1-phosphate and ATP (Turner & Turner, 1975).

Glucose 1-P + ATP →ADP-glucose + PPi

The role of AGPase as a major enzyme controlling starch synthesis has been demonstrated in many different plant species (Denyer *et al.*, 1995; Neuhaus & Stitt, 1990; Tiessen *et al.*, 2002).

ADP-glucose functions as the glucosyl donor for glucan synthesis by starch synthase. The catalytic activity of AGPase is allosterically regulated. This enzyme plays a key role in the modulation of photosynthetic efficiency in source tissues and also determines the level of storage starch in sink tissues, thus influencing overall crop yield potential (Salamone *et al.*, 2000). Mutants deficient in AGPase have reduced levels of starch (Salamone *et al.*, 2000). All of the AGPases from higher plants are heterotetramers composed of two distinct types of subunits: small subunits (ApS1 and ApS2) and large subunits (ApL1-ApL4). The small subunit of AGPase is highly conserved, whereas the similarity among the different large subunits is lower. It has been speculated that the two plant subunits were originally derived from the same gene. It has also been suggested that the major function of the large subunit (LS) is to regulate the activity of AGPase, whereas the small subunit (SS) is primarily involved in catalysis (Crevillen *et al.*, 2005).

All six Arabidopsis AGPase subunit mRNAs were detected in leaves,

inflorescences, fruits, and roots of mature plants, with *ApS1*, *ApL1*, *ApL3*, and *ApL4* showing elevated mRNA steady state levels, whereas *ApS2* and *ApL2* mRNA accumulated at very low levels (Crevillen *et al.*, 2005). Both the *ApS1* and *ApS2* genes are expressed in the main starch-producing tissues of the plant, however, *ApS1* expression level is up to 2 orders of magnitude higher than that of *ApS2*, suggesting that ApS1 plays a critical role in starch synthesis in all organs of the plant. Hence, ApS1 is the major SS isoform responsible for AGPase activity and starch synthesis in all tissues of the plant. The expression of APS1 was detected only in the early stages of pollen development, in the microspores and bicellular pollen (Crevillen *et al.*, 2005) (Table 1) and increasing the range and level of its expression during pollen development may contribute to improved starch synthesis.

1.7 **Hypothesis/Objectives**

Anther/pollen development is especially susceptible to high temperatures in many commercially important crops. Since carbohydrate metabolism is essential for normal pollen development and function, and appears to improve pollen heat tolerance, it is hypothesized that over-expressing carbohydrate metabolism genes throughout pollen development will improve pollen performance and seed set under HTS.

Therefore, the main objective of this study was to determine if Arabidopsis plants expressing APS1, STP6, PFK3, SUS1, cwINV2 or HXK1 under the control of a pollen-specific promoter expressing highly throughout pollen development would

improve pollen performance and fruit set under high temperature stress. This research should not only determine if the candidate genes can increase pollen heat tolerance, but should also provide insight into the role of these genes in anther/pollen development and function.

2. Materials and Methods

2.1 Plant Material and Tissue Collection

2.1.1 Plant Material

Arabidopsis (*Arabidopsis thaliana* Columbia) plants were used in all the experiments. After a cold treatment of the Arabidopsis seeds in the soil for 2 days at 4°C, they were grown in growth cabinets at a day/night temperature of 22/22°C for 4-5 weeks before being used for Arabidopsis transformation. All transformed Arabidopsis seedlings were also grown in the soil under the above conditions for 1-2 weeks before being subjected to the heat treatments (described below).

2.1.2 Tissue Collection

2.1.2.a Buds

After 2 weeks of heat treatment at 32°C, 8 (2 x 4) biggest floral buds from each plant were collected with tweezers, frozen immediately with liquid nitrogen and stored at –80°C for later analysis.

2.1.2.b Silique counting

Following heat treatment, the number of healthy looking siliques and the total number of potential siliques were counted manually. Siliques which were aborted, short, deflated or distorted significantly were considered as unhealthy looking. A photograph of some transgenic and untransformed lines following a HTS treatment is shown in Figure 3 to illustrate typical results.

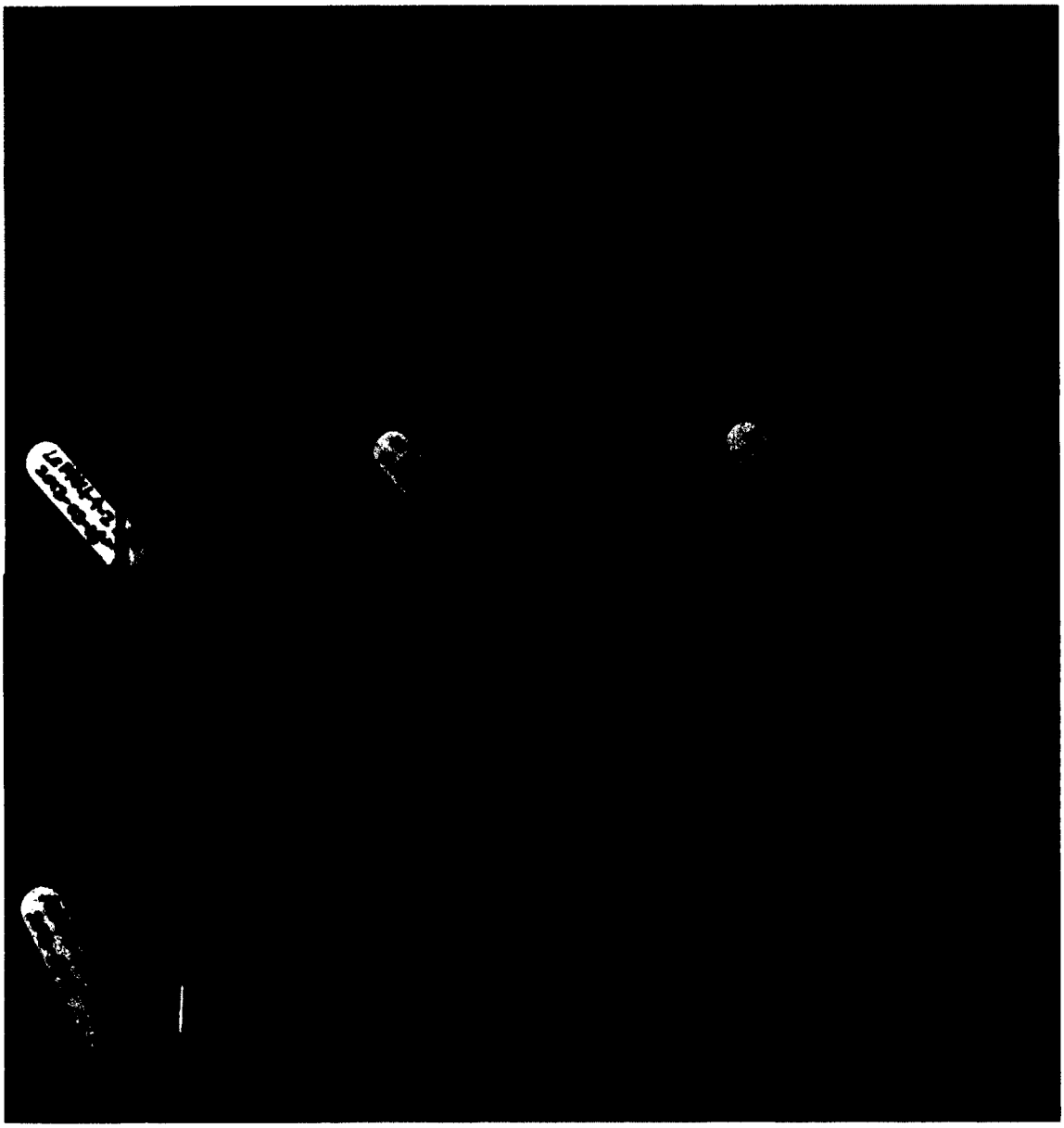

Figure 3. Different T_1 lines of PSP-AtSTP6, PSP-AtSUS1, PSP-AtPFK3 and PSP-AtAPS1 are compared to WT and heat tolerant LeFRK. All lines had been heat stressed for 14 days at 32°C. Red arrows point at healthy looking siliques.

2.1.2.c Seeds

When mature plants and seed pods were completely dry, each plant was cut just above soil level and placed into a Glassine bag whose weight had been obtained in advance. The total weight of each plant was determined. Seeds were collected by tapping the Glassine bag, cleaned, weighed and stored in small labeled envelopes. The percentage of healthy seeds (light brown and plump) was determined visually under a dissection microscope.

2.2 Optimization of Heat Treatment Conditions

2.2.1 Soil Tests

Both ProMix (Premier Horticulture Inc. USA) and regular soil (in-house premixed soil) were divided into two portions. One portion was autoclaved. The four different soils (ProMix + Autoclave, ProMix – Autoclave, Regular Soil + Autoclave, Regular Soil – Autoclave) were incubated at 30°C for 14 days in a growth chamber with the normal watering regime. The soil condition (humidity level, algae level) was observed for each soil.

To test the effect of the soils on plant growth, 15 seedlings of untransformed (WT) Arabidopsis were transplanted to each different soil. The size of the seedlings' rosette on the differently treated soils was compared to each other after 14 days in a growth cabinet at 22°C.

2.2.2 Heat Treatment Temperature Determination

All heat treatment tests were performed with untransformed WT plants for 2 weeks at a constant day/night temperature. Seven plants were heat stressed at 31°C, 28 at 32°C, 22 at 33°C, and 12 WT at 34°C. The soil was kept moist during the entire heat treatments. After 2 weeks, the number of healthy looking siliques was recorded at the different temperature regimes and pictures were taken with a Nikon D50 digital camera. The temperature treatment showing a consistent and significant reduction in silique production was selected to distinguish heat tolerant plants from heat sensitive plants.

2.3 Promoter selection

Transgenic plants shown previously to express transcriptional fusions between the green fluorescent protein (GFP) and the promoters of the different pollen-specific genes PSP (AT1G63060), PTIP (AT3G47440), PADF (AT5G52360), PCUP (AT5G38760), and PPI (AT4G08670) were heat stressed at day/night temperatures of 30°C/30°C for 48h. GFP activity resulting from the different promoter fusions was observed in mature pollen grains obtained from open flowers.

Since the *AtSTP2* (AT1G07340) gene was shown to express highly at the early stages of pollen development (Truernit *et al.*, 1999), the activity of a PSTP2-GFP fusion which had also been introduced into Arabidopsis was compared to the PSP-GFP fusion (which showed the widest range of expression in pollen) throughout pollen development at room temperature. GFP activity of each promoter in microspore/pollen at different

developmental stages (Bud length = 1.0 mm, 1.5 mm, 2.0 mm and open flower) was also compared to untransformed plants (WT). GFP activity was observed with an AxioVision 4.5 fluorescence microscope. All photographs were taken under the 40X objective with a 200 ms exposure time. (Photographs for the developmental study were provided by Madeleine Lévesque-Lemay).

2.4 Constructs

The PSP (At1g63060) promoter was found to express highly during pollen development under normal and high temperatures and was chosen to direct the expression of the 6 carbohydrate metabolism genes.

2.4.1 Gene Isolation and Cloning

Arabidopsis anther cDNA available in the lab was used as template for PCR amplification of the 6 candidate genes (*AtAPS1*, *AtcwINV2*, *AtHXK1*, *AtPFK3*, *AtSTP6* and *AtSUS1*). For all genes except *AtHXK1*, oligonucleotide primers were designed to amplify the complete coding sequence while introducing a *Bam* HI restriction site at the 5' end and a *Sac* I restriction site at the 3' end of each corresponding PCR product (Table 2). In the case of *AtHXK1*, the reverse primer introduced a *Spe* I site at the 3' end of the PCR fragment. PCR conditions were as follows: 98°C for 30 s, after which 2.5 units of Taq polymerase (Invitrogen, USA) were added, followed by 35 cycles of 98°C for 30 s, 56°C for 30 s, 72°C for 150 s, and a final extension of 10 min at 72°C. The PCR mixture

Table 2. Designed primers used to amplify the complete coding sequence of the candidate genes.

Oligonucleotide	Sequence
AtSTP-F1	**GGATCC**TTAAACATGGCTGTTGTTGTGTC
AtcwINV2-F2	**GGATCC**ATGAGTGCTCCAAAGTTTGGTTATG
AtAPS1-F1	**GGATCC**ACTACAATGGCGTCTGTATCTGCAATTGG
AtPFK3-F1	**GGATCC**TTATGAGTACTGTGGAGAGTAGCAAACCGAAG
AtSUS1-F1	**GGATCC**ATGGCAAACGCTGAACGTATGATAAC
AtHXK1-F3	**GGATCC**GGAAAAATGGGTAAAGTAGCTGTTGG
AtHXK1-R5	**ACTAGT**TTAAGAGTCTTCAAGGTAGAGAGAGTG
3 Race-SacI Reverse	CGC**GAGCTC**GAATTAATACGACTCACTATAGG

The *Bam* HI restriction site introduced at the 5' end of the fragment and the *Sac* I or *Spe* I restriction sites introduced at the 3' end are shown in bold.

contained lX buffer with 2.5 mM $MgCl_2$ and 0.2 mM dNTPs (Invitrogen, USA), and 20 pmol of a candidate gene-specific forward primer and of the 3' end RACE product-specific reverse primer. The resulting PCR fragments were ligated into pGEM-T Easy (Promega Corp. USA) and sequence verified (Ottawa Hospital Research Institute).

The candidate genes *AtAPS1, AtPFK3, AtSTP6* were digested with *Bam* HI and *Sac* I and introduced individually between the PSP promoter and the nopaline synthase polyadenylation signal of the PSP-GFP binary vector described above thereby replacing the GFP gene. The same approach was used for *AtHXK1* except for the fact that the gene fragment was cloned into PSP-GFP using the *Bam* HI and *Spe* I restriction sites.

Since there were either internal *Bam* HI and/or *Sac* I sites in the candidate genes *AtcwINV2* (Figure 7) and *AtSUS1* (Figure 9), they could not be cloned directly into the binary vector.

To construct PSP-AtSUS1, the *AtSUS1* cDNA was first digested with *Bam* HI and *Sac* I, resulting in one long and two short *Bam* HI-*Sac* I fragments. The *AtSUS1* gene was also digested separately with *Sac* I, resulting in a single restriction *Sac* I fragment. The long *Bam* HI-*Sac* I fragment was first introduced between the PSP promoter and the nopaline synthase polyadenylation signal of the PSP-GFP binary vector thereby replacing the GFP gene and creating the intermediate vector PSP-AtSUS1-Sac. The AtSUS1 *Sac* I fragment was then introduced into the *Sac* I site of the PSP-AtSUS1-Sac vector upstream of the nopaline synthase polyadenylation signal re-creating the *AtSUS1* gene and resulting in PSP-AtSUS1.

To construct PSP-AtcwINV2, the *AtcwINV2* cDNA was first digested with *Bam* HI and *Sac* I, resulting in one *Bam* HI-*Sac* I fragment and one *Bam* HI fragment. The *Bam* HI-*Sac* I fragment was first introduced between the PSP promoter and the nopaline synthase polyadenylation signal of the PSP-GFP binary vector replacing the GFP gene generating vector PSP-AtcwINV2-Bam. The *Bam* HI fragment containing the rest of the *AtcwINV2* gene was then introduced into PSP-AtcwINV2-Bam yielding PSP-AtcwINV2. No transformed *Agrobacterium* colonies could be obtained with this version of the PSP-AtcwINV2 vector suggesting that the PSP promoter functioned sufficiently in *Agrobacterium* for the cwINV to be detrimental to its growth. To prevent expression of the invertase in *Agrobacterium*, the *Bam* HI fragment of the AtcwINV2 cDNA of PSP-AtcwINV2 was replaced with the equivalent genomic *Bam* HI fragment which contains an intron as follows. The genomic *Bam* HI fragment was PCR amplified from Arabidopsis genomic DNA with primers AtcwINV2-F2 (5'-GGATCCATGAGTGCTCCAAAGTTTGGTTATG-3') and AtcwINV2-R1 (5'-GCGGATCCGTTCTCGCCATTATCG-3'). PCR conditions were as follows: 98°C for 30 s, after which 2.5 units of Taq polymerase (Invitrogen, USA) were added, followed by 35 cycles of 98°C for 30 s, 56°C for 30 s, 72°C for 80 s, and a final extension of 10 min at 72°C. The PCR mixture contained lX buffer with 2.5 mM $MgCl_2$ and 0.2 mM dNTPs (Invitrogen, USA), and 20 pmol of each primer (Sigma-Aldrich, Canada). The resulting PCR fragments were ligated into pGEM-T Easy (Promega Corp. USA) and sequence verified (Ottawa Hospital Research Institute). The *Bam* HI + intron

fragment was then introduced into the original PSP-AtcwINV2 vector replacing the cDNA fragment with the genomic fragment and producing PSP-AtcwINV2i.

2.4.2 Map of Each Construct

A plasmid map of each construct was built using the SeqMan Software (DNASTAR, USA).

2.5 Arabidopsis Transformation

2.5.1 *Agrobacterium* Transformation

Agrobacterium tumefaciens strain EHA105 competent cells were transformed with the individual binary vectors according to Holsters *et al.*'s protocol (1978) and *Agrobacterium* transformants were screened on 2YT medium (16 g/L of bacto-tryptone (Becton Dickinson, USA), 10 g/L of bacto-yeast (Becton Dickinson, USA), 5 g/L of NaCl, 15 g/L of bacto-agar (Becton Dickinson, USA)) containing 50 mg/ml kanamycin and 25 mg/ml chloramphenicol. The individual *Agrobacterium* strains were then used for plant transformation after verifying the integrity of the binary plasmid using construct-specific restriction enzyme digests.

2.5.2 Arabidopsis Transformation

Each construct was introduced separately into *Arabidopsis thaliana* according to the floral dip method described by Clough & Bent (1998). Seeds from the transformed

plants were plated on solid MS medium (0.5X Murashige and Skoog Basal Salt Mixture (PhytoTechnology Laboratories, USA), 1% Sucrose, 0.8% PhytoBlend (Caisson laboratories Inc., USA) containing 50 mg/ml kanamycin and kanamycin resistant seedlings were then transferred to soil for further analysis.

2.6 Transgenic Plant Analysis

2.6.1 Genomic PCR

To verify transgene integration into the Arabidopsis genome, a leaf sample (2 cm^2) was collected from each primary transformant and untransformed plants (WT), frozen instantly in liquid nitrogen and stored at -80°C. The Illustra DNA Extraction Kit (GE Healthcare, Canada) was used to extract leaf genomic DNA and a construct-specific fragment spanning part of the PSP promoter and the candidate carbohydrate metabolism gene was PCR amplified from 1 µl of genomic DNA according to the following conditions: 94°C for 2 min, after which 1.25 units of Taq polymerase (Invitrogen, USA) were added, followed by 35 cycles of 94°C for 30 s, 59.5°C for 30 s, 72°C for 80 s, and a final extension of 10 min at 72°C. The PCR mixture contained lX buffer with 2.5 mM $MgCl_2$ and 0.2 mM dNTPs (Invitrogen, USA), and 20 pmol of a candidate gene-specific reverse primer and the PSP promoter-specific forward primer AtPSP1-F2 (or AtPSP1-F1 for *AtAPS1*). The sequences for the primers used in RT-PCR are showed in Table 3. PCR products were separated on a 1.1% agarose gel, stained with ethidium bromide and photographed.

Table 3. Designed primers used for genomic PCR and transgene-specific RT-PCR.

Oligonucleotide	**Sequence**
AtPSP1-F2	ATCCTCAGCAAATTAATTTTCACC
AtPSP1-F1	AAAAAGCTTCATTAGCTCACAAATCTAGTGTATCACC
AtAPS1-R1	TATAGTTAGCACCAAGAGGCACAG
AtSTP6-R1	CAACGAGGTAAATCCCAACGAACG
AtPFK3-R1	CCATTCTCTATACTCTCGGCTTCC
AtHXK1-R6	CTAATTCCCTCTGTCTACCTTCTG
AtSUS1-R1	TGGCCTCAAACTCTTCATACAG
AtcwINV2-R2	CCCAACCGTCAGGAGTGTAACC

2.6.2 RT-PCR

2.6.2.a Transgene-specific RT-PCR

To verify that the different genetic constructs were expressed in the transgenic lines, RNA was first isolated from 4 floral buds (just prior to opening) from individual transgenic lines using the RNeasy Plant Mini Kit (Qiagen, Mississauga, Canada). Total RNA concentration was quantified with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc. USA). The QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, Canada) was then used to synthesize cDNA using approximately 800 ng of total RNA. Contaminating genomic DNA in the RNA samples was removed using the gDNA wipeout buffer provided with the reverse transcription kit. In order to differentiate between the transcripts resulting from the genetic constructs and the endogenous transcripts, a forward primer specific to the PSP 5' untranslated region (AtPSP1-F2) was used in combination with a reverse candidate gene-specific primer for the RT-PCR reactions (Table 3). Untransformed Arabidopsis was used as a negative control to ensure that the endogenous transcripts were not amplified with this approach. PCR was performed with 1 µl of cDNA as follows: 94°C for 2 min, after which 2.5 units of Taq polymerase (Invitrogen, USA) were added, followed by 35 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 80 s, and a final extension of 10 min at 72°C. The PCR mixture contained lX buffer with 2.5 mM $MgCl_2$ and 0.2 mM dNTPs (Invitrogen, USA), and 20 pmol of each primer (Sigma-Aldrich, Canada). PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed.

2.6.2.b Semi-quantitative RT-PCR

In order to compare the relative expression level of each candidate gene (endogenous + transgene) in transformed lines, candidate gene-specific primer pairs were used for semi-quantitative RT-PCR (Table 4). All gene-specific reverse primers were designed to amplify a genomic DNA region that contains an intron in order to differentiate a fragment amplified from gDNA as opposed to cDNA. No genomic DNA PCR fragments were ever detected in the semi-quantitative RT-PCR reactions. Untransformed Arabidopsis was used as negative control and 18S ribosomal RNA was used as an internal quantitative PCR control. PCR was performed with 1 µl of cDNA as follows: 94°C for 2 min, after which 2.5 units of Taq polymerase (Invitrogen, USA) were added, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at gene-specific temperatures for 30 s, (62°C for (*AtSTP6* and *AtcwINV2i*) or 61°C for (*AtASP1* and *AtSUS1*) or 59°C for (*AtHXK1*) or 63°C for (*AtPFK3*)), extension at 72°C for various amounts of time (90 s for (*AtSTP6*) or 50 s for (*AtAPS1*) or 60 s for (*AtHXK1* and *AtSUS1*) or 80 s for (*AtPFK3*) or 70 s for (*AtcwINV2i*)), and a final extension of 10 min at 72°C. The PCR mixture contained lX buffer with 2.5 mM $MgCl_2$ and 0.2 mM dNTPs (Invitrogen, USA), and 20 pmol of each primer (Sigma-Aldrich, Canada). PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed.

2.6.3 Heat Treatment

Younger plants had previously been shown to be more susceptible to HTS (results

Table 4. Designed primers used for semi-quantitative RT-PCR.

Oligonucleotide	Sequence
AtAPS1-R1	TATAGTTAGCACCAAGAGGCACAG
AtSTP6-R1	CAACGAGGTAAATCCCAACGAACG
AtPFK3-R1	CCATTCTCTATACTCTCGGCTTCC
AtHXK1-R6	CTAATTCCCTCTGTCTACCTTCTG
AtSUS1-R1	TGGCCTCAAACTCTTCATACAG
AtcwINV2-R2	CCCAACCGTCAGGAGTGTAACC
AtAPS1-F1	GGATCCACTACAATGGCGTCTGTATCTGCAATTGG
AtSTP6-F1	GGATCCTTAAACATGGCTGTTGTTGTGTC
AtPFK3-F1	GGATCCTTATGAGTACTGTGGAGAGTAGCAAACCGAAG
AtHXK1-F3	GGATCCGGAAAAATGGGTAAAGTAGCTGTTGG
AtSUS1-F1	GGATCCATGGCAAACGCTGAACGTATGATAAC
AtcwINV2-F2	GGATCCATGAGTGCTCCAAAGTTTGGTTATG
18S1-F	CTTCGGGATCGGAGTAATGA
18S1-R	GTGCCAGCGGAGTCCTATAA

not shown), therefore all plants were heat stressed just as they started to bolt. *LeFRK1* (*L. esculentum* fructokinase 1), which was identified in tomato plants and phosphorylates exclusively fructose, was shown to improve pollen tolerance to heat in transgenic Arabidopsis plants expressing the *LeFRK1* gene under the control of the *LeFRK4* promoter (Dr. David Granot, personal communication). These LeFRK transgenic lines were used as positive controls for the heat treatments. Generally, WT and LeFRK lines were included in the same flats as the transgenic lines.

The harder we heat stress the transformed lines, the more confidence we should have in the promising lines. Transgenic T_0 lines (PSP-AtAPS1, PSP-AtHXK1, PSP-AtPFK3, PSP-AtSTP6 and PSP-SUS1), untransformed plants (WT) and heat tolerant transformants (LeFRK) were initially heat stressed at a day/night temperature of 33/33°C. However, after 14 days, the number of healthy looking siliques was very low for all lines and not enough seeds had been produced to allow later T_1 screening. Therefore, all primary transformants as well as the WT and LeFRK lines were incubated at a day/night temperature of 32/32°C for another 7 days in order to increase seed production. After T_0 screening, T_1 progeny of promising transgenic lines, along with untransformed plants (WT) and heat tolerant transformants (LeFRK), were heat stressed at a day/night temperature of 32/32°C for 14 days.

Given the delays that occurred in the vector construction of PSP-AtcwINV2i, individual transformed plants for PSP-AtcwINV2i were only heat stressed at a day/night temperature of 32/32°C for 14 days and no T_1 screening was performed. Unfortunately,

all WT and LeFRK plants were contaminated in that experiment and could not be used for the T_0 heat treatment. Therefore, in the absence of controls, results for the PSP-AtcwINV2i T_0 lines could only be compared to each other.

After the 2-week heat treatment, photographs of each transformant were taken and the number of healthy looking siliques of each transformant was counted manually and the whole plant and seeds were weighed. Results for the T_0 and T_1 analyses are presented for individual lines to provide a better indication of the individual variations in phenotype. Averaging the results from all T_0 or T_1 lines for each individual construct was also performed, but did not provide additional information and therefore these results were not presented.

3. Constructs

3.1 Results

3.1.1 Pollen-specific Promoter Selection

In order to determine whether the available pollen-specific promoters express at high temperature, analyses were performed on 5 candidate promoters (PCUP, PSP, PADF, PPI and PTIP). The comparison of promoter activity in mature pollen grains after a 48 h heat treatment at 30°C is shown in Figure 4. There were no significant differences between the activity of any of the promoters at high temperature and that observed previously under normal growing conditions (results not shown).

The promoter for *AtSTP2* that putatively expresses at high levels early in pollen development was also evaluated and compared to the PSP promoter at different pollen developmental stages (Figure 5).

The results of the developmental screening showed that at normal temperatures, the PSTP2 promoter fragment used did not drive high GFP expression during pollen development, although its activity did appear higher in the earlier stages. Accordingly, the PSP promoter was chosen to direct the expression of the 6 carbohydrate metabolism genes.

3.1.2 Candidate Gene Constructs

All 6 candidate carbohydrate metabolism genes were isolated by PCR and their sequence was verified. The genes were cloned separately into a plant transformation

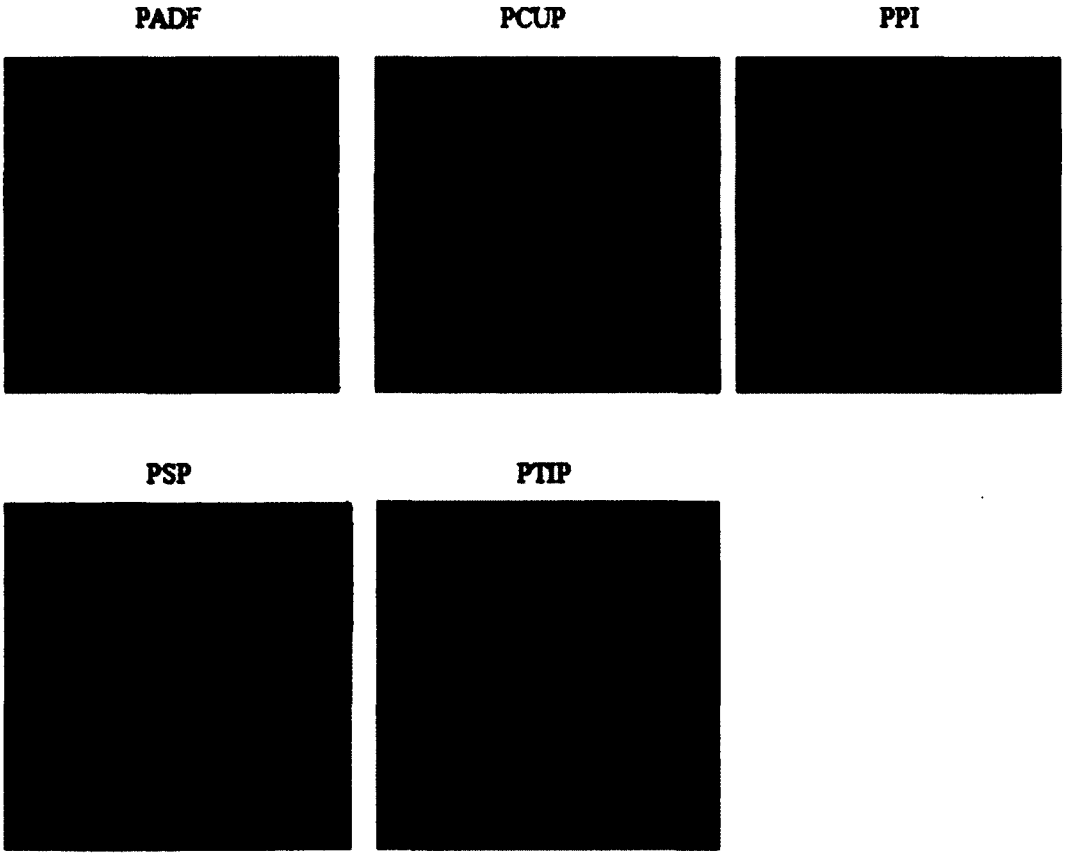

Figure 4. GFP activity of individual promoters in pollen grains from open flowers after a 48-hour heat treatment at 30°C. All pictures were taken under a 40X objective with a 200 ms exposure time.

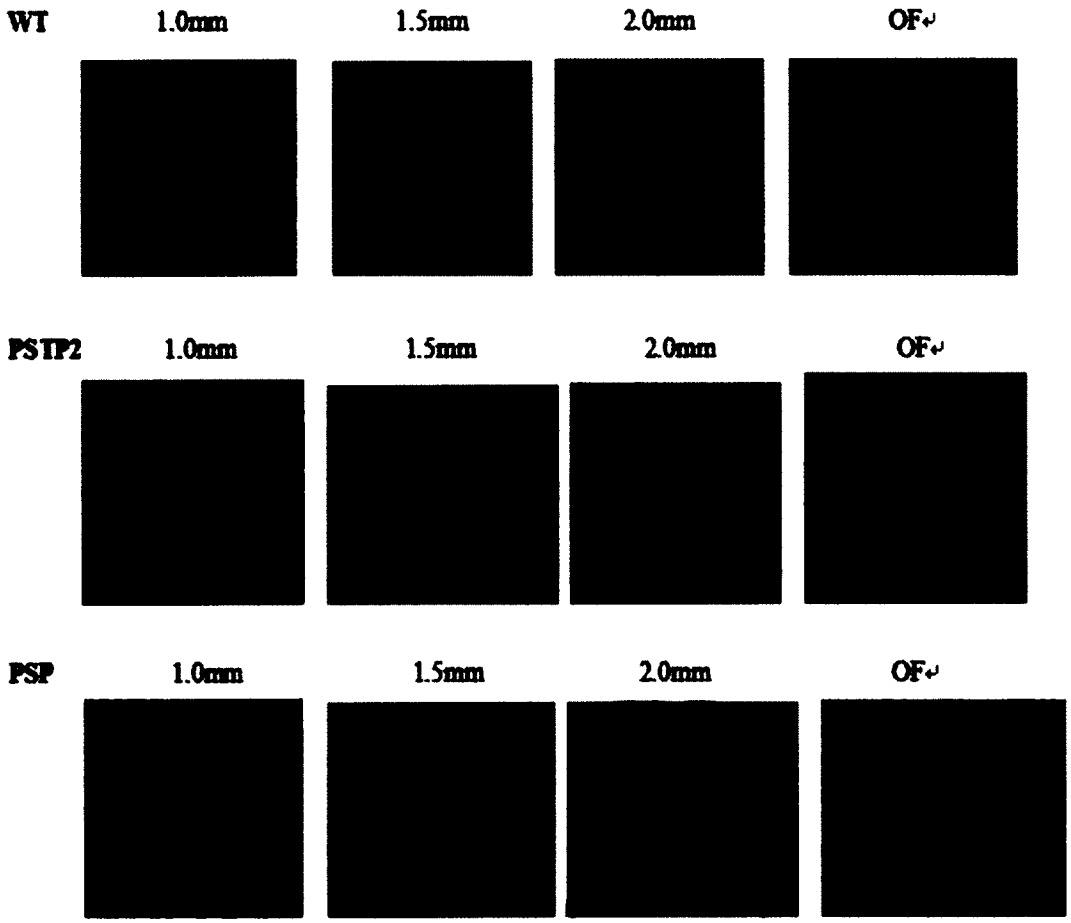

Figure 5. GFP activity in anthers of transgenic plants containing the PSTP2-GFP or PSP-GFP compared to untransformed plants (WT) and grown at normal temperature. Sizes above photographs refer to Arabidopsis flower bud length. OF: open flower. All pictures were taken under a 40X objective with a 200 ms exposure time.

binary vector (pRD420) and the maps of the final constructs are shown below (Figures 6-11).

3.2 Discussion

A group of putative pollen-specific promoters from *Arabidopsis thaliana* was selected based on their tissue-specificity and their expression level and timing using microarray data from different pollen developmental stages reported by Honys and Twell (2004). This group consisted of promoters for genes *PSP* (At1g63060), *PTIP* (At3g47440), *PADF* (At5g52360), *PCUP* (At5g38760), and *PPI* (At4g08670). To test the activity of these promoters, they were fused to the green fluorescent protein (GFP) gene and independently transformed into Arabidopsis. All Arabidopsis transformants showed pollen-specific GFP activity (results not shown). Since the goal of this study is to overexpress carbohydrate metabolism genes in the pollen under heat stress, it was important to verify whether the activity of these promoters was affected by high temperatures. Comparing promoter-driven GFP activity in mature pollen grains after heat stress, PCUP-GFP and PSP-GFP showed strong activity, PADF-GFP and PPI-GFP showed considerably less activity, whereas PTIP-GFP showed low activity (Figure 4). These results are in close agreement with the results obtained under normal growing temperatures (results not shown) and therefore demonstrate that under the conditions tested high temperature may not affect the activity of the selected pollen-specific promoters.

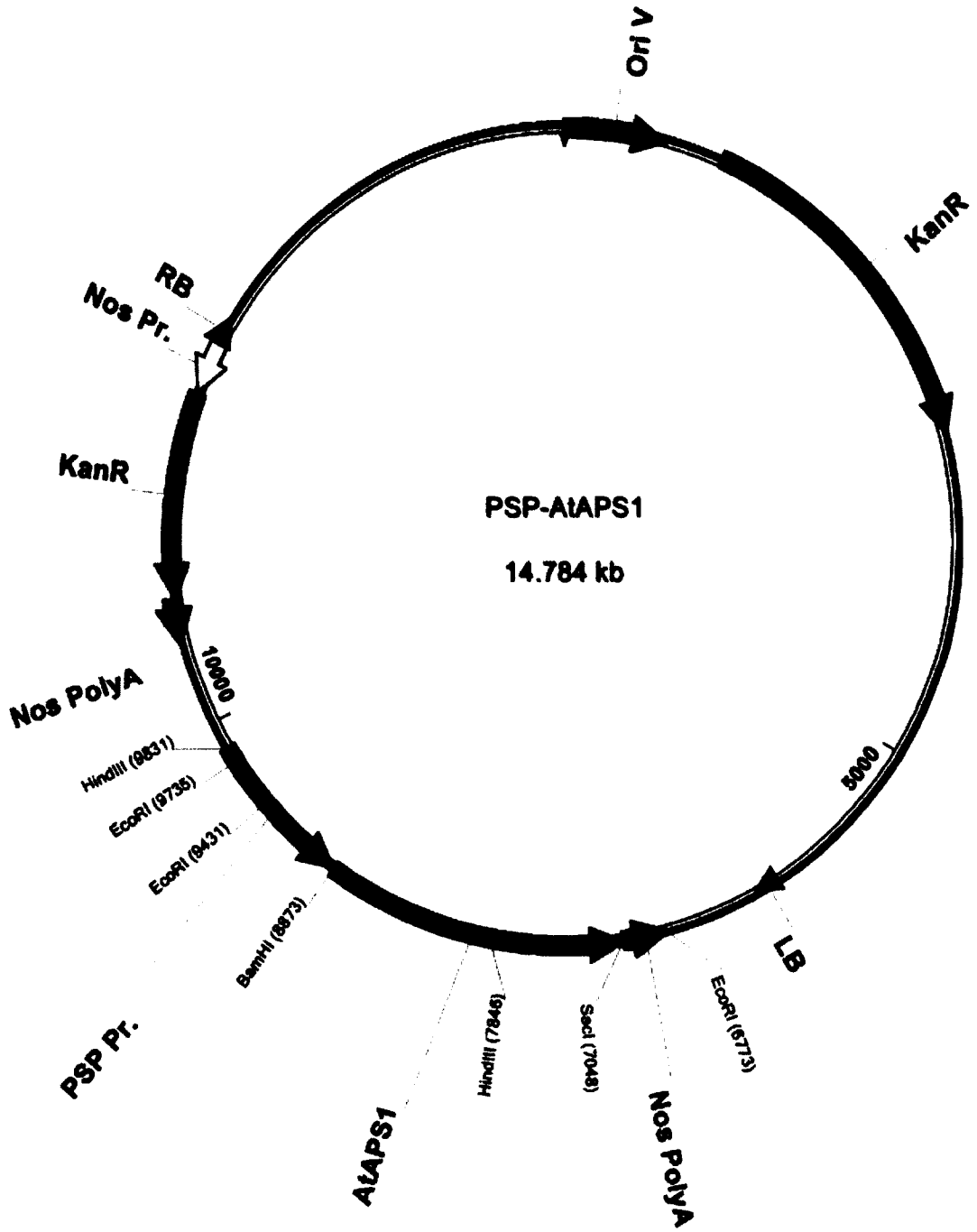

Figure 6. Plasmid map of PSP-AtAPS1. Ori V—Replication origin; KanR—Kanamycin resistance gene; LB—T-DNA left border; Nos PolyA—Nopaline synthase polyadenylation signal; AtAPS1—*Arabidopsis thaliana* ADP-glucose Pyrophosphorylase Small subunit 1; PSP Pr.—*Arabidopsis thaliana* pollen-specific protein (PSP) promoter; Nos Pr.—Nopaline synthase promoter; RB—T-DNA right border.

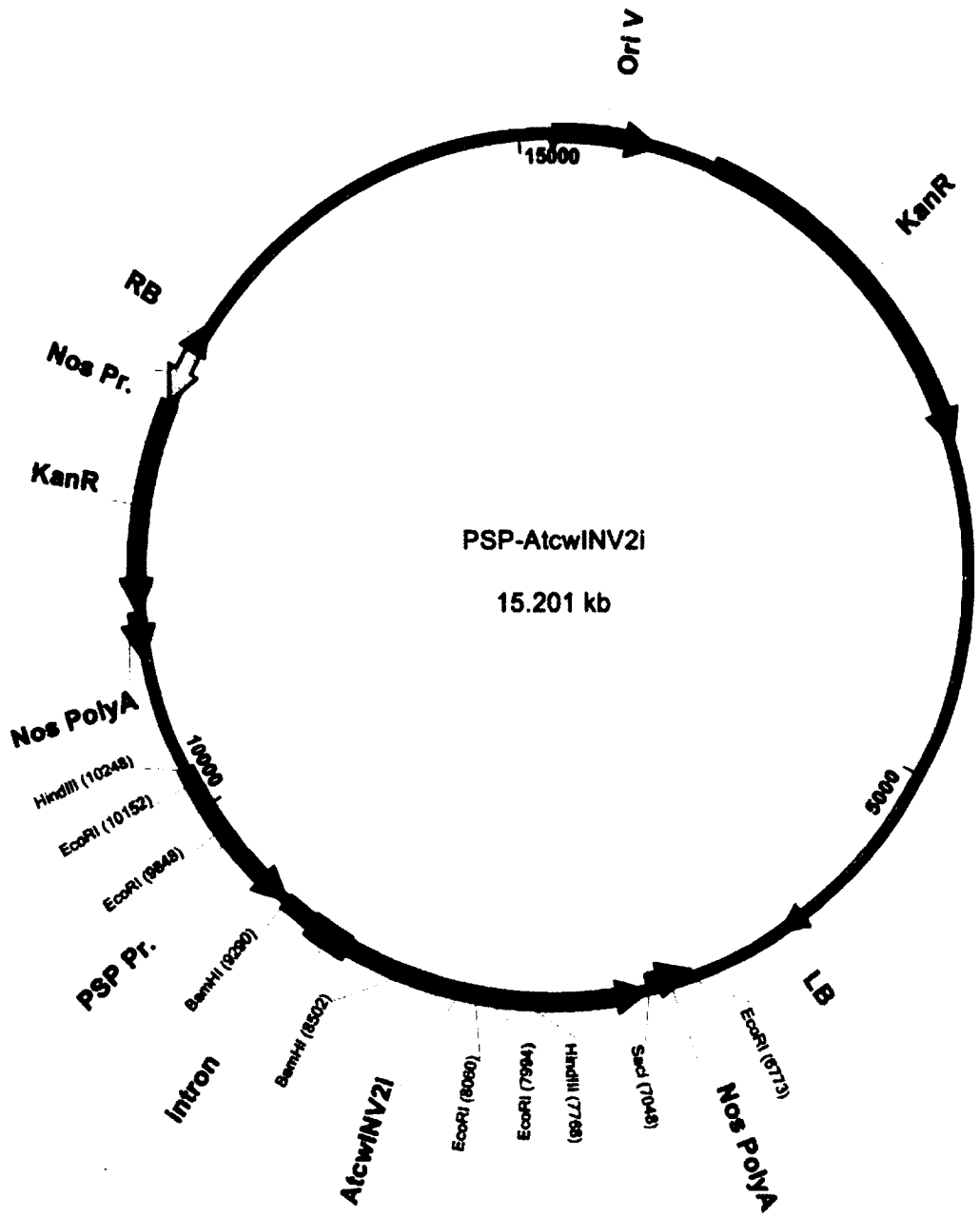

Figure 7. Plasmid map of PSP-AtcwINV2i. Ori V—Replication origin; KanR—Kanamycin resistance gene; LB—T-DNA left border; Nos PolyA—Nopaline synthase polyadenylation signal; AtcwINV2i—*Arabidopsis thaliana* cell wall Invertase 2 + intron; PSP Pr.—*Arabidopsis thaliana* pollen-specific protein (PSP) promoter; Nos Pr.—Nopaline synthase promoter; RB—T-DNA right border.

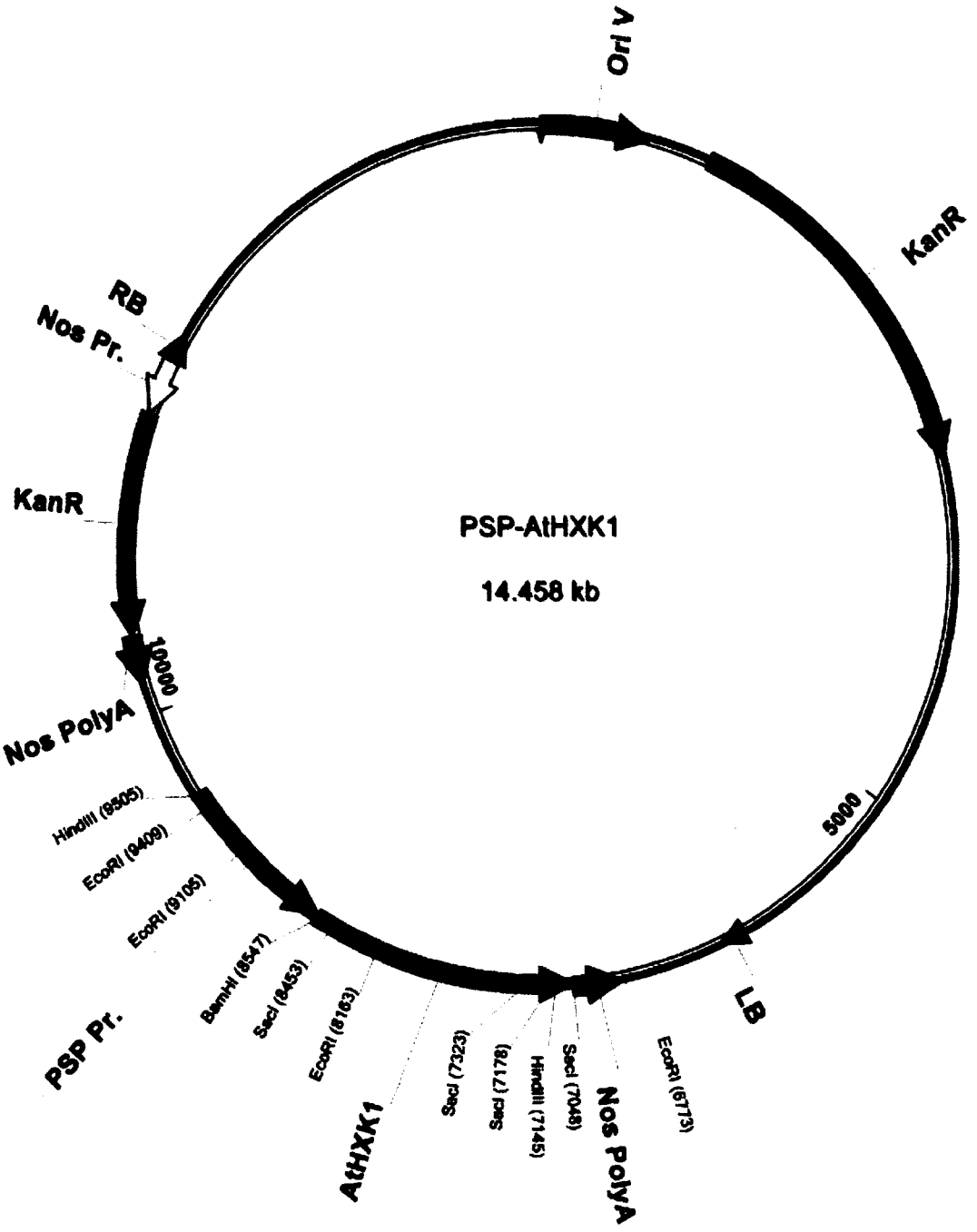

Figure 8. Plasmid map of PSP-AtHXK1. Ori V—Replication origin; KanR—Kanamycin resistance gene; LB—T-DNA left border; Nos PolyA—Nopaline synthase polyadenylation signal; AtHXK1—*Arabidopsis thaliana* Hexokinase 1; PSP Pr.—*Arabidopsis thaliana* pollen-specific protein (PSP) promoter; Nos Pr.—Nopaline synthase promoter; RB—T-DNA right border.

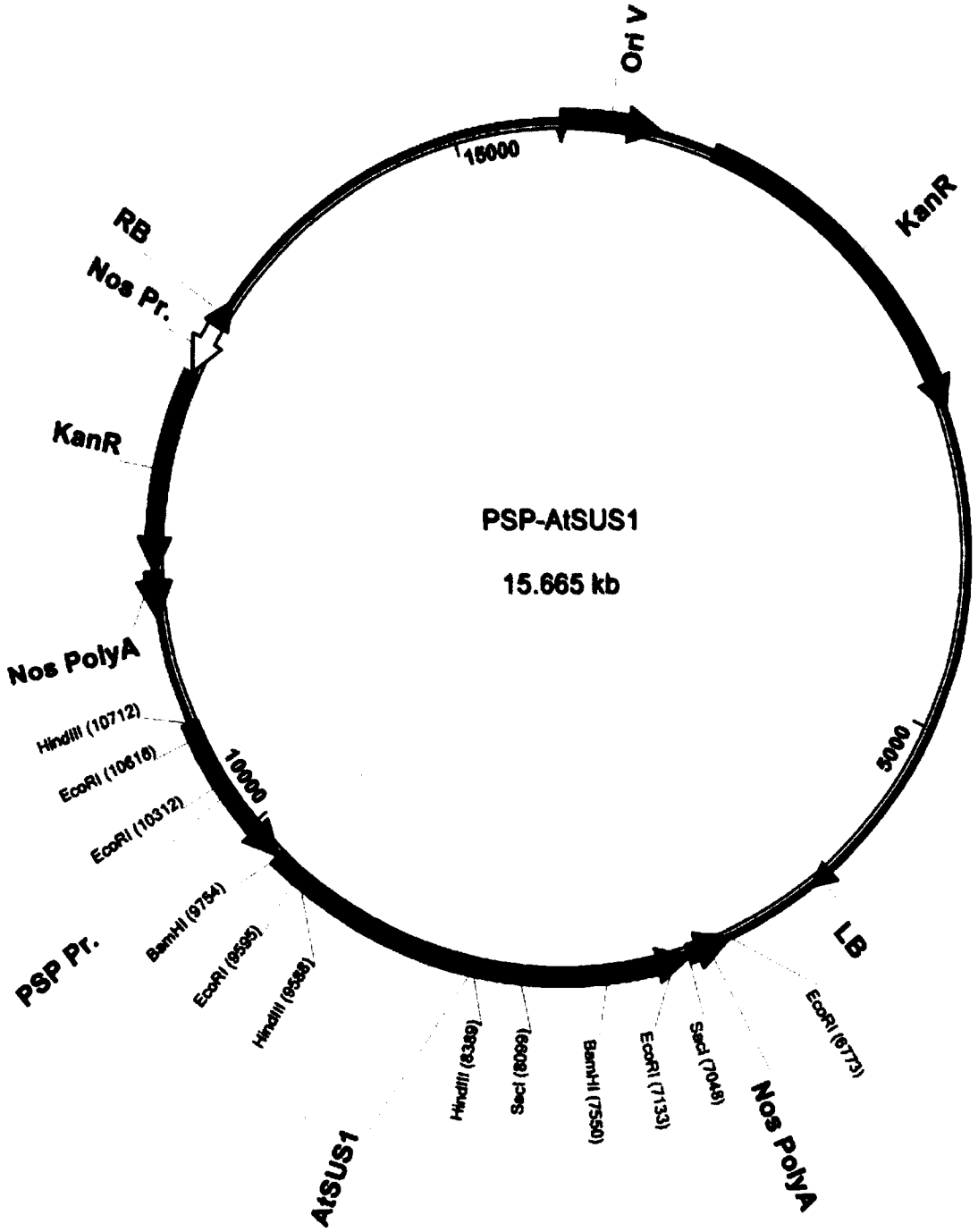

Figure 9. Plasmid map of PSP-AtSUS1. Ori V—Replication origin; KanR—Kanamycin resistance gene; LB—T-DNA left border; Nos PolyA—Nopaline synthase polyadenylation signal; AtSUS1—*Arabidopsis thaliana* Sucrose Synthase 1; PSP Pr.—*Arabidopsis thaliana* pollen-specific protein (PSP) promoter; Nos Pr.—Nopaline synthase promoter; RB—T-DNA right border.

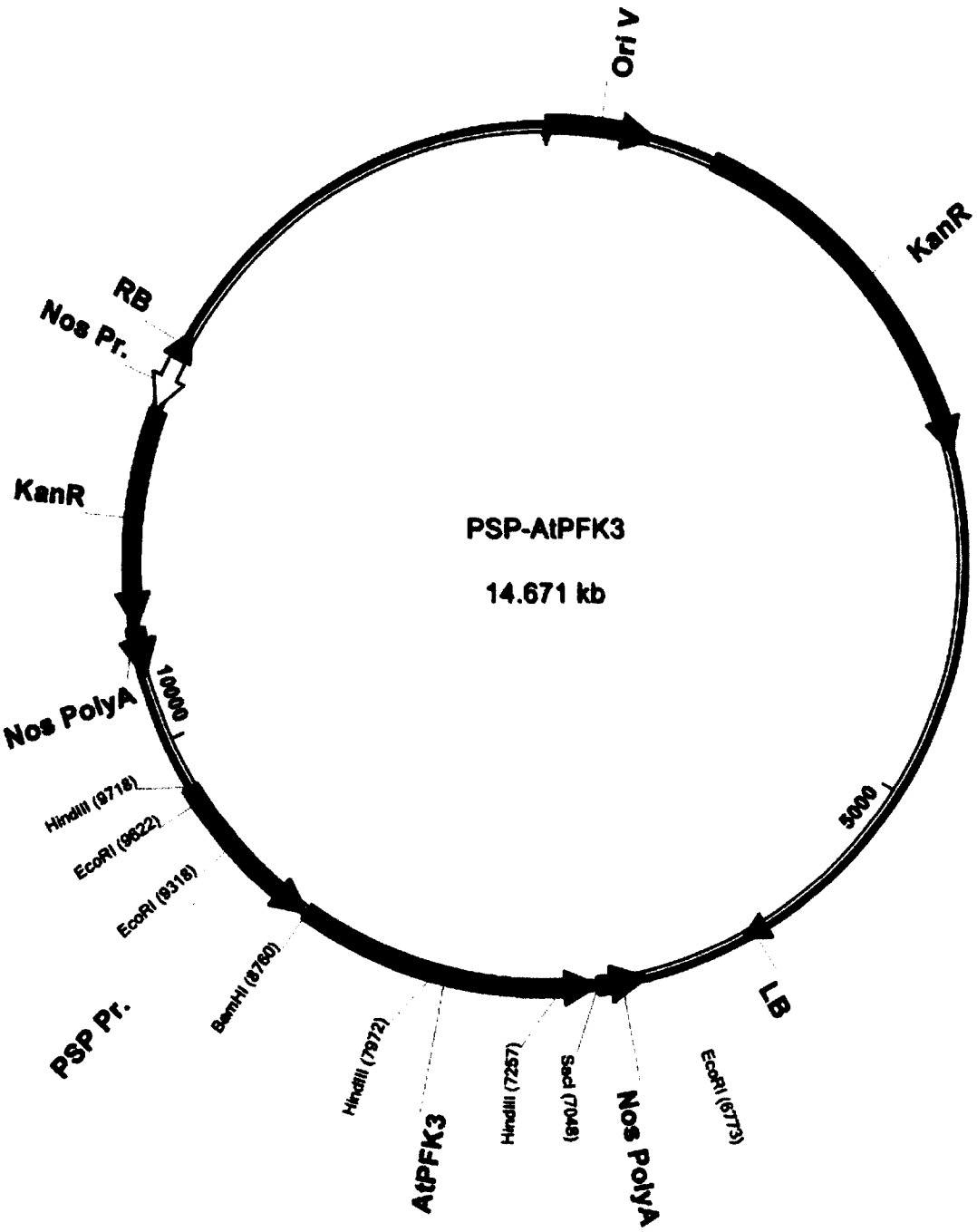

Figure 10. Plasmid map of PSP-AtPFK3. Ori V—Replication origin; KanR—Kanamycin resistance gene; LB—T-DNA left border; Nos PolyA—Nopaline synthase polyadenylation signal; AtPFK3—*Arabidopsis thaliana* PhosphoFructoKinase 3; PSP Pr.—*Arabidopsis thaliana* pollen-specific protein (PSP) promoter; Nos Pr.—Nopaline synthase promoter; RB—T-DNA right border.

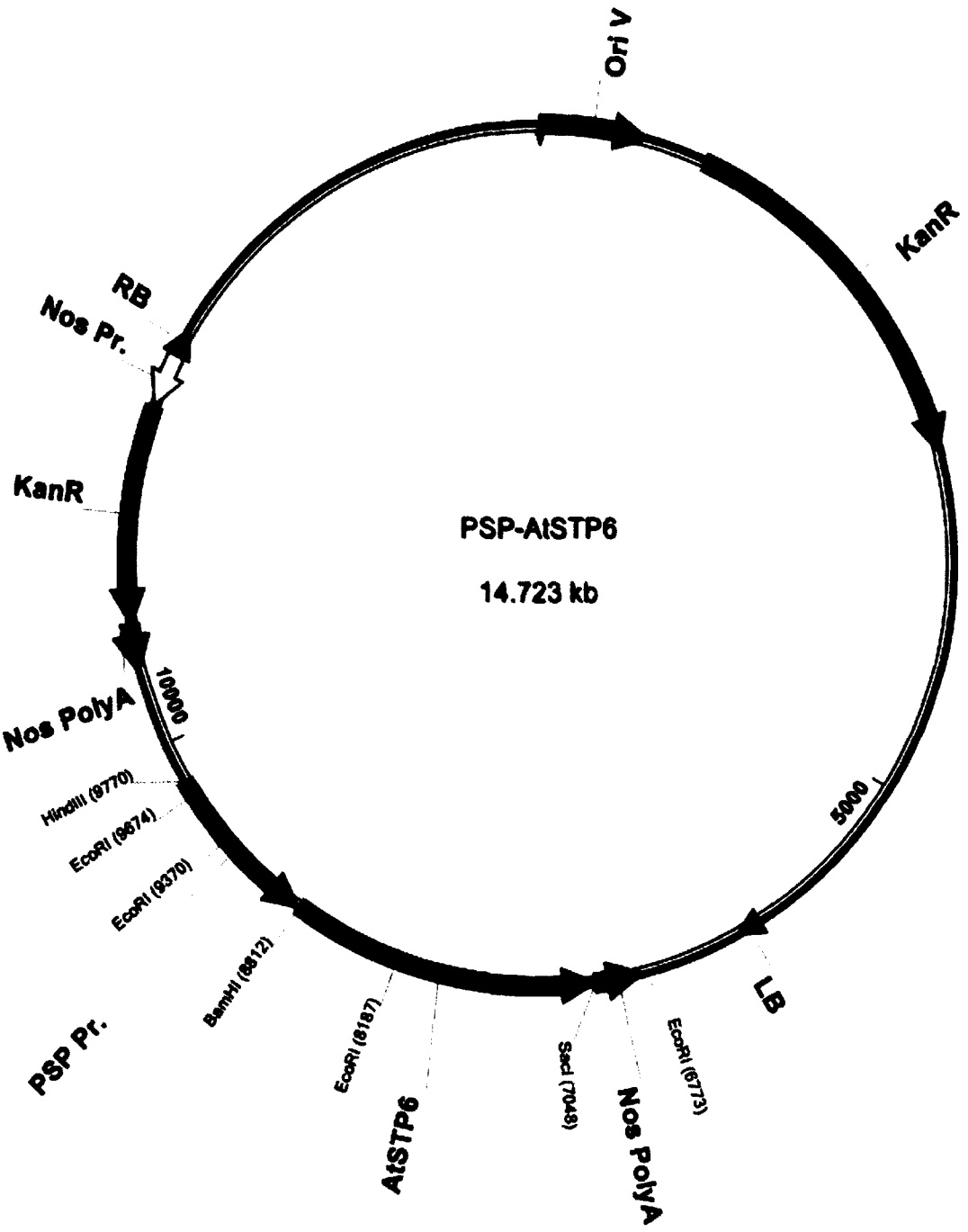

Figure 11. Plasmid map of PSP-AtSTP6. Ori V—Replication origin; KanR—Kanamycin resistance gene; LB—T-DNA left border; Nos PolyA—Nopaline synthase polyadenylation signal; AtSTP6—*Arabidopsis thaliana* Sugar Transport Protein 6; PSP Pr.—*Arabidopsis thaliana* pollen-specific protein (PSP) promoter; Nos Pr.—Nopaline synthase promoter; RB—T-DNA right border.

Generally, the earlier phases of pollen development are more susceptible to abiotic stress (Sakata *et al.*, 2010). Accordingly, in order to affect carbohydrate metabolism at this sensitive stage it is important to utilize a promoter which functions well during microspore development. The promoter for *AtSTP2* (At1g07340) was shown to express early in pollen development (Truernit *et al.*, 1999) and these results are in agreement with the microarray data (Honys and Twell, 2004). Therefore, the activity of the PSTP2 promoter was compared to the PSP promoter which displayed the highest level of expression early in development of the 5 promoters tested above (results not shown). The results of the developmental screening showed that the GFP activity driven by PSP is much higher than that observed for PSTP2 throughout pollen development except for the earliest stage (Figure 5).

The coding regions for the six selected carbohydrate genes were obtained from Arabidopsis anther cDNA using PCR while adding convenient restriction sites for cloning into the binary vector. *AtAPS1*, *AtPFK3* and *AtSTP6* were cloned as single fragments, whereas *AtHXK1*, *AtSUS1* and *AtcwINv2* required additional cloning steps. Furthermore, *AtcwINV2* seemed deleterious to *Agrobacterium* and an intron was introduced into the sequence to prevent expression in the bacterium. Challenges in the construction of PSP-AtHXK1 and PSP-AtcwINV2i resulted in delays which meant that no analyses were performed on their T_1 progeny.

3.3 Conclusion

The PSP promoter displayed the highest level of expression over the broadest pollen developmental range and did not seem to be affected by HTS. The six candidate genes were therefore introduced individually downstream of this promoter within a binary vector to be used for *Agrobacterium*-mediated Arabidopsis transformation.

4. Optimization of heat treatment condition

4.1 Results

4.1.1 Soil Tests

In order to determine which type of soil can hold water properly at high temperature, analyses were performed on 4 differently treated soils (ProMix + Autoclave, ProMix – Autoclave, Regular Soil + Autoclave, Regular Soil – Autoclave). Comparing soil conditions after a 14 day heat treatment at 30°C with regular watering, the ProMix – Autoclave and Regular Soil – Autoclave were wet and covered with green algae, Regular Soil + Autoclave was moist, whereas ProMix + Autoclave was dry. Thus, Regular Soil + Autoclave can hold water properly at high temperature and limits algal contamination (results not shown).

The same soil batches were also tested for their ability to support plant growth. Comparing relative size of Arabidopsis rosettes after growing 14 days at 22°C, the size of rosettes on ProMix – Autoclave and Regular Soil – Autoclave was small, rosettes on ProMix + Autoclave were of medium size, whereas the largest rosettes were observed on Regular Soil + Autoclave (Table 5, Figure 12). Therefore, Regular Soil + Autoclave best supported Arabidopsis plant growth and was selected for all the experiments described below.

4.1.2 Temperature Determination for Heat Treatment

In order to determine which temperature should be used to distinguish heat

Table 5. Arabidopsis plants grown under different soil conditions.

Soil Type	Soil Condition	Relative Size of Rosette
ProMix + Autoclaved	Moist (A little bit dry)	Medium
ProMix – Autoclaved	Wet	Small
Regular Soil + Autoclaved	Moist	Big
Regular Soil – Autoclaved	Wet	Small

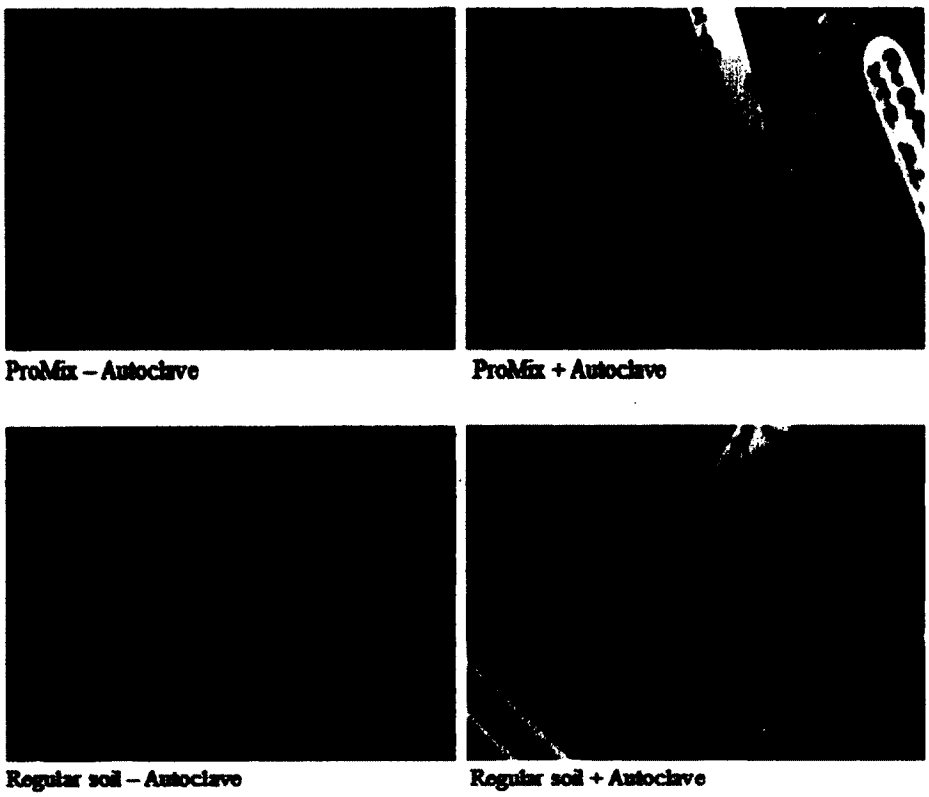

Figure 12. WT Arabidopsis seedlings on 4 differently treated soils after 14 days at 22°C.

tolerant plants from heat sensitive plants based on silique and seed production, analyses were performed at 4 different temperatures (31°C, 32°C, 33°C and 34°C). The number (Figure 13) and percentage (Figure 14) of healthy looking siliques of untransformed Arabidopsis plants at different temperatures was compared.

As the treatment temperature increases above 31°C, the total number of healthy looking siliques decreases greatly with some siliques still being produced at 32°C (Figure 13).

Similarly, as the temperature goes up, the proportion of healthy looking siliques decreases significantly above 31°C (Figure 14).

4.2 Discussion

4.2.1 Soil Tests

In the field, the concurrence of multiple abiotic stresses is more common than only one particular stress. Heat stress and drought stress are two different abiotic stresses that usually occur simultaneously (Wang & Huang, 2004). Drought stress is defined as a condition where the water supply to keep the soil moist is deficient for a long period of time. It also has detrimental effects on plant reproductive processes causing pollen sterility and leading to poor seed set. For example, a study demonstrated that starch accumulation was reduced in developing pollen under drought stress during meiosis and anthesis in both wheat and rice (Saini & Westgate, 2000). In addition, exposure of *Poa pratensis* to the combination of heat stress and water stress was shown to have a

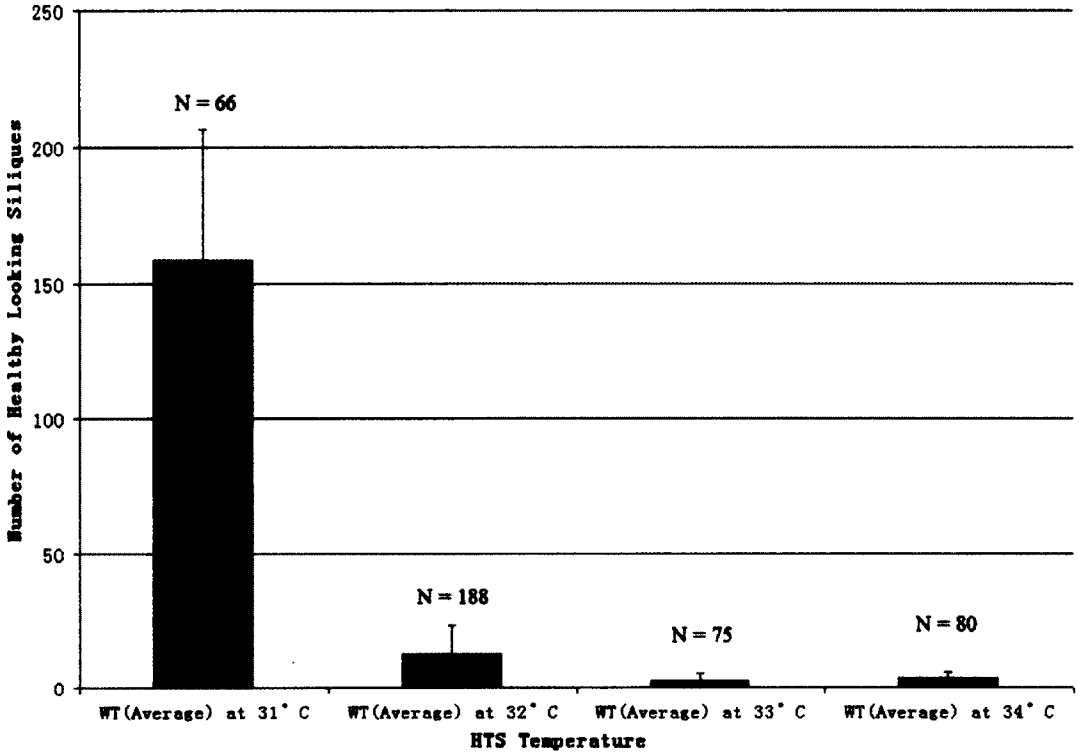

Figure 13. The average number of healthy looking siliques of untransformed plants (WT) at different temperature treatments. All WT lines were heat stressed for 14 days. (HTS: High Temperature Stress)

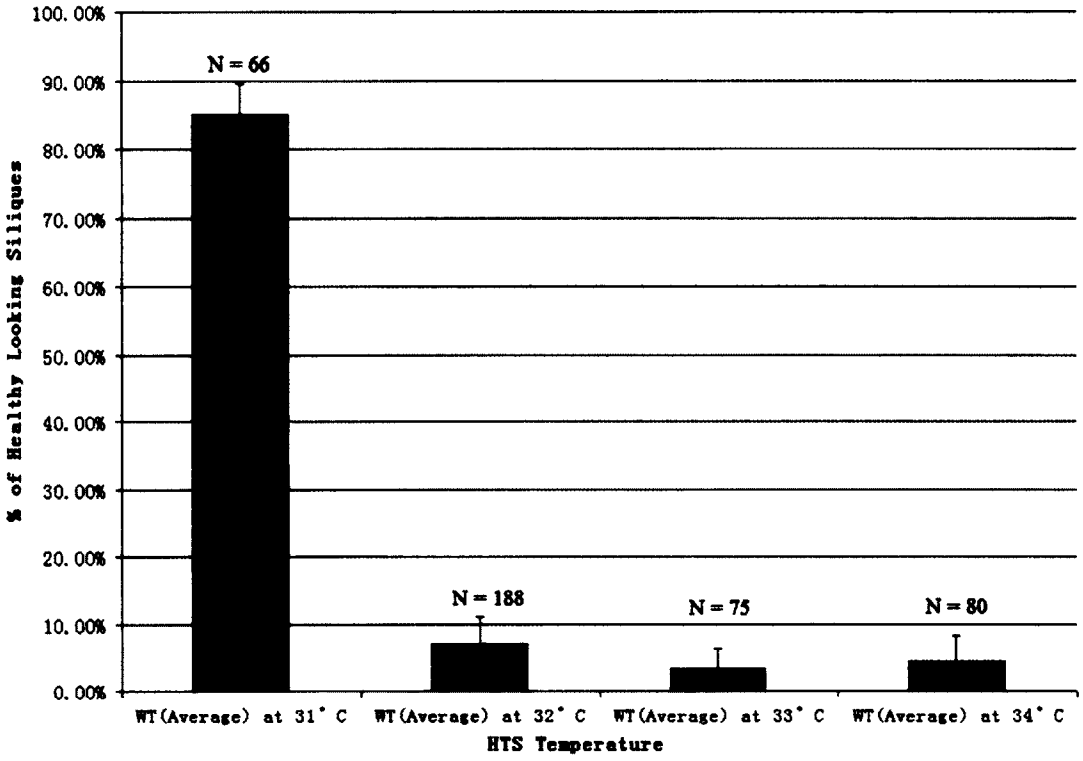

Figure 14. The average percentage of healthy looking siliques of untransformed plants (WT) at different temperature treatments. All WT lines were heat stressed for 14 days. (HTS: High Temperature Stress)

significantly greater negative effect on plant growth and productivity than a single stress (Wang & Huang, 2004). Since this research focuses on pollen development under high temperature stress only, it is important to avoid any additional stress. It was therefore necessary to evaluate different soil conditions to ascertain which conditions would allow both good water retention and optimal plant growth.

ProMix and Regular Soil were the two premixed soils available and they contain different proportions of sphagnum peat moss, coarse perlite, vermiculite, limestone, macronutrients and micronutrients, which not only contribute to water retention in the soil, but also provide necessary nutrients for plant growth. Autoclaving was also evaluated as a method of reducing microbial contamination which can be an issue when the soil is kept moist. Thus, 4 differently treated soils (ProMix + Autoclave, ProMix – Autoclave, Regular Soil + Autoclave, Regular Soil – Autoclave) were tested to determine which type of soil can hold water properly under HTS and best supports plant growth. The largest Arabidopsis rosettes were observed with autoclaved soils (Table 5, Figure 12) suggesting that microbial contamination can be a significant deterrent to plant growth under these growth conditions. Regular Soil + Autoclave gave the biggest Arabidopsis rosettes consistent with the fact that it was also shown to hold water properly without algal contamination under HTS (results not shown). The better water retention the soil has the less chance the plant will be subjected to drought stress.

4.2.2 Temperature Determination for Heat Treatment

In this research, the optimal temperature of the heat stress will be the one that can best distinguish heat tolerant from heat sensitive plants. Phenotypic differences associated with seed production such as silique number and seed weight are a common measure of the effects of heat stress on reproductive development (e.g. Young *et al.*, 2004). The fewer siliques and seeds produced by WT plants under heat stress, the greater the chance that a positive effect will be detected in a transgenic plant. However, too great of a heat stress can simply eliminate all seed production.

Heat treatment has been performed on Arabidopsis plants in many studies with the intensity of the heat stress varying in length and temperature depending on the goal of the study. A short intense HTS treatment at around 40°C has often been applied in order to monitor the response of plants to the stress (Kim *et al.*, 2001; Larkindale & Knight, 2002; Rizhsky *et al.*, 2004). Alternatively, a longer period of moderately high temperature (around 30°C) was commonly used to heat stress transgenic plants overexpressing a specific gene, which may improve heat tolerance, to verify the stability and the long-term effect of the gene (Kurek *et al.*, 2007; Panchuk *et al.*, 2002). Although no reports dealing specifically with the effect of heat stress on Arabidopsis pollen development could be found, it was recently shown that growing Arabidopsis at a constant temperature of 30°C could efficiently discriminate the positive effects of a pollen-expressed *LeFRK1* gene on Arabidopsis seed production (Dr. D. Granot, personal communication).

Thus, 4 different temperatures (31°C, 32°C, 33°C and 34°C) were applied to

untransformed Arabidopsis (WT) plants for the same period of time (14 days). The number and percentage of the healthy looking siliques decreased greatly above 31°C with some siliques still being produced at the higher temperatures (Figure 13, 14). The seed production data (results not shown) led to a similar conclusion.

4.3 Conclusion

In conclusion, Regular Soil + Autoclave best met the requirements for water retention without microbial or algal contamination while maintaining good plant growth and was therefore selected for all experiments. All HTS treatments were performed at temperatures superior to 31°C as this temperature was found to allow considerable healthy silique and seed production in WT Arabidopsis in our hands.

5. PSP-AtAPS1

5.1 Results

The PSP-AtAPS1 construct is meant to overexpress in pollen the Arabidopsis ADP-glucose pyrophosphorylase small subunit 1, a key enzyme in starch biosynthesis. A total of 15 T_0 transformant lines (PSP-AtAPS1), 4 untransformed lines (WT) and 4 heat tolerant transgenic lines (LeFRK) were heat stressed together and evaluated. To verify transgene integration and expression, four transgenic lines were selected for analysis. After T_0 screening, T_1 progeny of promising transgenic lines PSP-AtAPS1-3 and PSP-AtAPS1-4 were tested for improved heat tolerance.

5.1.1 Verification of Transgenic Lines

5.1.1.a Genomic PCR

Genomic PCR was performed to verify the integration of PSP-AtAPS1 into Arabidopsis (Figure 15). The expected PSP-AtAPS1 fusion-specific fragment was clearly observed in all 4 transgenic lines.

5.1.1.b Transgene-specific *AtAPS1* RT-PCR

To verify *AtAPS1* transgene expression, RT-PCR was performed on Arabidopsis floral buds using construct-specific primers. The expected fragment (403 bp) was only observed in the transgenic lines thus confirming the expression of the transgene (Figure 16). Some variation could be observed in the expression level of the *AtAPS1* transgene among the different transgenic lines. As with the other candidate genes (see below),

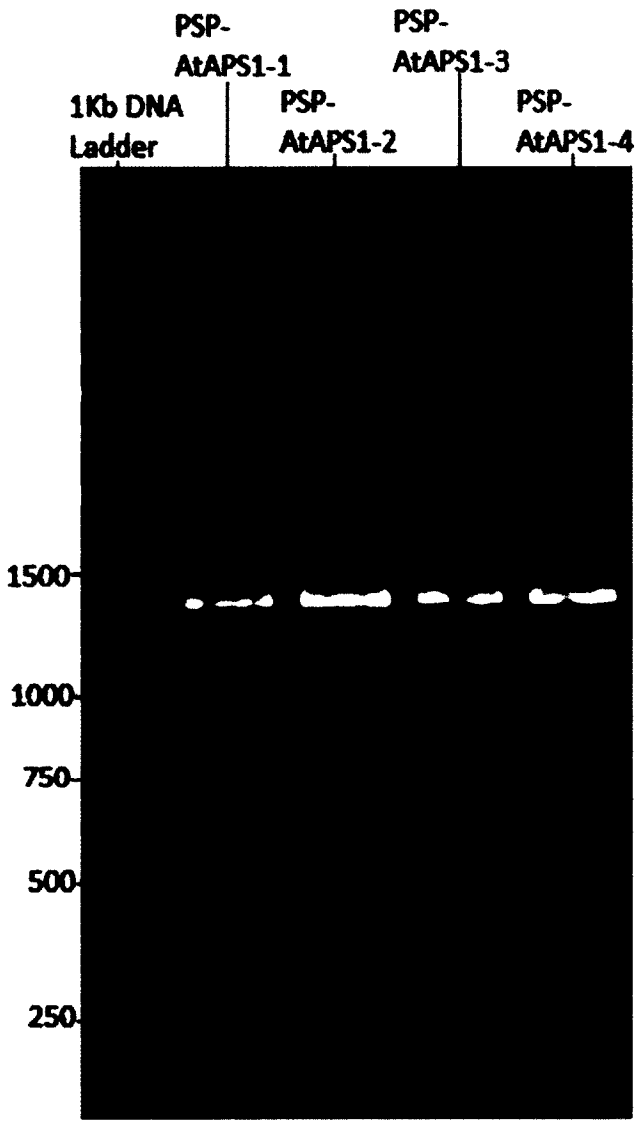

Figure 15. Agarose gel electrophoresis of genomic PCR fragment from individual PSP-AtAPS1 (PSP promoter-*Arabidopsis thaliana* ADP-glucose Pyrophosphorylase Small subunit 1, expected size: 1332 bp) T_0 lines. The sizes of some standard DNA fragments are indicated in base pairs.

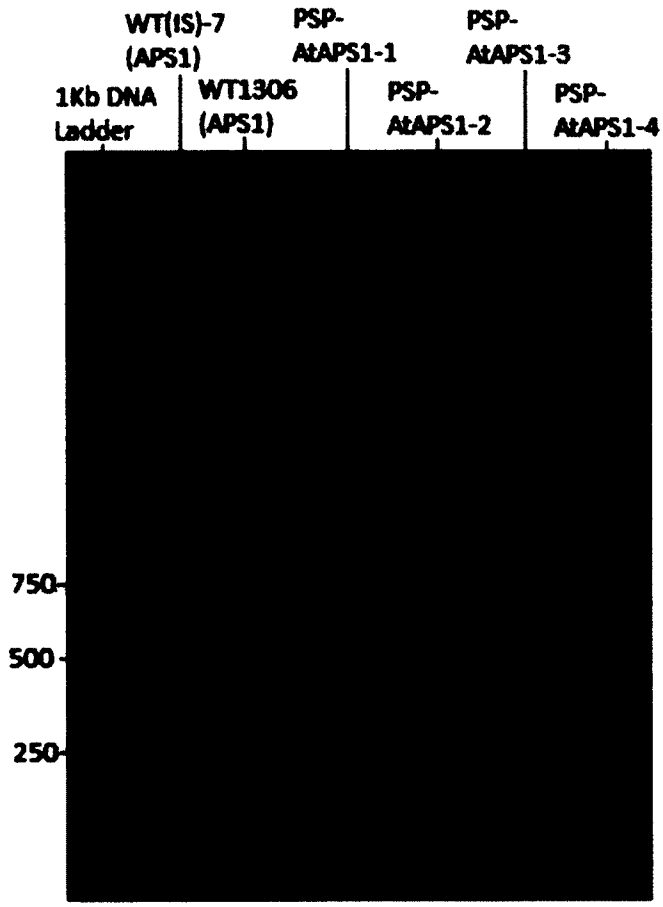

Figure 16. Agarose gel electrophoresis of transgene-specific RT-PCR fragments (Expected size: 403 bp) obtained with cDNA from heat stressed mature floral buds of individual PSP-AtAPS1 T_0 lines and WT lines. The sizes of some standard DNA fragments are indicated in base pairs.

transgene-specific RT-PCR always yielded two bands, since they all share the PSP promoter the doublet may be due to alternative transcription start sites, although no evidence for this could be found from the limited public sequence data.

5.1.1.c Semi-quantitative *AtAPS1* RT-PCR

As mentioned above, some differences were observed in transgene expression levels among the different PSP-AtAPS1 T_0 lines. In order to investigate these differences, semi-quantitative RT-PCR (measuring simultaneously the expression of the endogenous gene and the transgene) was performed using an internal ribosomal RNA PCR control (Figure 17). Although there was little correlation between the results of the transgene-specific RT-PCR and the semi-quantitative RT-PCR, the fact that all the transgenic lines appear to express more *AtAPS1* mRNA than WT, which seemed unaffected by HTS conditions, provides additional support for the expression of the transgene.

5.1.2 Phenotypic Analysis of PSP-AtAPS1 Transgenic Lines (T_0 Generation)

Comparisons of the number and percentage of healthy looking siliques (after 14 days at 33°C), as well as seed weight (after an additional 7 days at 32°C) for PSP-AtAPS1 versus WT and LeFRK lines are shown in Figures 18, 19 and 20.

5.1.2.a Silique Number

Figure 18 displays the total number of healthy looking siliques counted for each

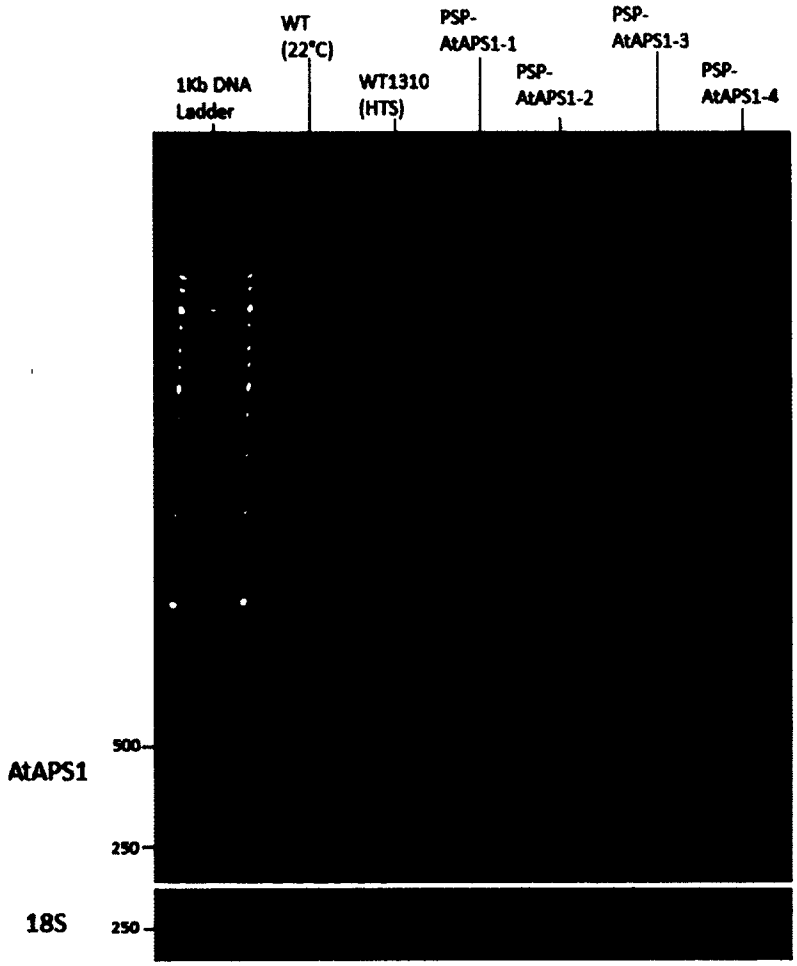

Figure 17. Agarose gel electrophoresis of semi-quantitative RT-PCR fragments (expected size: 376 bp) obtained with cDNA from mature floral buds of individual PSP-AtAPS1 (HTS) T_0 lines and untransformed (WT (22°C) and WT1310 (HTS)) lines. Ribosomal RNA (18S) was used as an internal control. The sizes of some standard DNA fragments are indicated in base pairs.

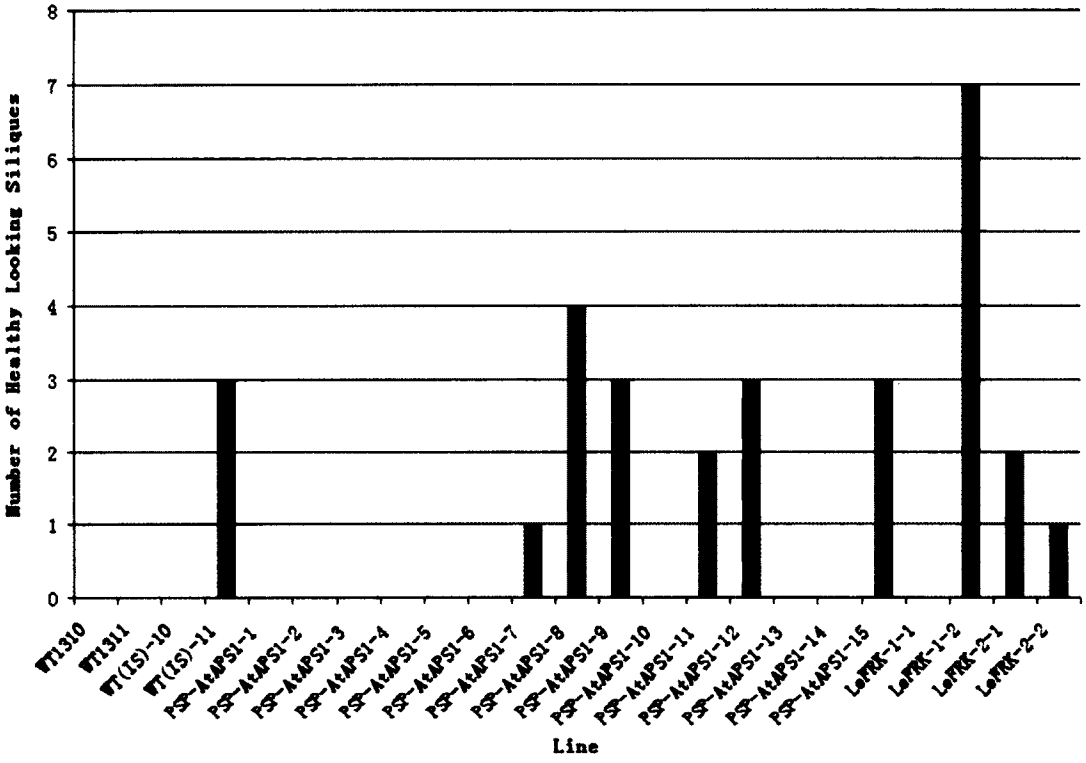

Figure 18. The number of healthy looking siliques for individual PSP-AtAPS1 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. The 15 PSP-AtAPS1, 4 LeFRK and 4 WT lines were located in the same flat.

individual transgenic PSP-AtAPS1 T_0 line, as well as the numbers for WT and LeFRK plants undergoing heat stress under the same conditions. Generally, the number of healthy looking siliques was low for all plants examined and no PSP-AtAPS1 transformant appeared to perform very well under heat stress. Presenting this data as a proportion of healthy looking siliques versus the total number of potential siliques (Figure 19) led to a similar conclusion.

5.1.2.b Seed Weight

Although the number of healthy looking siliques can be low, a plant under heat stress can have a lot of "abnormal" looking siliques that can still produce healthy seeds. When comparing the weight of healthy seeds as a function of plant weight, two transgenic lines (PSP-AtAPS1-3 and PSP-AtAPS1-4) showed the highest values of all lines tested, including the LeFRK lines (Figure 20). The progeny of these two lines was selected for further heat stress treatments.

5.1.3 Phenotypic Analysis of Selected PSP-AtAPS1 Transgenic Lines (T_1 Generation)

Following the initial PSP-AtPSP1 T_0 screening, kanamycin resistant T_1 progeny of PSP-AtAPS1-3 (10 lines) and PSP-AtAPS1-4 (9 lines), along with 4 untransformed lines (WT) and 19 heat tolerant lines (LeFRK) were heat stressed. Generally, the values for the number and percentage of healthy looking siliques, as well as seed weight for all lines (Figures 21, 22, 23) were higher than those observed previously (Figures 18, 19, 20) and

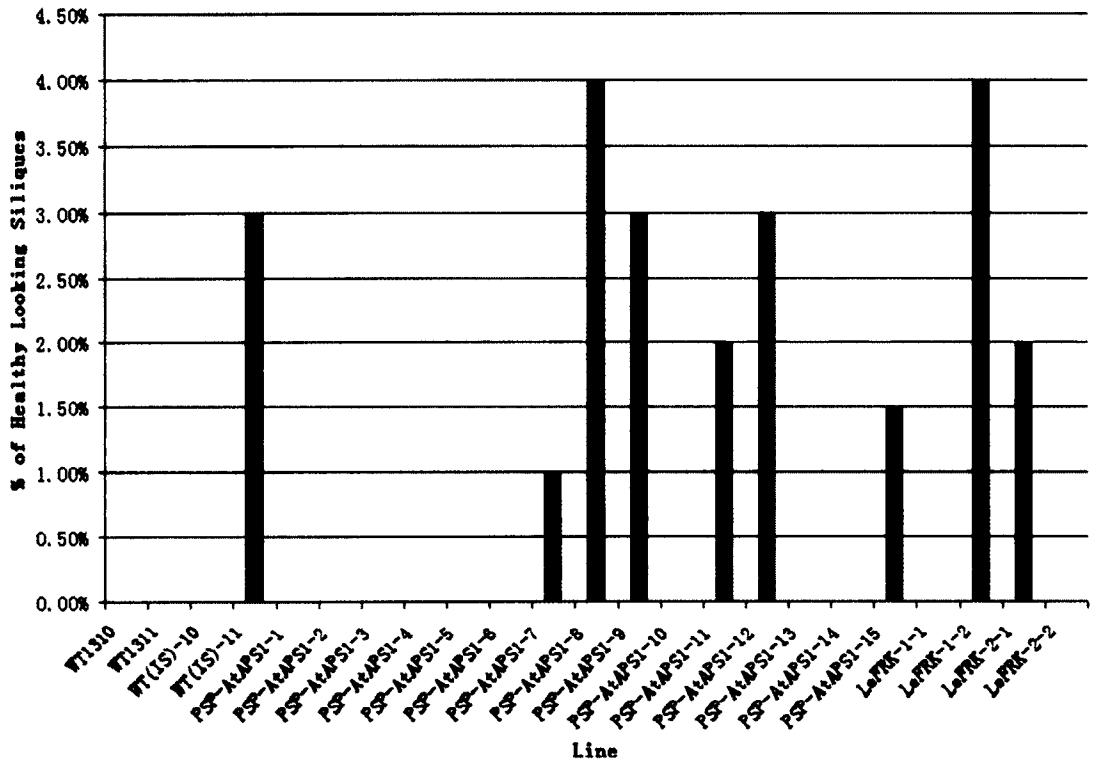

Figure 19.The percentage of healthy looking siliques for individual PSP-AtAPS1 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. The 15 PSP-AtAPS1, 4 LeFRK and 4 WT lines were located in the same flat.

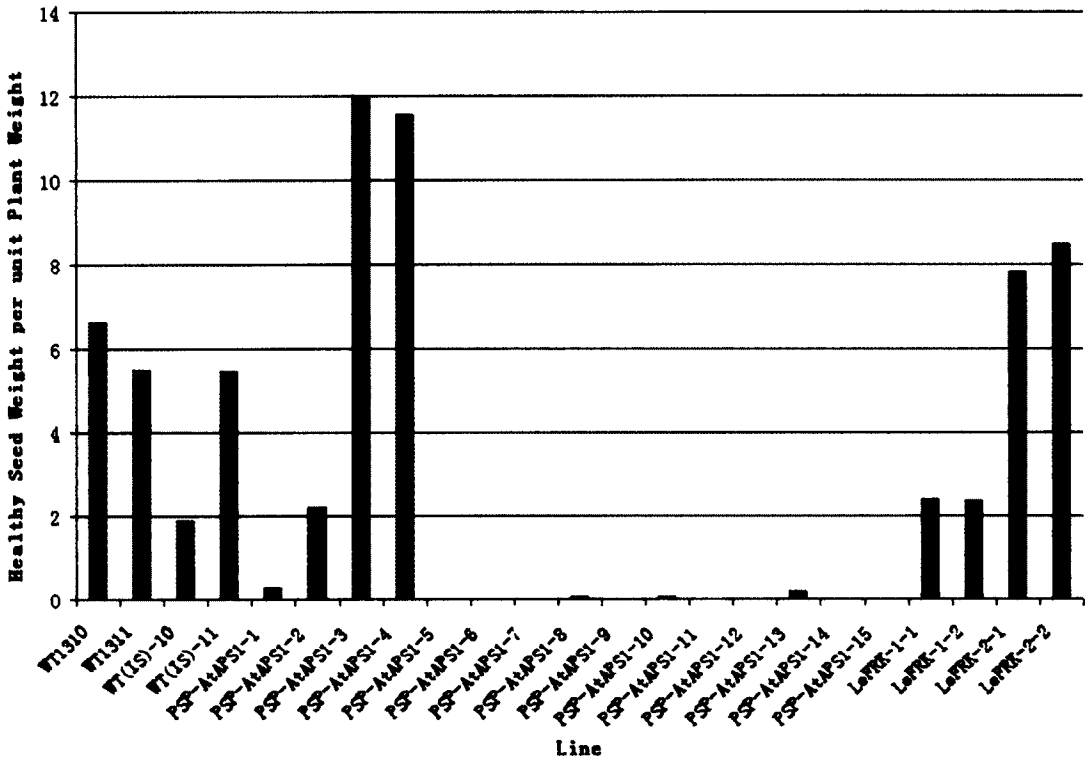

Figure 20. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) of each PSP-AtAPS1 T_0 line is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 7 days at 32°C following the 14 day heat treatment at 33°C. 15 PSP-AtAPS1, 4 LeFRK and 4 WT lines were located in the same flat.

this likely reflects the fact that the heat stress treatment consisted of 14 days at 32°C (as opposed to 14 days at 33°C followed by 7 days at 32°C). The results are described in detail below.

5.1.3.a Silique Number

The PSP-AtAPS1-3 lines generally possessed the highest number of healthy looking siliques (Figure 21). Interestingly, WT1305-1 had a higher number of healthy looking siliques than the average of the 19 LeFRK lines.

When the percentage of healthy looking siliques versus the total number of potential siliques was calculated (Figure 22), the values for the PSP-AtAPS1-4 lines were closer to those of PSP-AtAPS1-3 lines indicating that the PSP-AtAPS1-4 lines initiated fewer siliques overall.

5.1.3.b Seed Weight

Unexpectedly, in spite of the fact that they produced no healthy looking siliques, WT1311-1 and WT(IS)-5-1 had the highest healthy seed weight per unit plant weight of all lines with the exception of the PSP-AtAPS1-3-4 line (Figure 23). Given the fact that the mature plants for these two WT lines were not greatly smaller than the other lines (results not shown), this would tend to indicate that none of the transgenic lines tested produced considerably more healthy seeds than WT.

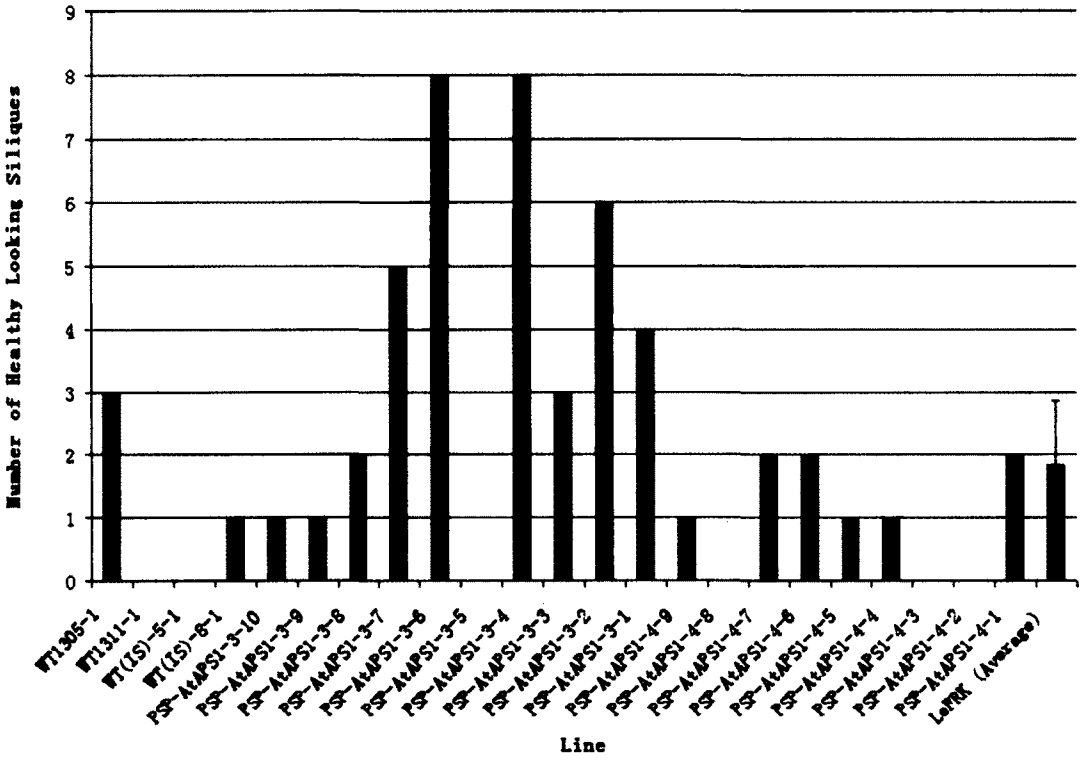

Figure 21. The number of healthy looking siliques for individual T_1 progeny of lines PSP-AtAPS1-3 and PSP-AtAPS1-4 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The number of healthy looking siliques for the LeFRK lines was averaged. 19 PSP-AtAPS1 and 4 WT lines were located in the same flat.

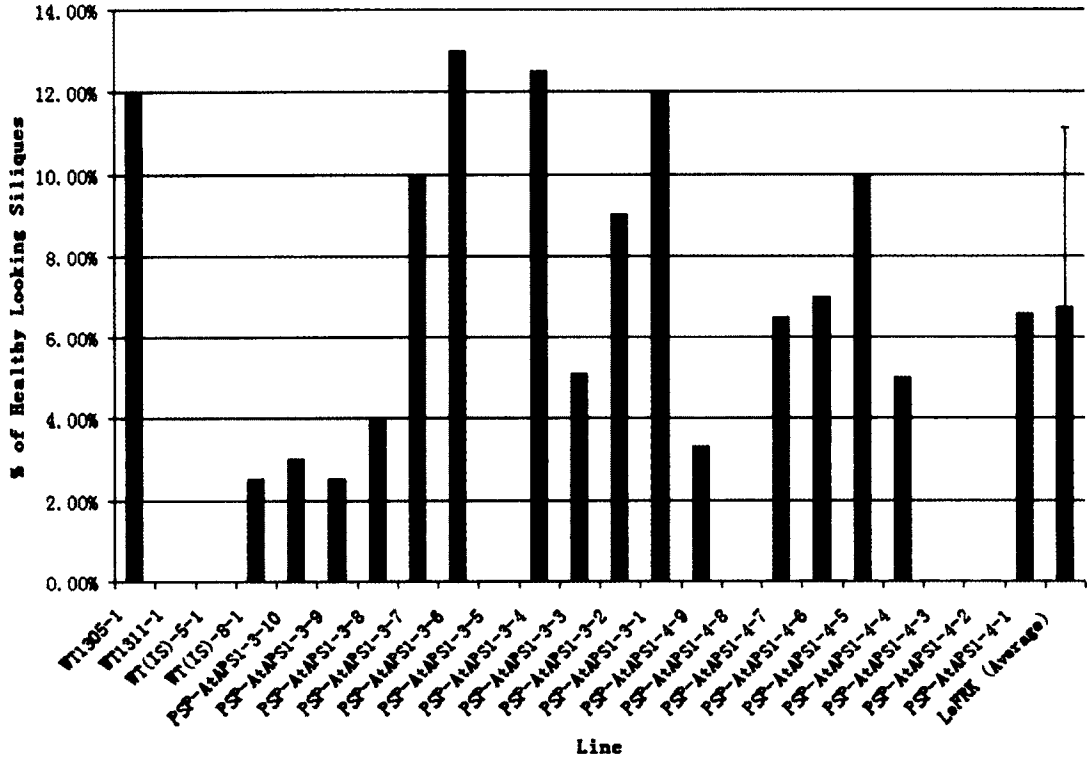

Figure 22. The percentage of healthy looking siliques for individual T_1 progeny of lines PSP-AtAPS1-3 and PSP-AtAPS1-4 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The percentage of healthy looking siliques for the LeFRK lines was averaged. 19 PSP-AtAPS1 and 4 WT lines were located in the same flat.

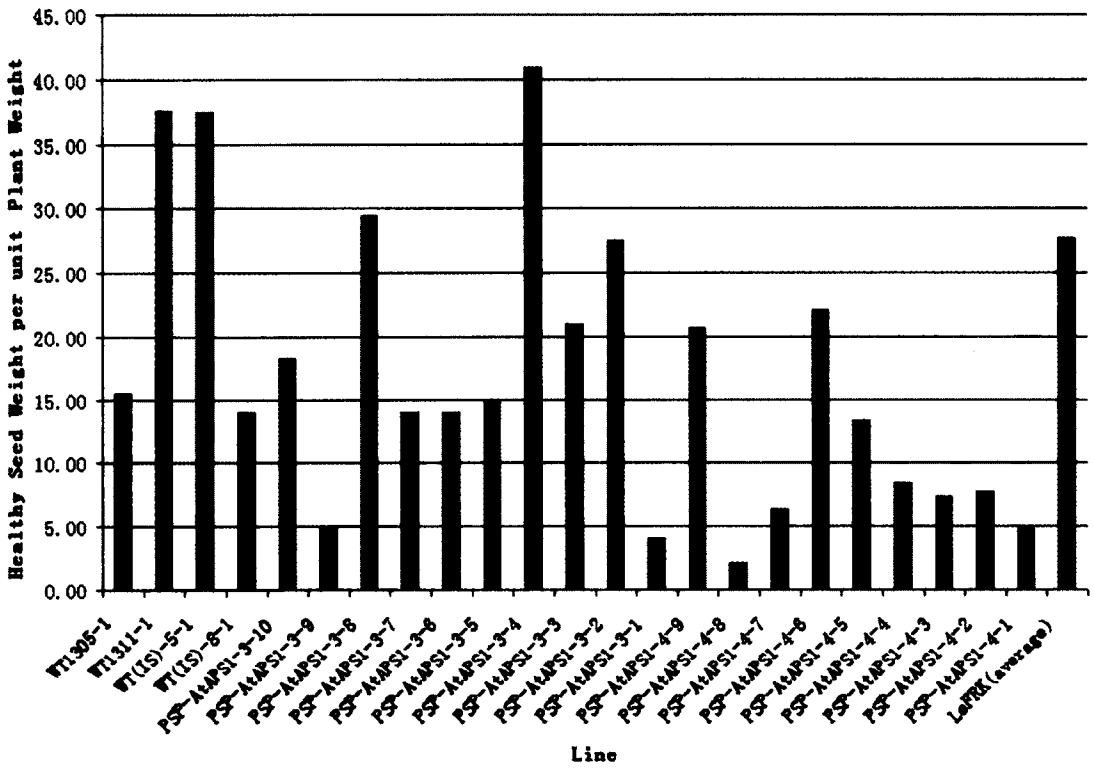

Figure 23. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) for individual T_1 progeny of lines PSP-AtAPS1-3 and PSP-AtAPS1-4 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The healthy seed weight per unit plant weight for the LeFRK lines was averaged (standard deviation = 43.9). 19 PSP-AtAPS1 and 4 WT lines were located in the same flat.

5.2 Discussion

ADP-glucose pyrophosphorylase (AGPase) is a major enzyme regulating starch synthesis in many different plant species (Denyer *et al.*, 1995; Neuhaus & Stitt, 1990; Tiessen *et al.*, 2002). The regulatory and rate-limiting step of starch biosynthesis is the synthesis of ADP-glucose by AGPase (Turner & Turner, 1975). All of the AGPases from higher plants are heterotetramers composed of two distinct types of subunits: small subunits (ApS1 and ApS2) and large subunits (ApL1-ApL4), whose mRNAs were detected in leaves, flowers, fruits and roots of mature plants. The large subunit (LS) primarily regulates the activity of AGPase, whereas the small subunit (SS) is mainly involved in catalysis (Crevillen *et al.*, 2005). Between two SS isoforms, ApS1 is the major one responsible for AGPase activity and starch synthesis in all tissues of the plant and its mRNA was detected only in the early stages of pollen development (Crevillen *et al.*, 2005). Therefore, *AtAPS1* was chosen as a candidate gene for this study.

To our knowledge, this is the first study reporting the overexpression of APS1 during pollen development in any species. However, APS1 has been the subject of numerous investigations. For example, one study showed that RNAi repression of *APS1* expression in vegetative tissues lead to a significant reduction in the starch level (Sanjaya *et al.*, 2011). Another study demonstrated that an *aps1* mutant, which had no AGPase activity, only accumulated approximately 2% of WT leaf starch (Bahaji *et al.*, 2011). Such studies confirm that APS1 is important to plant starch synthesis. Starch is the major storage polysaccharide accumulated in many different organs such as pollen grains. Stored starch

is used to support the high demand for carbohydrates during pollen tube germination and growth, and it is important for the ultimate success of the fertilization process. Studies in various plant species have demonstrated that the reduced starch level in the anther wall and developing pollen grains resulting from different abiotic stresses lead to lower numbers of pollen grains with decreased pollen viability, as well as low pollen germination (Firon *et al.*, 2006; Pressman *et al.*, 2002; Pressman *et al.*, 2006; Saini & Westgate, 2000). Therefore, overexpressing *AtAPS1* using a pollen-specific promoter whose activity is high under heat stress may maintain pollen starch production at a level necessary to support pollen germination and tube growth under these conditions.

In this study, *AtAPS1* is overexpressed by the PSP promoter throughout pollen development. Based on the available microarray data (Honys & Twell, 2004), *AtAPS1* mRNA is expressed moderately at the uninucleate microspore and bicellular pollen stages with no expression observed at the tricellular pollen and mature pollen grain stages (Table 1). Therefore, the PSP promoter should considerably enhance the level of expression of *AtAPS1* at all stages and especially the later stages. RT-PCR did reveal the presence of the *AtPS1* transcript in WT mature buds (Figure 16) which suggests that even if it is not expressed in the pollen, it is expressed in the rest of the bud. The transgenic lines all showed a significant increase in *AtPS1* expression at that stage, presumably from transgene expression in the pollen (Figure 17).

During T_0 screening, no PSP-AtAPS1 line had a higher number and percentage of healthy looking siliques than untransformed (WT) plants under HTS (Figures 18 and 19).

However, the result for healthy seed weight per unit plant weight showed that transgenic lines PSP-AtAPS1-3 and PSP-AtAPS1-4 had the highest values of all lines tested, including the LeFRK lines (Figure 20). This would suggest that the "abnormal" looking siliques in these lines produced a considerable amount of seeds under HTS. However, these results could not be confirmed in the analysis of the T_1 progeny of these two promising transgenic lines as they did not produce more healthy seeds than WT lines due to the good performance of WT1311-1 and WT(IS)-5-1 (Figure 23). Although overexpressing AtAPS1 does not seem to have a huge impact on pollen heat tolerance, it would be interesting to investigate whether the transgenic pollen grains have a greater amount of starch.

5.3 Conclusion

According to above results, the level of *AtPS1* in untransformed plants appears unchanged by HTS. Although more *AtAPS1* transcripts could be detected in the transgenic lines it did not appear to improve seed production under heat stress in Arabidopsis.

6. PSP-AtPFK3

6.1 Results

The PSP-AtPFK3 construct is intended to overexpress in pollen the Arabidopsis phosphofructokinase 3, a key regulatory enzyme in plant glycolysis. A total of 15 T_0 transformed lines (PSP-AtPFK3), 4 untransformed lines (WT) and 4 heat tolerant transformed lines (LeFRK) were heat stressed together and evaluated. Four transgenic lines were selected to verify transgene integration and expression. After T_0 screening, T_1 progeny of promising transgenic lines PSP-AtPFK3-7, PSP-AtPFK3-9 and PSP-AtPFK3-23 were tested for improved heat tolerance.

6.1.1 Verification of Transgenic Lines

6.1.1.a Genomic PCR

Genomic PCR was performed to verify the integration of PSP-AtPFK3 into transgenic Arabidopsis. The expected PSP-AtPFK3 fusion-specific fragment was observed in all transgenic lines except PSP-AtPFK3-12, which could be due to a rearrangement in the *AtPFK3* transgene preventing PCR as the T_0 plants were all kanamycin resistant (Figure 24). Differences in intensity may reflect differences in the number of integrated copies of the transgene.

6.1.1.b Transgene-specific *AtPFK3* RT-PCR

To verify *AtPFK3* transgene expression, RT-PCR was performed on Arabidopsis

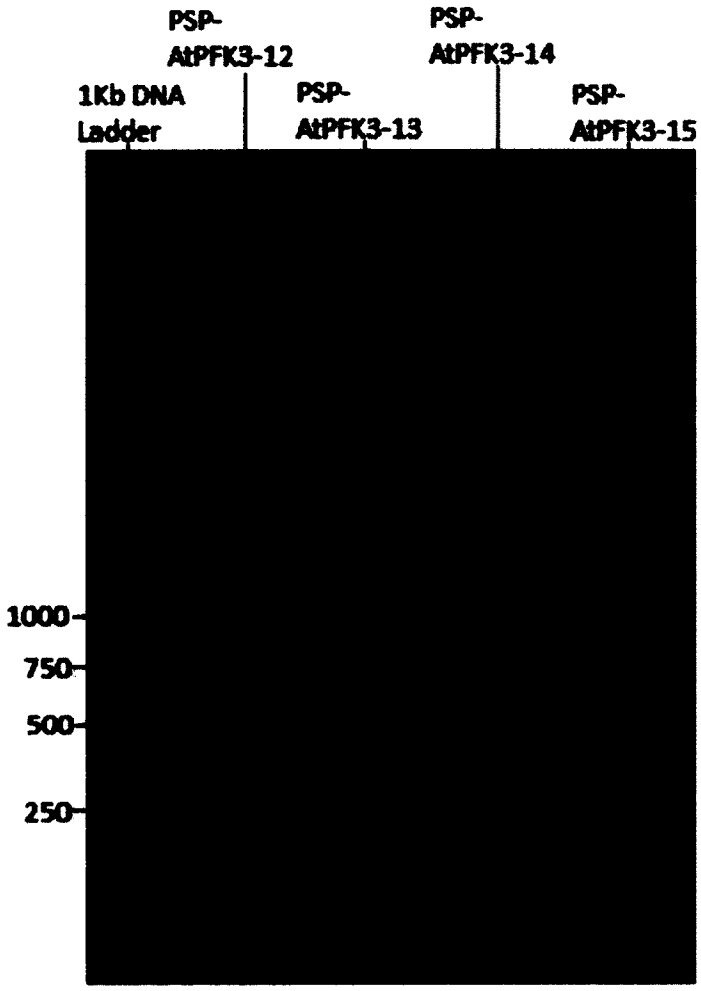

Figure 24. Agarose gel electrophoresis of genomic PCR fragment from individual PSP-AtPFK3 (PSP promoter-*Arabidopsis thaliana* Phosphofructokinase 3, expected size: 803 bp) T_0 lines. The sizes of some standard DNA fragments are indicated in base pairs.

floral buds using construct-specific primers. Transgene expression was confirmed in all lines except PSP-AtPFK3-12 supporting the possibility that the transgene was rearranged in this plant (Figure 25). Some variation could be observed in the expression level of the *AtPFK3* gene among the different transgenic lines.

6.1.1.c Semi-quantitative *AtPFK3* RT-PCR

Semi-quantitative RT-PCR was performed on the PSP-AtPFK3 lines to compare the combined expression of the endogenous gene and the transgene (Figure 26). No correlation between the results of the transgene-specific RT-PCR and the semi-quantitative RT-PCR was observed. The transgenic lines appear to express a little more *AtPFK3* mRNA than WT, which expresses at a relatively low level in both normal and HTS conditions, providing additional support for the expression of the transgene.

6.1.2 Phenotypic Analysis of PSP-AtPFK3 Transgenic Lines (T_0 Generation)

Comparisons of the number and percentage of healthy looking siliques, as well as seed weight for PSP-AtPFK3 versus WT and LeFRK lines are shown in Figures 27, 28, 29.

6.1.2.a Silique Number

Figure 27 displays the total number of healthy looking siliques counted for each individual transgenic PSP-AtPFK3 T_0 line, as well as the numbers for WT and LeFRK plants undergoing the same heat stress. Generally, the number of healthy looking siliques

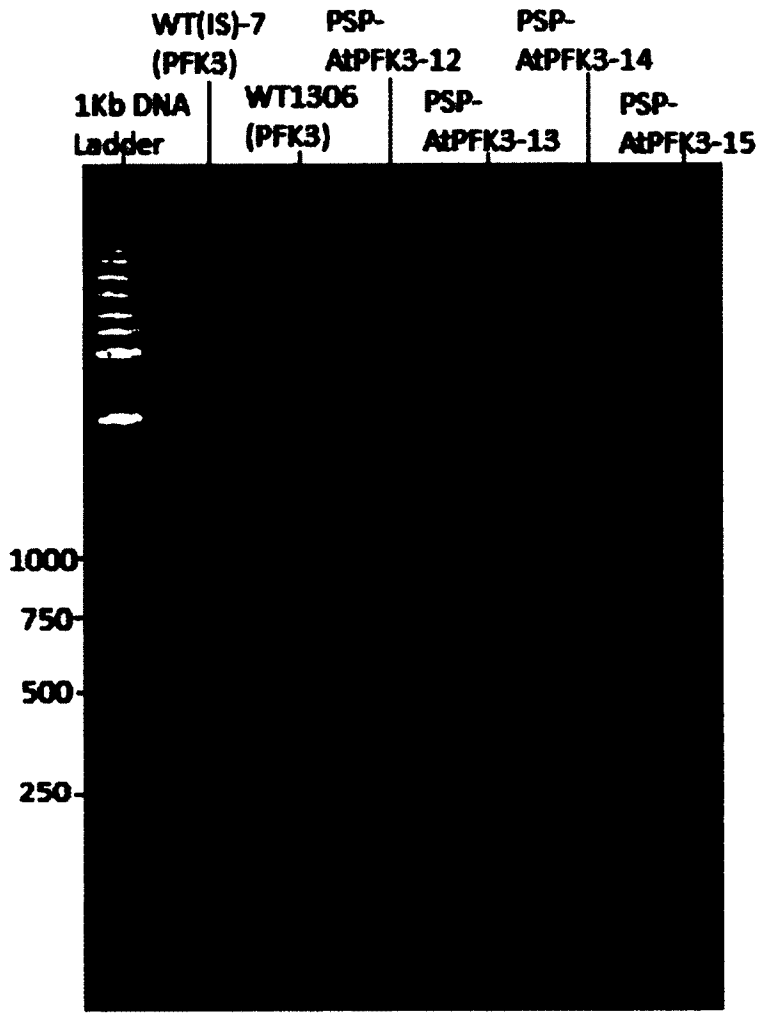

Figure 25. Agarose gel electrophoresis of transgene-specific RT-PCR fragments (Expected size: 803 bp) obtained with cDNA from heat stressed mature floral buds of individual PSP-AtPFK3 T_0 lines and WT lines. The sizes of some standard DNA fragments are indicated in base pairs.

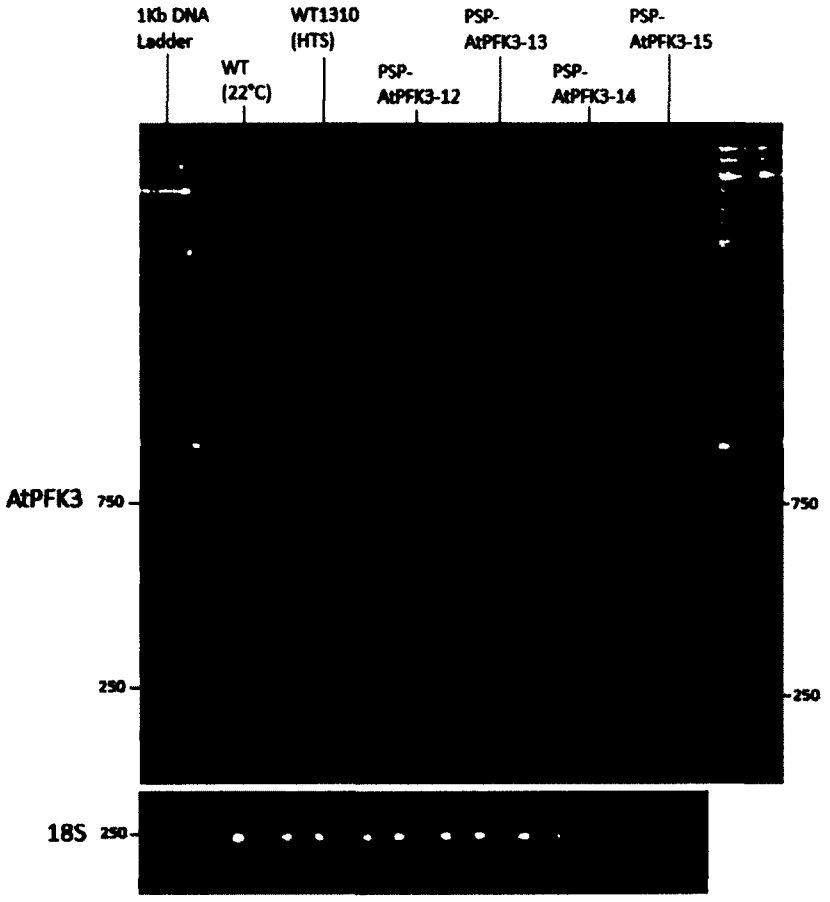

Figure 26. Agarose gel electrophoresis of semi-quantitative RT-PCR fragments (expected size: 776 bp) obtained with cDNA from mature floral buds of individual PSP-AtPFK3 (HTS) T_0 lines and untransformed (WT (22°C) and WT1310 (HTS)) lines. Ribosomal RNA (18S) was used as an internal control. The sizes of some standard DNA fragments are indicated in base pairs.

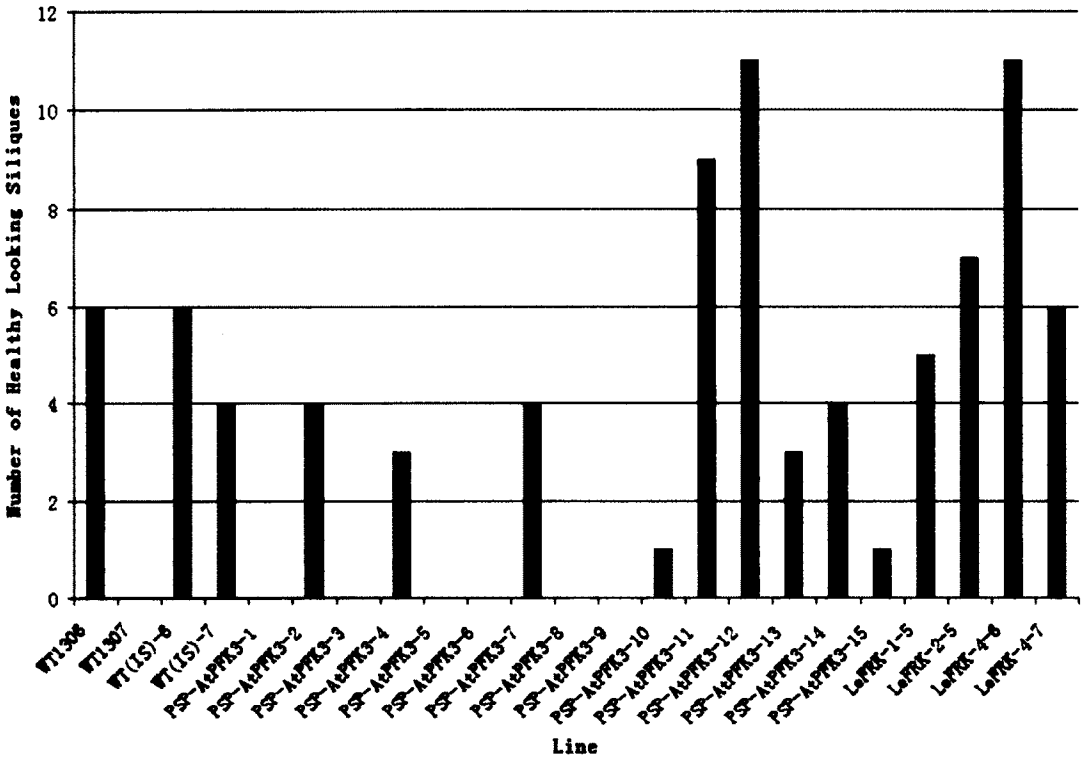

Figure 27. The number of healthy looking siliques for individual PSP-AtPFK3 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. The 15 PSP-AtPFK3, 4 LeFRK and 4 WT lines were located in the same flat.

was low for all plants examined, although PSP-AtPFK3-11 and PSP-AtPFK3-12 seemed to perform somewhat better. Comparing the proportion of healthy looking siliques versus the total number of potential siliques (Figure 28) led to a similar conclusion.

6.1.2.b Seed Weight

When comparing the weight of healthy seeds as a function of plant weight, transgenic lines PSP-AtPFK3-7 and PSP-AtPFK3-9 showed the highest values of all lines tested except LeFRK-2-5 (Figure 29). PSP-AtPFK3-23 from another HTS experiment also showed some promising results (results not shown). The progeny of these three lines was chosen for further heat stress treatments.

6.1.3 Phenotypic Analysis of PSP-AtPFK3 Transgenic Lines (T_1 Generation)

Following the initial PSP-AtPFK3 T_0 screening, kanamycin resistant T_1 progeny of PSP-AtPFK3-7 (7 lines), PSP-AtPFK3-9 (6 lines) and PSP-AtPFK3-23 (6 lines), along with WT and LeFRK lines were heat stressed. The results are described in detail below.

6.1.3.a Silique Number

Most transgenic lines had less healthy looking siliques than the best WT line, with the exception of lines PSP-AtPFK3-23-5 and PSP-AtPFK3-23-3 (Figure 30). In fact these two lines had better values than the highest LeFRK line (result now shown).

When the percentage of healthy looking siliques versus the total number of potential siliques was calculated, lines PSP-AtPFK3-23-5 and PSP-AtPFK3-23-6 displayed the

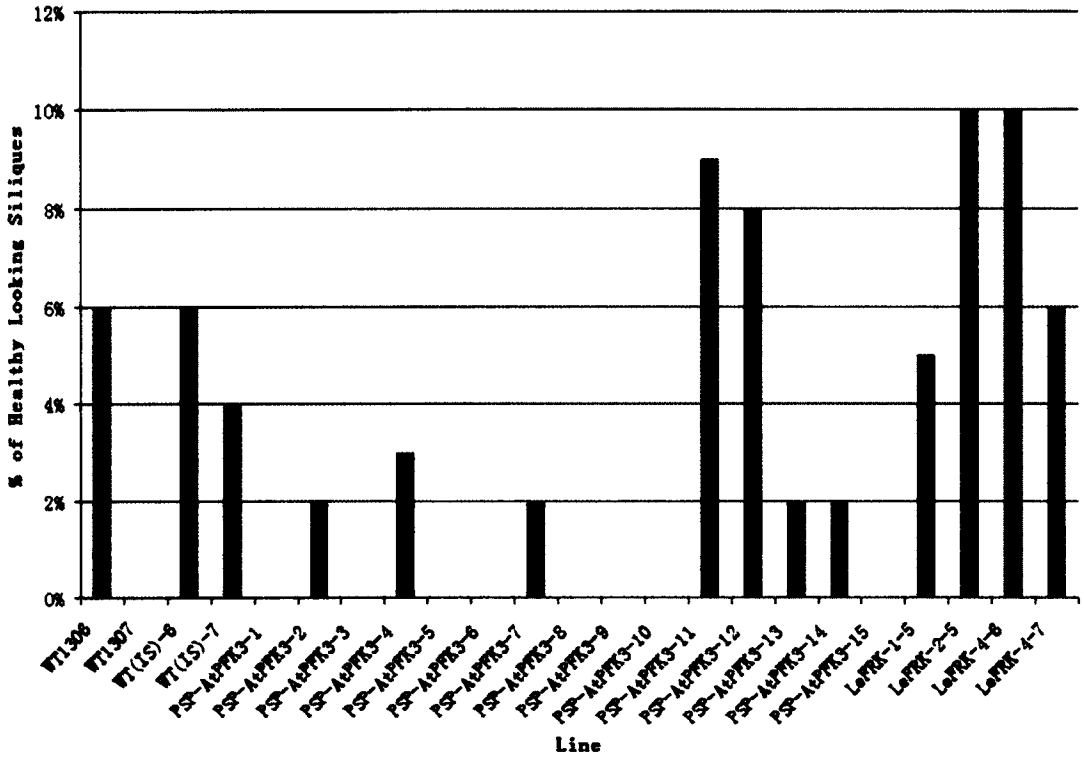

Figure 28.The percentage of healthy looking siliques for individual PSP-AtPFK3 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. The 15 PSP-AtPFK3, 4 LeFRK and 4 WT lines were located in the same flat.

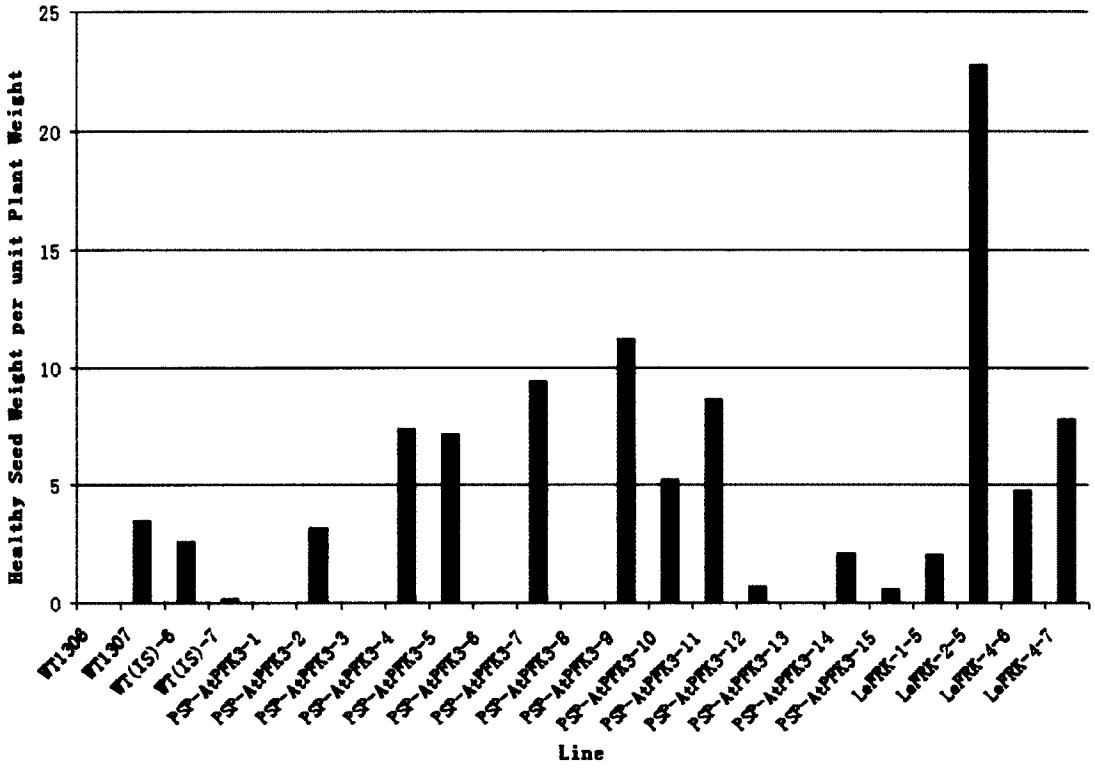

Figure 29. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) of each PSP-AtPFK3 T_0 line is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 7 days at 32°C following 14 days of heat treatment at 33°C. 15 PSP-AtPFK3, 4 LeFRK and 4 WT lines were located in the same flat.

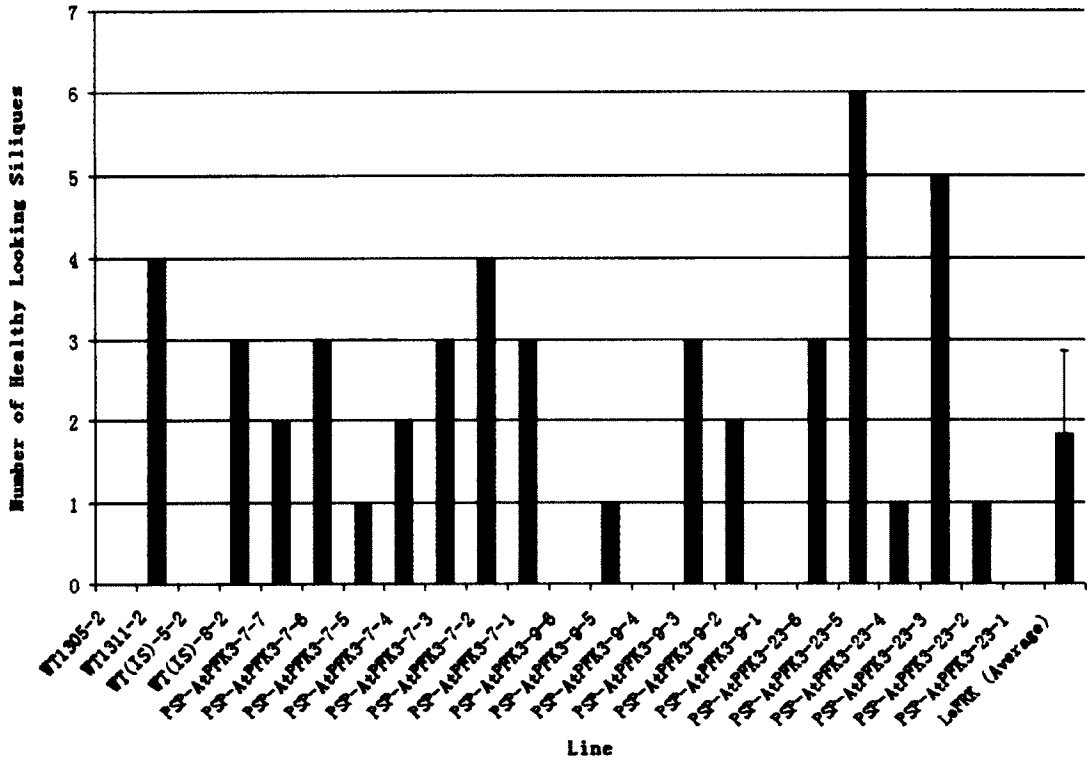

Figure 30. The number of healthy looking siliques for individual T_1 progeny of lines PSP-AtPFK3-7, PSP-AtPFK3-9 and PSP-AtPFK3-23 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The number of healthy looking siliques for the LeFRK lines was averaged. 19 PSP-AtPFK3 and 4 WT lines were located in the same flat.

highest values (Figure 31).

6.1.3.b Seed Weight

Unexpectedly, the untransformed lines had among the highest values for healthy seed weight per unit plant weight of all lines tested (Figure 32). The size of mature plants of these WT lines was similar to that of the transgenic lines (results not shown) indicating that none of the transgenic lines tested produced more healthy seeds than the best WT line.

6.2 Discussion

Phosphofructokinase (PFK) is a key regulatory enzyme of glycolysis which is the main pathway of carbohydrate degradation (Mustroph *et al.*, 2007) and is important for adaptation to stress conditions (Churchill *et al.*, 1994; Plaxton, 1996; Shenton & Grant, 2003). PFK can be regulated to determine the rate of glycolysis since PFK catalyzes the first unique step (Turner & Turner, 1975). According to bioinformatic and phylogenetic analyses, there are seven sequences encoding ATP-dependent PFK (*AtPFK1-7*) in Arabidopsis and they are all expressed in floral tissues (Mustroph *et al.*, 2007; Winkler *et al.*, 2007). Among all AtPFK isoforms, *AtPFK3* has the highest level expression in pollen (Honys & Twell, 2004) and it also has been shown to be induced following HTS (Mustroph *et al.*, 2007). Therefore, *AtPFK3* was selected as a candidate for pollen overexpression.

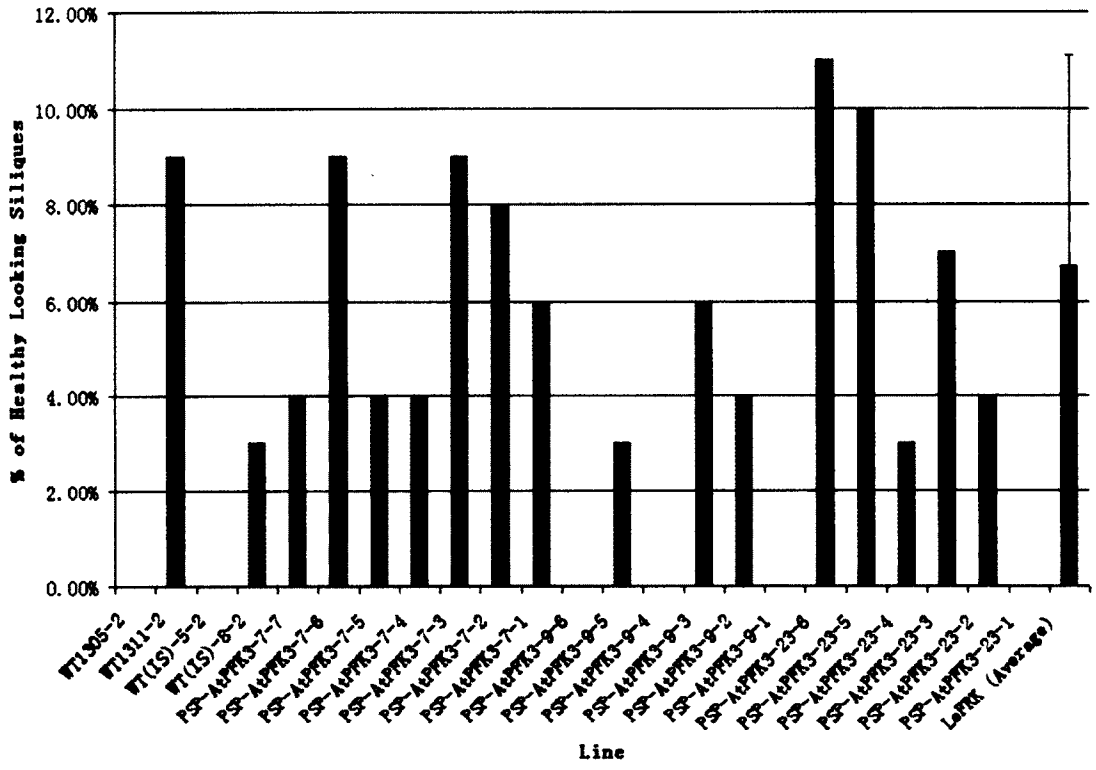

Figure 31. The percentage of healthy looking siliques for individual T_1 progeny of lines PSP-AtPFK3-7, PSP-AtPFK3-9 and PSP-AtPFK3-23 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The percentage of healthy looking siliques for the LeFRK lines was averaged. 19 PSP-AtPFK3 and 4 WT lines were located in the same flat.

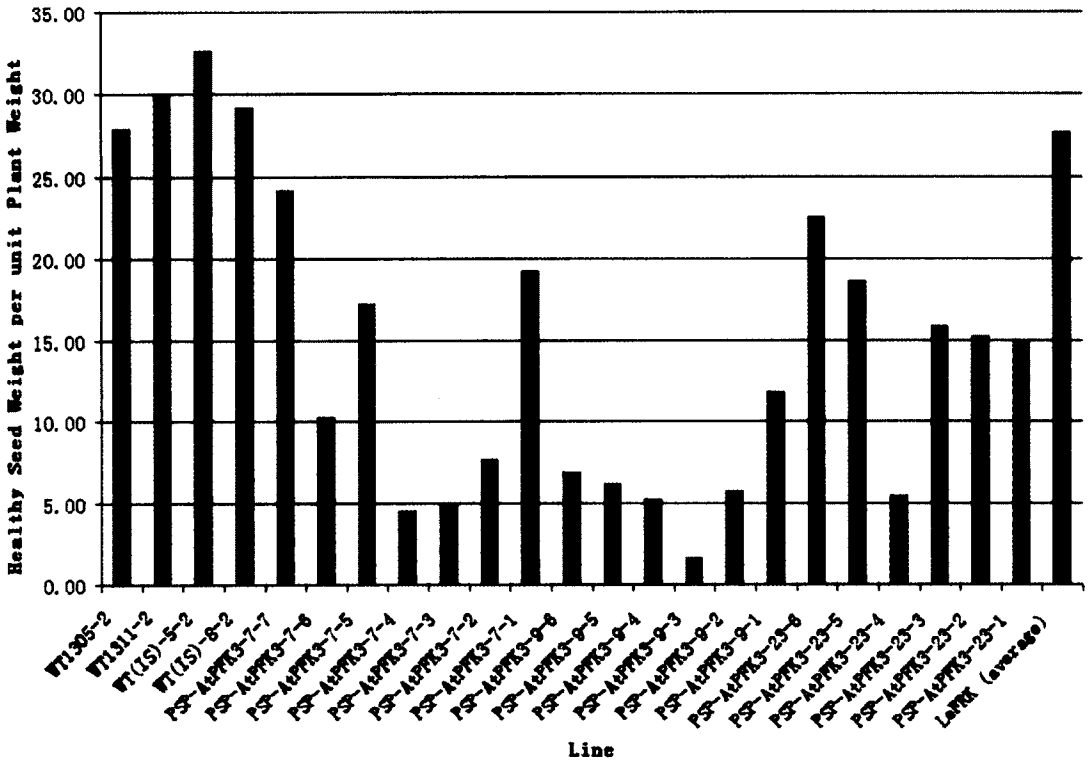

Figure 32. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) for individual T_1 progeny of lines PSP-AtPFK3-7, PSP-AtPFK3-9 and PSP-AtPFK3-23 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The healthy seed weight per unit plant weight for the LeFRK lines was averaged (Standard deviation = 43.9). 19 PSP-AtPFK3 and 4 WT lines were located in the same flat.

To my knowledge, PFK3 has never been overexpressed in any species. Although transgene integration and expression was confirmed in three of the four transgenic lines selected, the combined expression of the endogenous gene and the transgene in those three lines was only slightly higher than WT. According to the available microarray data (Honys and Twell, 2004), the level of the *AtPFK3* transcript peaks at the tricellular pollen stage with moderate expression occurring at the other stages (Table 1). Therefore the PSP promoter should have significantly increased the level of expression of *AtPFK3* at all stages. It is possible that the level of this transcript is somehow tightly regulated in pollen.

During T_0 screening, all 15 transgenic lines possessed a lower number and percentage of healthy looking siliques than most of WT and LeFRK lines, except PSP-AtPFK3-11 and PSP-AtPFK3-12 (Figures 27 and 28). However, these results were not consistent with the results of healthy seed weight per unit plant weight, where transgenic lines PSP-AtPFK3-7 and PSP-AtPFK3-9 showed the highest values of all lines tested except LeFRK-2-5 (Figure 29). Evidently, there is little correlation between the appearance of the siliques and seed production as plants with fewer healthy looking siliques can produce more seeds than those with more healthy looking siliques. Screening the T_1 progeny of three promising transgenic lines, PSP-AtPFK3-7, PSP-AtPFK3-9 and PSP-AtPFK3-23, did not yield results superior to the WT lines confirming that they did not display improved heat tolerance as measured by seed production.

6.3 Conclusion

The level of *AtPFK3* mRNA was found to be relatively low in WT plants and did not vary greatly under HTS. The level of *AtPFK3* did not increase substantially in the transgenic lines given the strength of the PSP promoter and may indicate a tight regulation of this transcript's abundance in pollen. No major improvements in seed production were observed in the transgenic PSP-AtPFK3 lines under heat stress.

7. PSP-AtSTP6

7.1 Results

PSP-AtSTP6 is designed to overexpress the Arabidopsis sugar transport protein 6 in pollen. Sugar transporters play a critical role in the membrane transport of sugars and their cell-to-cell and long-distance distribution throughout the plant (Williams *et al.*, 2000). A total of 15 PSP-AtSTP6 T_0 transformed lines were subjected to HTS. Four transgenic lines were selected to verify transgene integration and expression. After screening, T_0 lines PSP-AtSTP6-5 and PSP-AtSTP6-14 were chosen for additional HTS experiments.

7.1.1 Verification of Transgenic Lines

7.1.1.a Genomic PCR

The integration of PSP-AtSTP6 into transgenic plants was analyzed using genomic PCR (Figure 33). The expected PSP-AtSTP6 fusion-specific fragment was only observed in the PSP-AtSTP6-14 transgenic line. Since kanamycin selection is a proven effective way to select transformed Arabidopsis plants, the absence of the expected PSP-AtSTP6 fragment may be due to a gene rearrangement.

7.1.1.b Transgene-specific *AtSTP6* RT-PCR

AtSTP6 transgene expression was only observed in line PSP-AtSTP6-14 (Figure 34) suggesting that if a DNA rearrangement prevented the genomic PCR above it was also

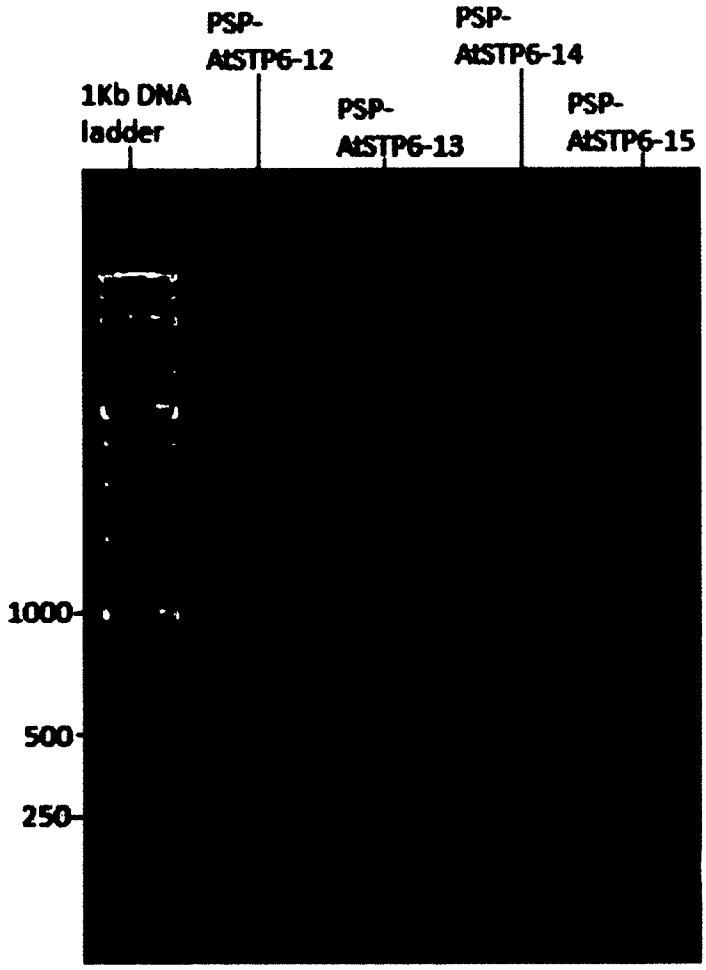

Figure 33. Agarose gel electrophoresis of genomic PCR fragment from individual PSP-AtSTP6 (PSP promoter-*Arabidopsis thaliana* Sugar Transport Protein 6, expected size: 1054 bp) T_0 lines. The sizes of some standard DNA fragments are indicated in base pairs.

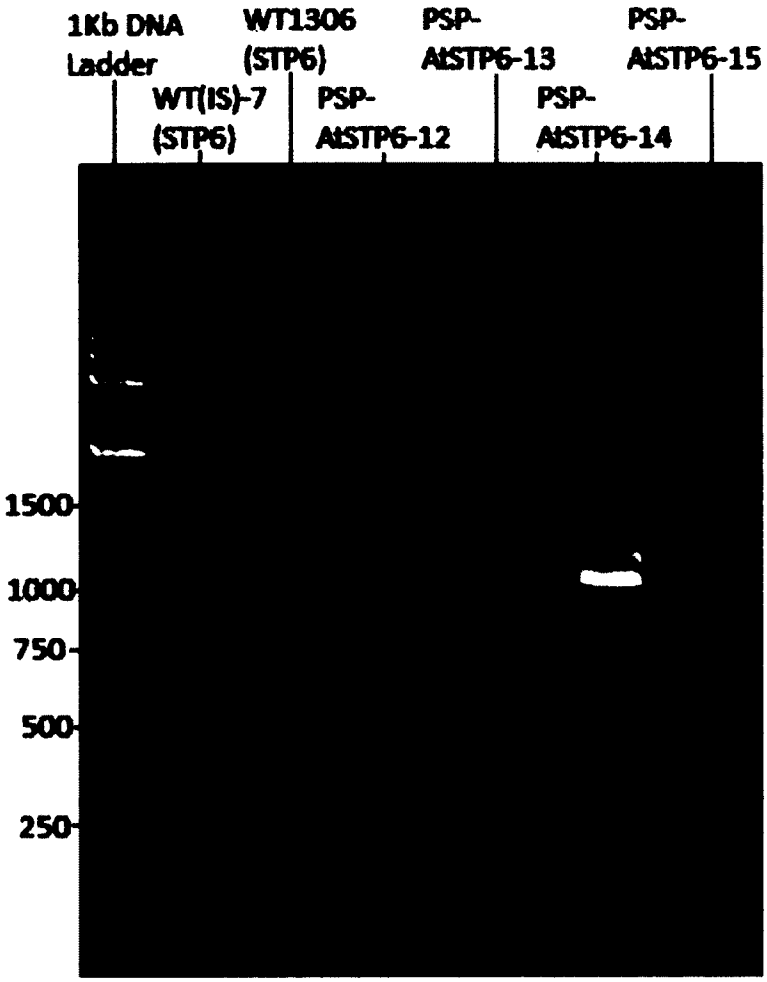

Figure 34. Agarose gel electrophoresis of transgene-specific RT-PCR fragments (Expected size: 1054 bp) obtained with cDNA from heat stressed mature floral buds of individual PSP-AtSTP6 T_0 lines and WT lines. The sizes of some standard DNA fragments are indicated in base pairs.

detrimental to the expression of the gene.

7.1.1.c Semi-quantitative *AtSTP6* RT-PCR

The quantity of *AtSTP6* was considerably reduced under heat stress in the WT line and in the T_0 lines which showed no transgene expression (Figure 35). The PSP-AtSTP6-14 T_0 line therefore displays a higher level of *AtSTP6* expression than would normally be found under heat stress.

7.1.2 Phenotypic Analysis of PSP-AtSTP6 Transgenic Lines (T_0 Generation)

Comparisons of the number and percentage of healthy looking siliques, as well as seed weight for PSP-AtSTP6 transformants versus WT and LeFRK lines are shown in Figures 36, 37, 38.

7.1.2.a Silique Number

PSP-AtSTP6-6, PSP-AtSTP6-9, PSP-AtSTP6-10 showed the highest numbers of healthy looking siliques of all plants tested (Figure 36). The same conclusion could be drawn from the data of the proportion of healthy looking siliques versus the total number of potential siliques (Figure 37).

7.1.2.b Seed Weight

One WT line (WT(IS)-8) showed the highest healthy seed weight per unit plant weight of all lines examined, including all LeFRK lines (Figure 38). Low values were

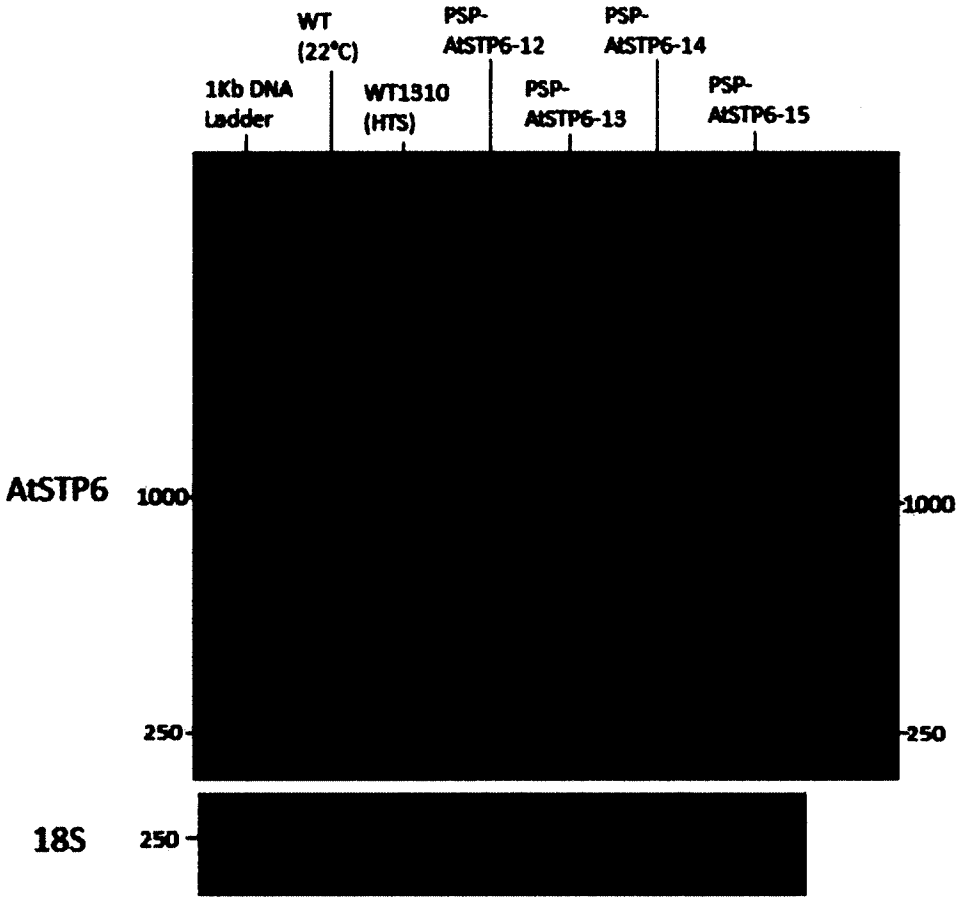

Figure 35. Agarose gel electrophoresis of semi-quantitative RT-PCR fragments (expected size: 1027 bp) obtained with cDNA from mature floral buds of individual PSP-AtSTP6 (HTS) T_0 lines and untransformed (WT (22°C) and WT1310 (HTS)) lines. Ribosomal RNA (18S) was used as an internal control. The sizes of some standard DNA fragments are indicated in base pairs.

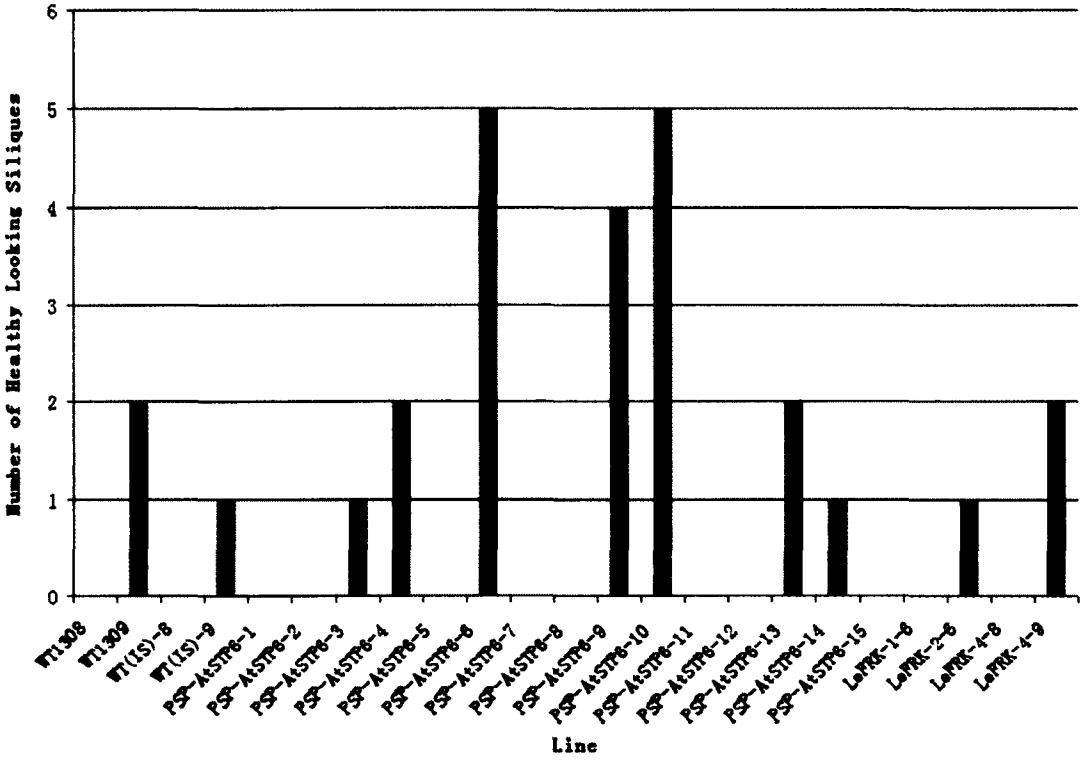

Figure 36. The number of healthy looking siliques for individual PSP-AtSTP6 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. The 15 PSP-AtSTP6, 4 LeFRK and 4 WT lines were located in the same flat.

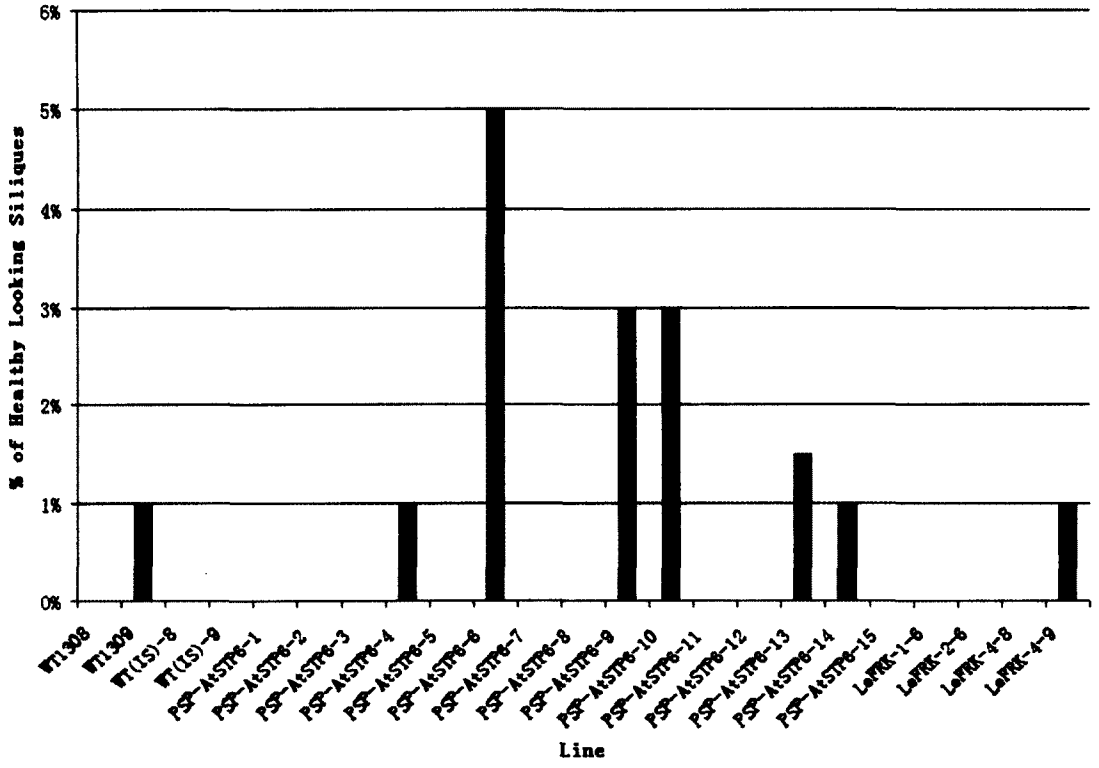

Figure 37.The percentage of healthy looking siliques for individual PSP-AtSTP6 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. The 15 PSP-AtSTP6, 4 LeFRK and 4 WT lines were located in the same flat.

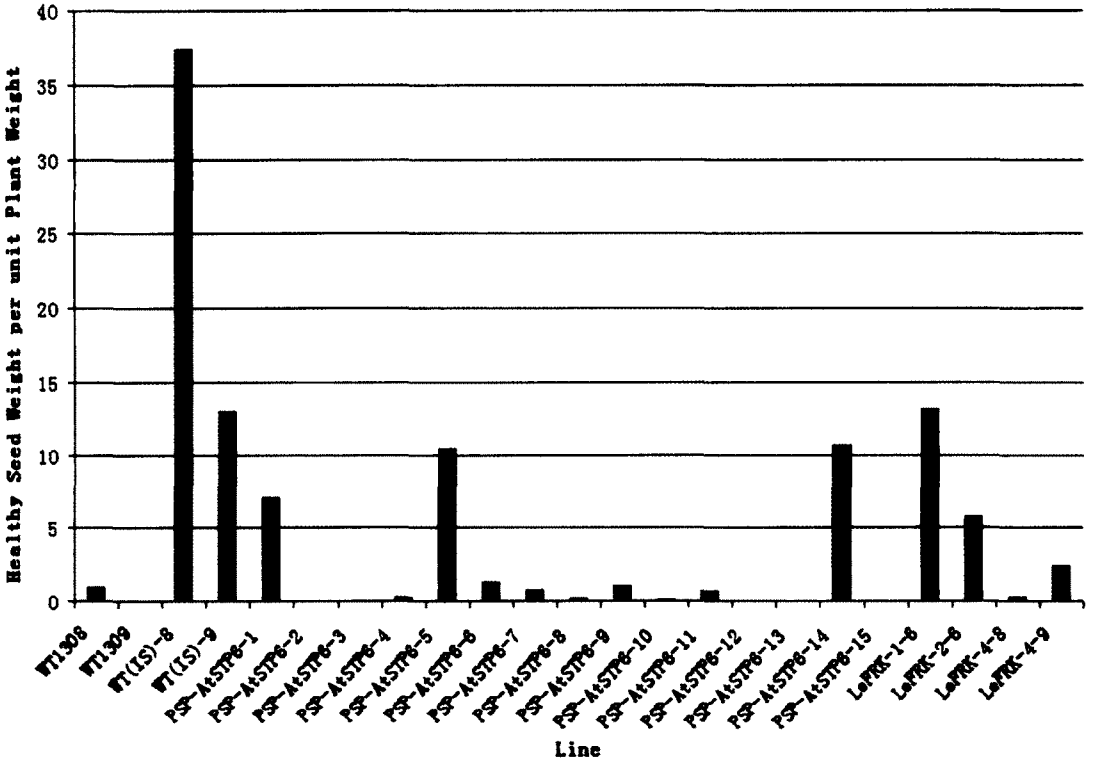

Figure 38. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) of each PSP-AtSTP6 T_0 line is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 7 days at 32°C following 14 days of heat treatment at 33°C. 15 PSP-AtSTP6, 4 LeFRK and 4 WT lines were located in the same flat.

observed for all PSP-AtSTP6 T_0 lines including the lines showing more healthy looking siliques described above. Lines PSP-AtSTP6-5 and PSP-AtSTP6-14 (which displayed an increased level of *AtSTP6* transcript) had the best values and were selected for further heat stress treatments.

7.1.3 Phenotypic Analysis of PSP-AtSTP6 Transgenic Lines (T_1 Generation)

The results obtained for the HTS treatment of the kanamycin resistant T_1 progeny of PSP-AtSTP6-5 (12 lines) and PSP-AtSTP6-14 (7 lines) are described in detail below.

7.1.3.a Silique Number

The PSP-AtSTP6-14 lines with healthy looking siliques had numbers greater than those of the WT lines (Figure 39). However, this was true of only 2 of the 12 PSP-AtSTP6-5 lines. The data representing the proportion of healthy looking siliques versus the total number of potential siliques gave similar results (Figure 40).

7.1.3.b Seed Weight

Unexpectedly, although no healthy looking siliques were produced by WT1311-4, WT(IS)-5-4 and WT(IS)-8-4, they had the highest healthy seed weight per unit plant weight of all lines with the exception of the LeFRK lines (Figure 41). In fact, most transgenic lines did not produce seeds including the lines with healthy looking siliques.

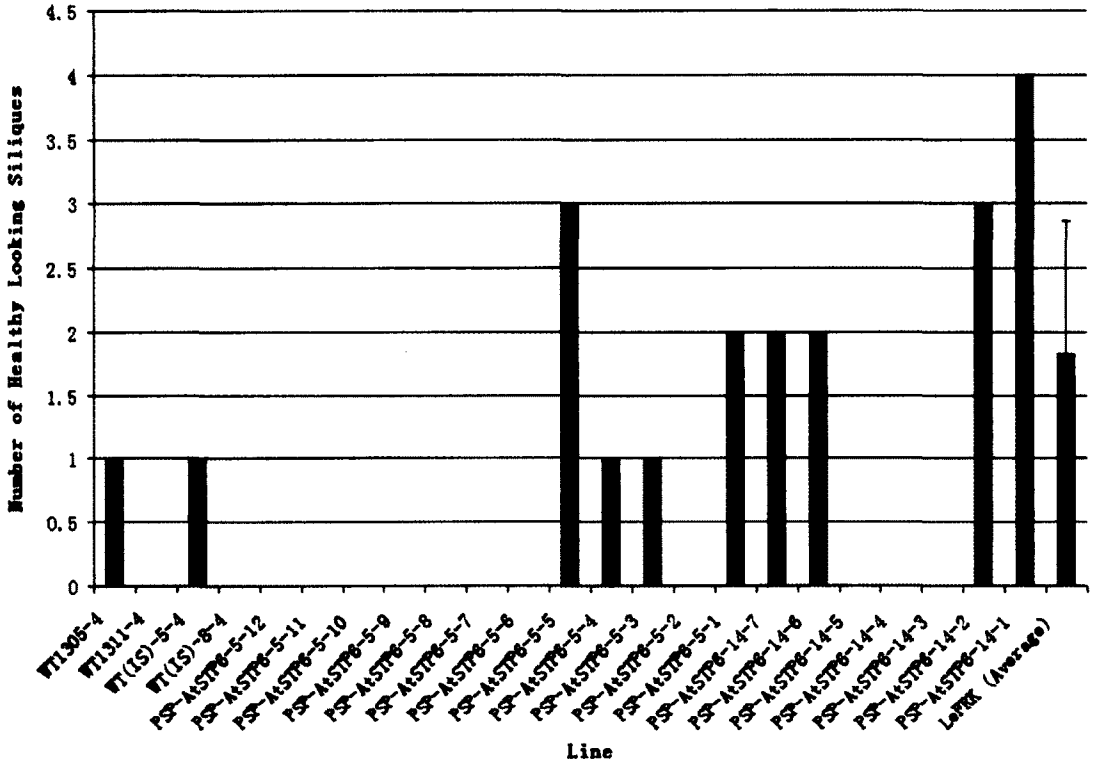

Figure 39. The number of healthy looking siliques for individual T_1 progeny of lines PSP-AtSTP6-5 and PSP-AtSTP6-14 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The number of healthy looking siliques for the LeFRK lines was averaged. 19 PSP-AtSTP6 and 4 WT lines were located in the same flat.

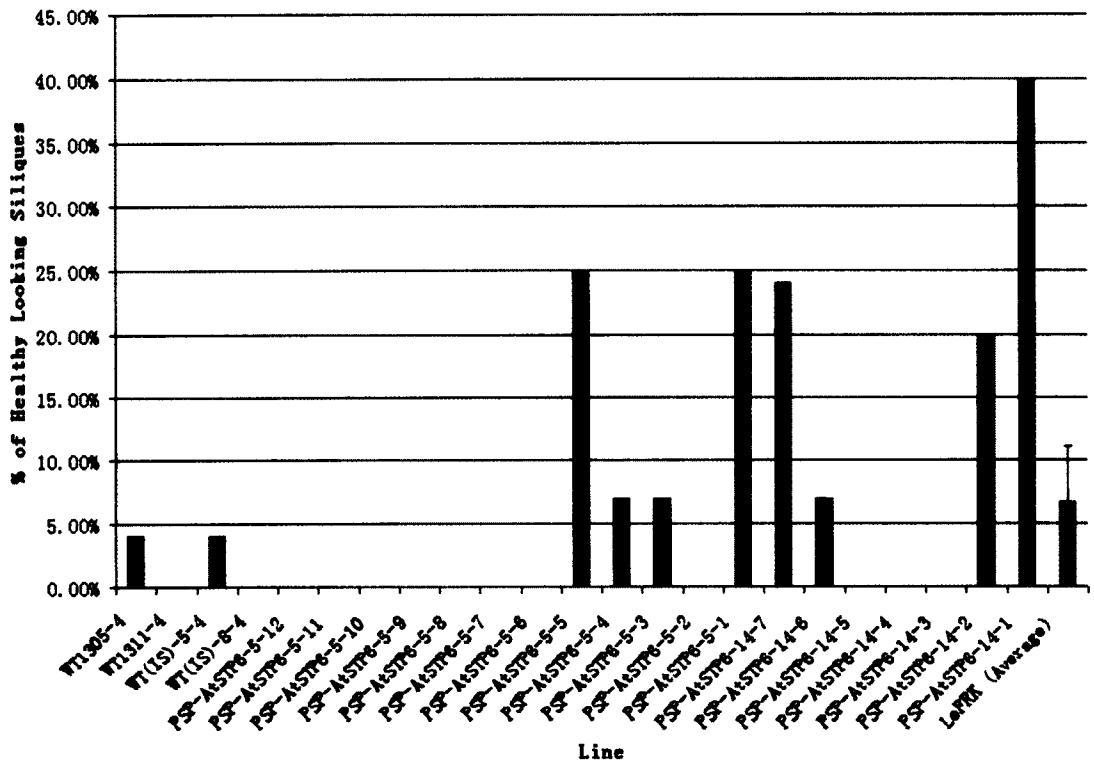

Figure 40. The percentage of healthy looking siliques for individual T_1 progeny of lines PSP-AtSTP6-5 and PSP-AtSTP6-14 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The percentage of healthy looking siliques for the LeFRK lines was averaged. 19 PSP-AtSTP6 and 4 WT lines were located in the same flat.

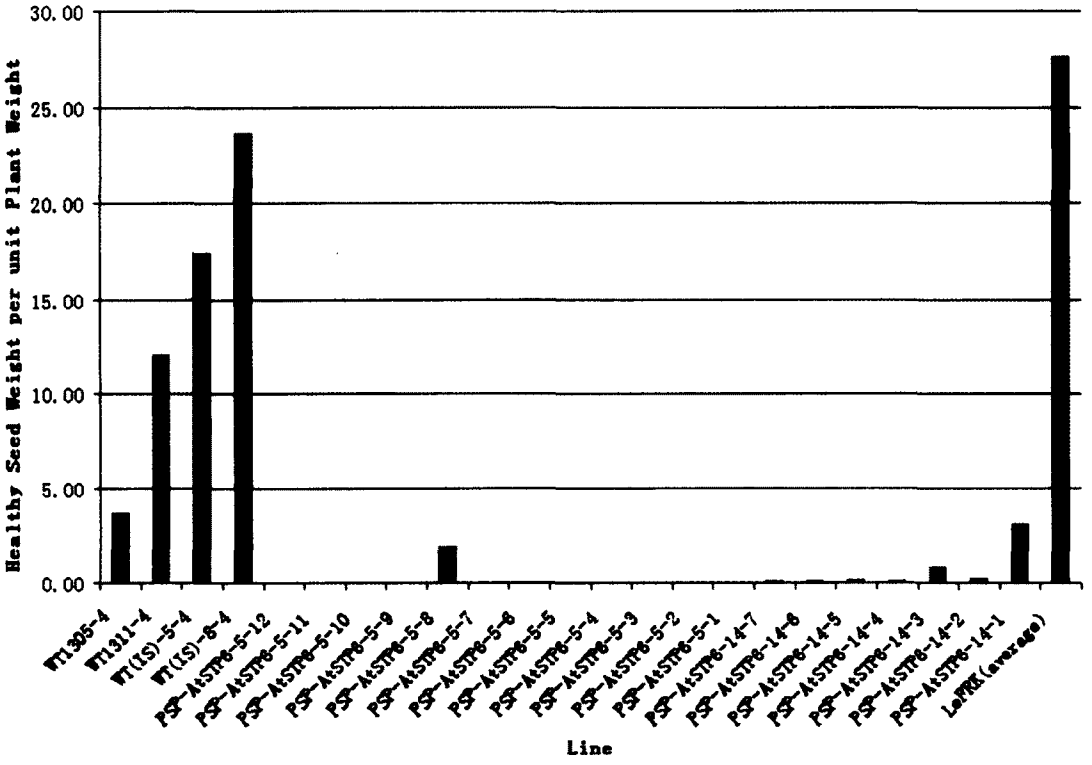

Figure 41. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) for individual T_1 progeny of lines PSP-AtSTP6-5 and PSP-AtSTP6-14 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The healthy seed weight per unit plant weight for the LeFRK lines was averaged (Standard deviation = 43.9). 19 PSP-AtSTP6 and 4 WT lines were located in the same flat.

7.2 Discussion

Sugar transport proteins (STPs) are key proteins catalyzing either passive diffusion or energy-dependent active transport to convey sugars across membrane barriers to allow accumulation of sugar substrates (Büttner, 2007). Developing pollen grains receive carbohydrates for growth and development from the anther wall and tapetum apoplast, which get their supply of sugar from carbon sources. STPs not only play a pivotal role in sugar distribution throughout the plant, but they are important for pollen development, germination and pollen tube growth (Williams *et al.*, 2000). Abiotic stresses such as drought stress have a negative impact on sugar accumulation, causing pollen sterility and low seed number (Ji *et al.*, 2010). HTS was also shown to have a detrimental effect on these processes by disturbing the carbohydrate supply to the developing pollen grain (Wahid *et al.*, 2012). Maintaining sink strength in the anther/pollen is a key to preserving pollen fertility and seed production under abiotic stresses (Ji *et al.*, 2010). To my knowledge, the effect of overexpressing a sugar transporter during pollen development has never been studied in any species. However, overexpression of STPs in tobacco, barley and rice plants was shown to maintain the level of sugar import from carbon source tissues under stress, thus leading to enhanced plant growth and higher yield (Khalil *et al.*, 2010; Sun *et al.*, 2011; Weichert *et al.*, 2010). In Arabidopsis, AtSTP13 is the only STP to have been overexpressed and this lead to increases in the rates of glucose uptake, resulting in higher sucrose levels. The transgenic seedlings were also found to be larger and have higher biomass than untransformed seedlings (Schofield *et al.*, 2009).

Among all AtSTP isoforms, AtSTP6 is highly expressed exclusively in the latest stages of pollen development (Büttner, 2007; Scholz-Starke *et al.*, 2003) and was therefore selected as the candidate STP gene for this study. According to the microarray expression data from Honys and Twell (2004), the highest *AtSTP6* mRNA expression is found at the tricellular pollen stage with moderate expression occurring at the other stages (Table 1). Therefore, overexpressing *AtSTP6* under the PSP promoter should significantly increase the level of the *AtSTP6* transcript at all stages and especially the UNM and MPG stages.

In this study, the level of the pollen-specific *AtSTP6* transcript in untransformed Arabidopsis plants was shown to be much reduced under HTS (Figure 35). This finding is consistent with the results from another study which pointed out that cold stress repressed the expression of the monosaccharide transporter *OsMST8* in rice, leading to low sugar level in anther wall and developing pollen grains (Parish *et al.*, 2012). Therefore, given the importance of sugar transport to proper pollen development, overexpressing *AtSTP6* throughout pollen development should help increase or maintain the carbohydrate supply in developing and mature pollen to improve pollen performance and germination under HTS. It is unknown why three of the four transgenic lines appeared to have transgene rearrangements, but PSP-AtSTP6-14, the only transgenic line where transgene integration and expression was confirmed, did show considerably higher expression of *AtSTP6* compared to untransformed lines (Figure 35). Nonetheless, it is important to keep in mind that it is possible that many of the PSP-AtSTP6 lines tested

did not express the transgene.

During T_0 screening, although PSP-AtSTP6-6, PSP-AtSTP6-9, PSP-AtSTP6-10 showed the highest numbers of healthy looking siliques of all plants tested (Figures 36 and 37), all transgenic lines had a low value of healthy seed weight per unit plant weight (Figure 38). The results indicating no transgenic lines produced more healthy seeds than WT lines were confirmed in the T_1 progeny of two promising transgenic lines PSP-AtSTP6-5 and PSP-AtSTP6-14 (Figure 41). Since PSP-AtSTP6-14 was shown to overexpress *AtSTP6* in pollen during heat stress, these findings may suggest there is little correlation between the level of *AtSTP6* and seed production, although a larger sample of transgenic plants with confirmed overexpression of *AtSTP6* in pollen would need to be studied to validate this possibility.

7.3 Conclusion

It was demonstrated that HTS has a detrimental effect on the expression of *AtSTP6* in Arabidopsis. It was also shown that the PSP promoter can increase the level of this transcript in pollen under HTS, although the above results seem to indicate that this does not have a big impact on improving heat tolerance of Arabidopsis as measured by seed production.

8. PSP-AtSUS1

8.1 Results

The PSP-AtSUS1 construct is meant to overexpress in pollen the Arabidopsis sucrose synthase 1, a key enzyme involved in sucrose metabolism. A total of 15 T_0 transformant were heat stressed together and evaluated. Four transgenic lines were chosen for verification of transgene integration and expression. After T_0 screening, promising transgenic T_0 lines PSP-AtSUS1-2, PSP-AtSUS1-8 and PSP-AtSUS1-12 were selected for further heat treatment

8.1.1 Verification of Transgenic Lines

8.1.1.a Genomic PCR

Genomic PCR was performed to verify the integration of PSP-AtSUS1 into transgenic Arabidopsis (Figure 42). The presence of PSP-AtSUS1 was confirmed in all lines except PSP-AtSUS1-7.

8.1.1.b Transgene-specific *AtSUS1* RT-PCR

RT-PCR was performed to verify *AtSUS1* transgene expression. Relatively high expression was confirmed in all the lines shown above to possess the PSP-AtSUS1 genomic fragment (Figure 43).

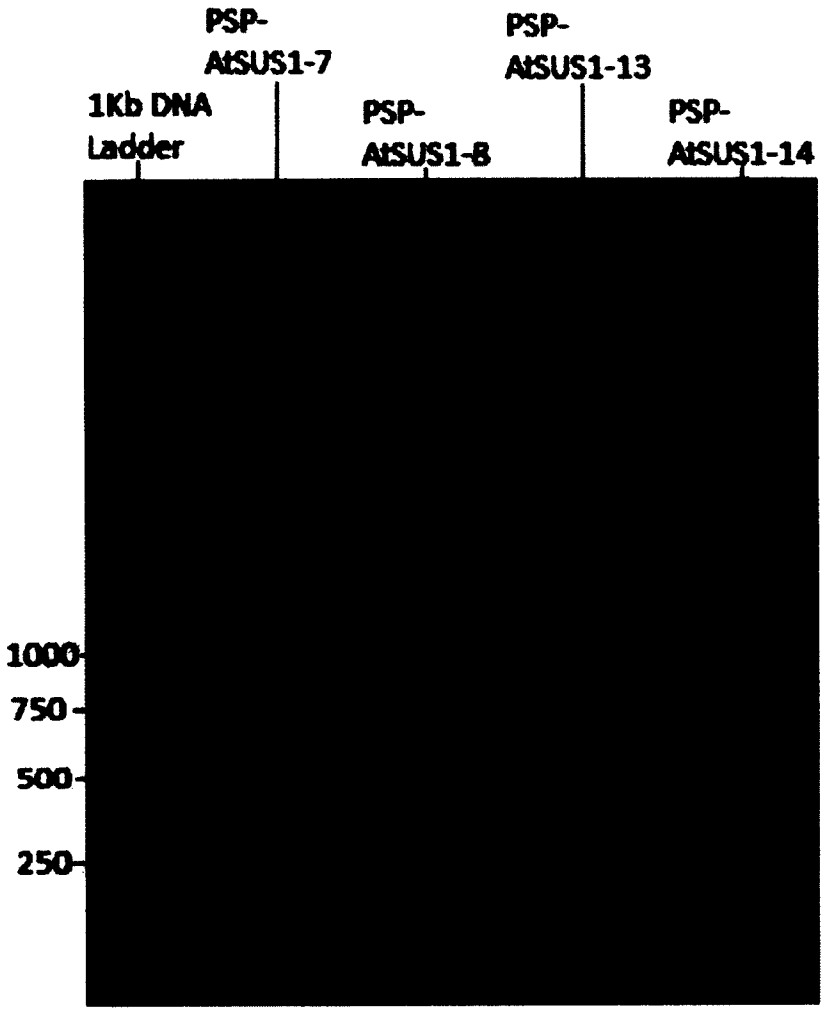

Figure 42. Agarose gel electrophoresis of genomic PCR fragment from individual PSP-AtSUS1 (PSP promoter-*Arabidopsis thaliana* Sucrose Synthase 1, expected size: 727 bp) T_0 lines. The sizes of some standard DNA fragments are indicated in base pairs.

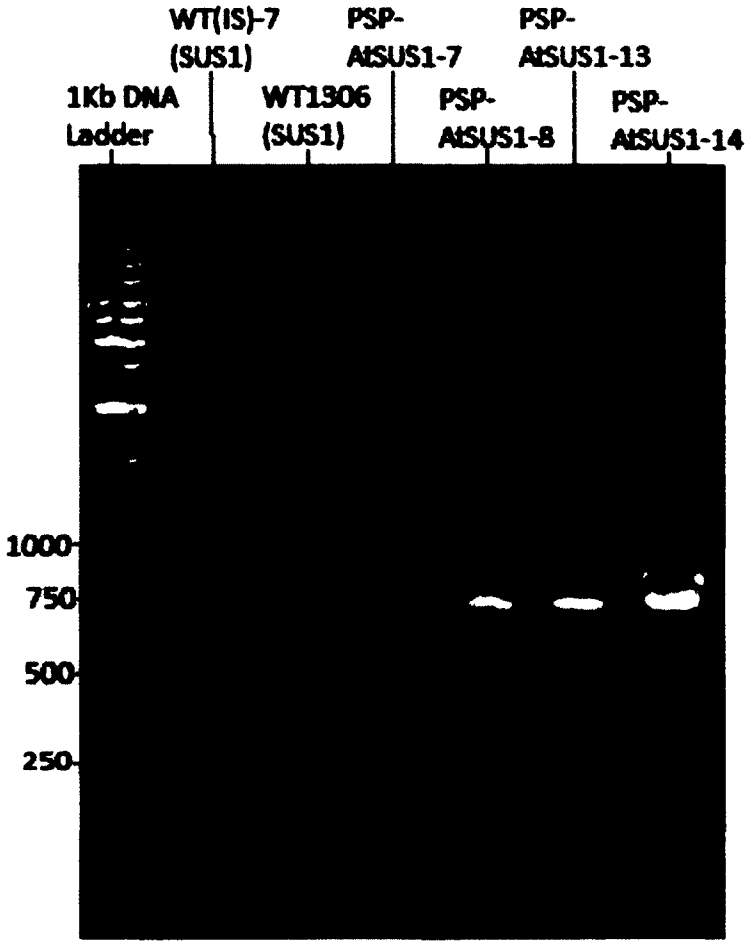

Figure 43. Agarose gel electrophoresis of transgene-specific RT-PCR fragments (Expected size: 727 bp) obtained with cDNA from heat stressed mature floral buds of individual PSP-AtSUS1 T_0 lines and WT lines. The sizes of some standard DNA fragments are indicated in base pairs.

8.1.1.c Semi-quantitative *AtSUS1* RT-PCR

Semi-quantitative evaluation of the combined endogenous and transgene *AtSUS1* expression revealed that the T_0 transgenic lines PSP-AtSUS1-8, PSP-AtSUS1-13 and PSP-AtSUS1-14 all displayed relatively higher expression levels than non-transformed plants (Figure 44). This fact provides additional support for the expression of the transgene.

8.1.2 Phenotypic Analysis of PSP-AtSUS1 Transgenic Lines (T_0 Generation)

Comparisons of the number and percentage of healthy looking siliques, as well as seed weight for PSP-AtSUS1 versus WT and LeFRK lines are shown in Figures 45, 46, 47.

8.1.2.a Silique Number

As seen in Figure 45, the total number of healthy looking siliques produced under heat stress by the PSP-AtSUS1 T_0 lines was generally inferior to that of the best WT line.

When the percentage of healthy looking siliques versus the total number of potential siliques was calculated (Figure 46), the results led to a very similar conclusion as in Figure 45.

8.1.2.b Seed Weight

When comparing the weight of healthy seeds per unit plant weight, one transgenic line (PSP-AtSUS1-12) possessed the highest values of all lines tested, including the

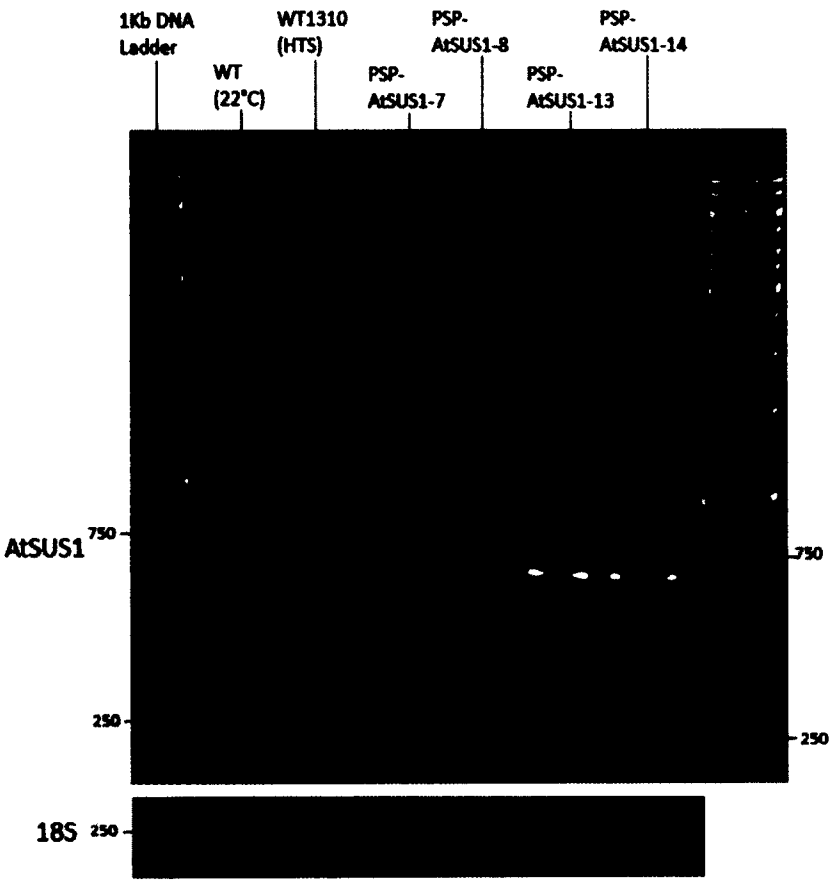

Figure 44. Agarose gel electrophoresis of semi-quantitative RT-PCR fragments (expected size: 700 bp) obtained with cDNA from mature floral buds of individual PSP-AtSUS1 (HTS) T_0 lines and untransformed (WT (22°C) and WT1310 (HTS)) lines. Ribosomal RNA (18S) was used as an internal control. The sizes of some standard DNA fragments are indicated in base pairs.

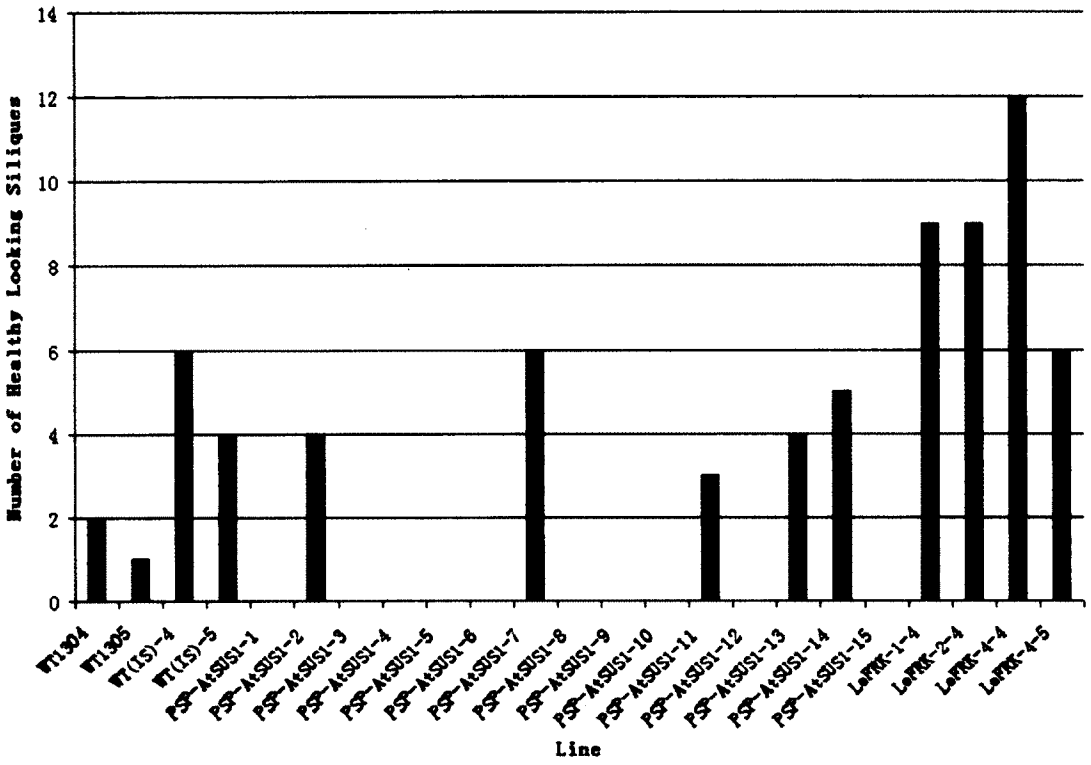

Figure 45. The number of healthy looking siliques for individual PSP-AtSUS1 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. 15 The PSP-AtSUS1, 4 LeFRK and 4 WT lines were located in the same flat.

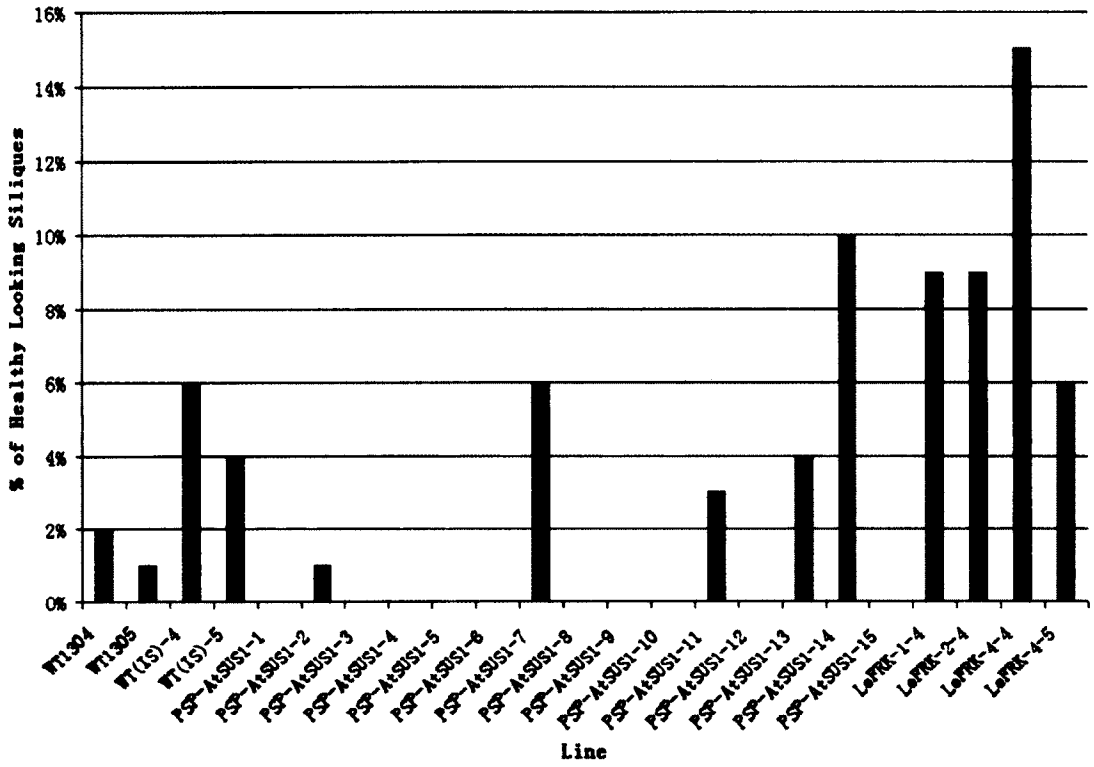

Figure 46. The percentage of healthy looking siliques for individual PSP-AtSUS1 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. The 15 PSP-AtSUS1, 4 LeFRK and 4 WT lines were located in the same flat.

LeFRK lines (Figure 47). In spite of producing only one healthy looking silique (Figure 45), WT1305 had the second highest values of all lines examined. Since the values for PSP-AtSUS1-2 and PSP-AtSUS1-8 were higher than most WT lines and LeFRK lines, they were chosen along with PSP-AtSUS1-12 for additional HTS treatments.

8.1.3 Phenotypic Analysis of PSP-AtSUS1 Transgenic Lines (T_1 Generation)

The performance of kanamycin resistant T_1 progeny of PSP-AtSUS1-2 (6 lines), PSP-AtSUS1-8 (6 lines) and PSP-AtSUS1-12 (7 lines) was evaluated under heat stress. The results are described in detail below.

8.1.3.a Silique Number

In general, the majority of the PSP-AtSUS1-12 and PSP-AtSUS1-8 T_1 lines possessed higher numbers of healthy looking siliques than most of WT lines and the average of the 19 LeFRK lines (Figure 48).

When comparing the percentage of healthy looking siliques, 3 of the 7 PSP-AtSUS1-12 T_1 transgenic lines had higher values than all tested lines (Figure 49).

8.1.3.b Seed Weight

Although some PSP-AtSUS1-12 T_1 lines produced relatively higher number of healthy looking siliques, only one line (PSP-AtSUS1-12-1) had higher healthy seed weight per unit plant weight than all WT lines (Figure 50). Interestingly, 3 of the 6 PSP-AtSUS1-8 lines possessed higher values than all WT lines. Lines PSP-AtSUS1-2-4

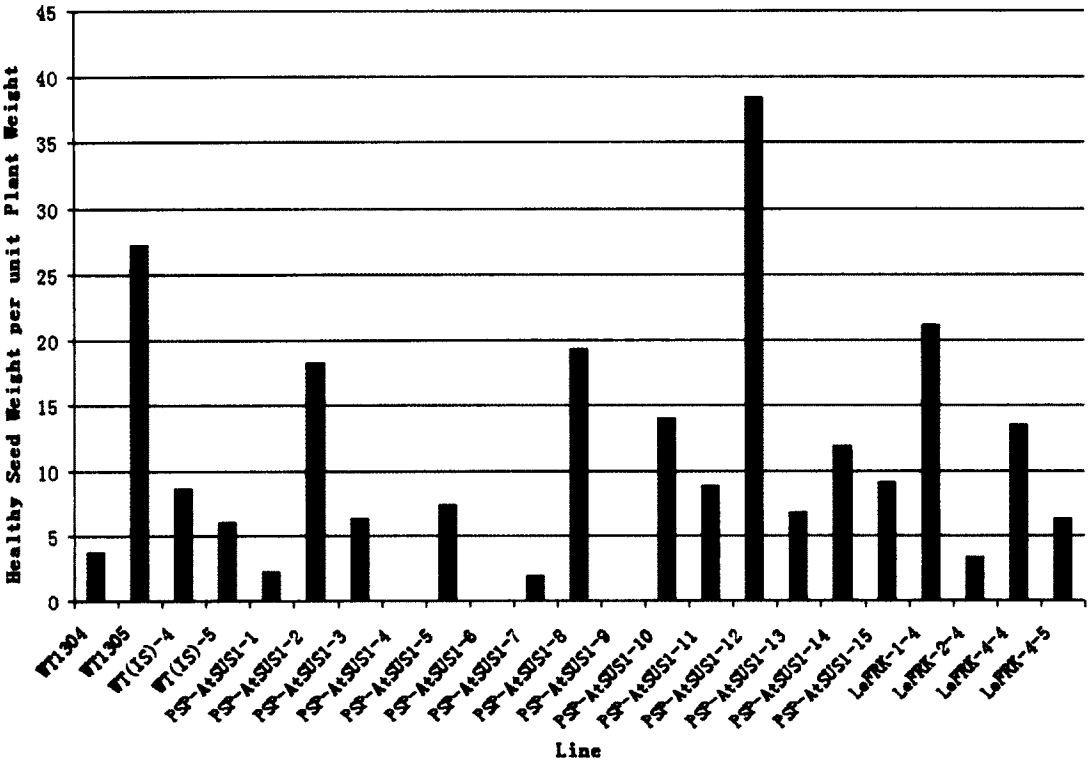

Figure 47. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) of each PSP-AtSUS1 T_0 line is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 7 days at 32°C following 14 days heat treatment at 33°C. 15 PSP-AtSUS1, 4 LeFRK and 4 WT lines were located in the same flat.

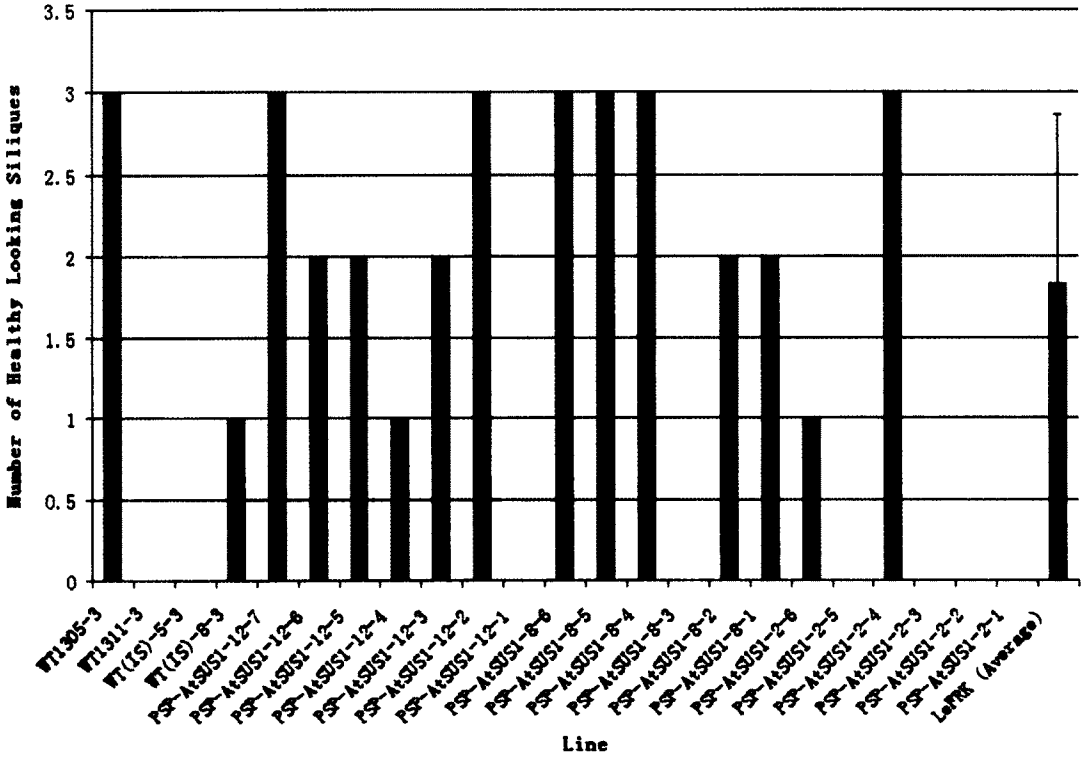

Figure 48. The number of healthy looking siliques for individual T_1 progeny of lines PSP-AtSUS1-2, PSP-AtSUS1-8 and PSP-AtSUS1-12 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The number of healthy looking siliques for the LeFRK lines was averaged. 19 PSP-AtSUS1 and 4 WT lines were located in the same flat.

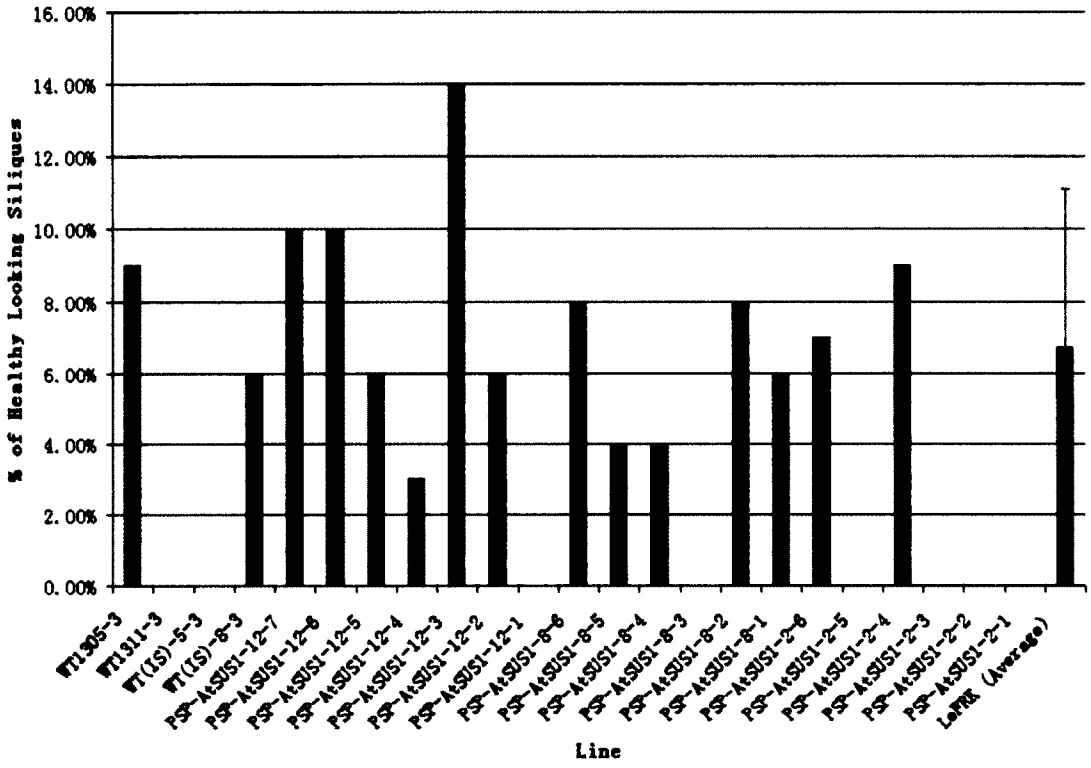

Figure 49. The percentage of healthy looking siliques for individual T_1 progeny of lines PSP-AtSUS1-2, PSP-AtSUS1-8 and PSP-AtSUS1-12 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The percentage of healthy looking siliques for the LeFRK lines was averaged. 19 PSP-AtSUS1 and 4 WT lines were located in the same flat.

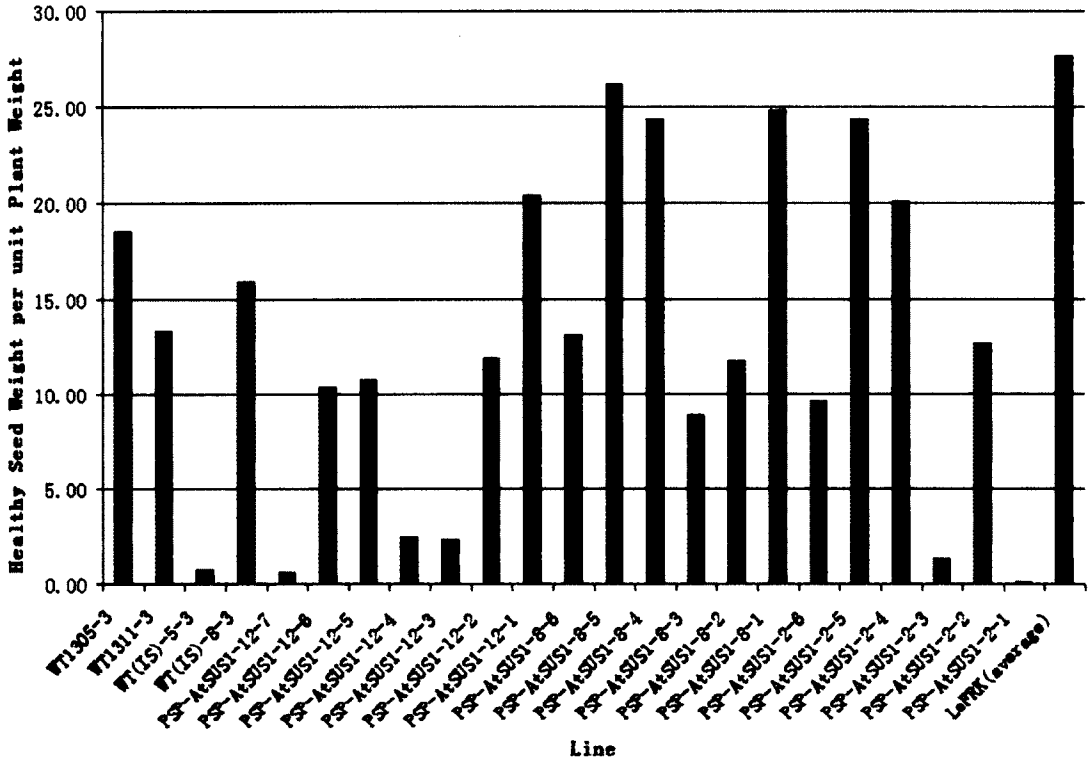

Figure 50. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) for individual T_1 progeny of lines PSP-AtSUS1-2, PSP-AtSUS1-8 and PSP-AtSUS1-12 is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 32°C. The healthy seed weight per unit plant weight for the LeFRK lines was averaged (Standard deviation). 19 PSP-AtSUS1 and 4 WT lines were located in the same flat.

and PSP-AtSUS1-2-5 also performed better than the best WT line.

8.2 Discussion

Sucrose synthase (SUS) is a key enzyme involved in sucrose metabolism by catalyzing the reversible conversion of sucrose and UDP to UDP-G (UDP-glucose) and fructose (Granot, 2008). Sucrose must be cleaved by either invertase or sucrose synthase in order to undergo further metabolism in sink tissues. SUS activity has been shown to play a major role in carbohydrate metabolism in many plants by controlling the mobilization of sucrose into various pathways important for the metabolic, structural, and storage functions of the plant cell (Baud *et al.*, 2004). Its activity also has been found to feed sucrose into metabolic pathways in sink tissues, such as pollen grains (Claeyssen & Rivoal, 2007). Although abiotic stresses, such as cold stress, and drought stress can have negative effects on the reproductive process, leading to poor seed set (Saini & Westgate, 2000; Thakura *et al.*, 2010; Zinn *et al.*, 2010), they can induce SUS expression (Baud *et al.*, 2004; Dejardin *et al.*, 1999; Kleines *et al.*, 1999). For example, *AtSUS2*, *AtSUS4*, *AtSUS5*, and *AtSUS6* mRNA levels in rosette leaves were unaffected under cold and drought stress, whereas a significant increase was observed in the expression levels of *AtSUS3* and *AtSUS1* (Baud *et al.*, 2004).

To my knowledge, the effect of overexpressing a sucrose synthase gene during pollen development has never been studied in any species. However, overexpressing the *SUS* gene was shown to improve plant growth and seed production (Coleman *et al.*, 2006;

Coleman *et al.*, 2009; Xu & Joshi, 2010; Xu *et al.*, 2012). For example, overexpression of the *Gossypium hirsutum SUS* gene in poplar trees increased cell wall cellulose content per dry weight by 2% to 6% and resulted in thicker xylem secondary cell wall (Coleman *et al.*, 2009). Another study showed that doubling the SUS activity in cotton improved early seed development with reduced seed abortion, leading to an enhanced seed weight of about 30% (Xu *et al.*, 2012). Overexpressing an aspen *SUS* gene (*PtrSUS1*) in *Arabidopsis* plants led to dramatically increased SUS activity, causing early flowering and faster growth in root and hypocotyls length. Both fresh and dry weights of whole plants were increased in transgenic lines (Xu & Joshi, 2010). Overexpression of SUS in tobacco plants was also shown to lead to significant increases in plant height (Coleman *et al.*, 2006). Suppression of the *SUS* gene has also been investigated in several other studies. One study showed that cotton with SUS suppression constructs repressed the SUS activity by 70% and led to shrunken seeds with 5% of wild-type seed weight, inhibiting fiber initiation and elongation (Ruan *et al.*, 2003). However, the result in cotton is not consistent with findings in Arabidopsis. Mutant Arabidopsis plants lacking individual SUS isoforms or a combination of four SUS isoforms were shown to have the same level of starch, sugar and cellulose content, lipid content, seed weight or seed composition as wild-type plants, suggesting the loss of SUS was compensated by INV isoforms (Barratt *et al.*, 2009; Bieniawska *et al.*, 2007).

There are 6 AtSUS isoforms identified in Arabidopsis and they were all shown to express weakly in seeds (Baud *et al.*, 2004). Among these sucrose synthase genes,

AtSUS1 is the only one with expression in the anther, albeit at a relatively low level (Table 1). The PSP promoter should dramatically increase the level of the *AtSUS1* mRNA at all stages and especially the MPG stage. The level of *AtSUS1* in WT plants was not greatly affected by HTS (Figure 34). The transgene integration and expression in lines PSP-AtSUS1-8, PSP-AtSUS1-13 and PSP-AtSUS1-14 was confirmed (Figures 42 and 43) and the combined expression of endogenous and transgene in those 3 lines was indeed shown to be higher than untransformed lines (Figure 44).

During T_0 screening, in spite of the low number and percentage of healthy looking siliques produced under heat stress, the weight of healthy seeds per unit plant weight for PSP-AtSUS1-8 was higher than most WT lines and LeFRK lines. This finding was confirmed again in the T_1 screening, as 50% of T_1 progeny of PSP-AtSUS1-8 possessed higher seed production than all WT lines (Figure 50). Although PSP-AtSUS1-8 overexpressing *AtSUS1* mRNA in pollen under HTS (Figure 44) was confirmed to have higher seed production than untransformed plants, the correlation between the level of *AtSUS1* mRNA and seed production is still low and needs further confirmation on a larger sample of transgenic plants.

8.3 Conclusion

The level of *AtSUS1* transcript was not greatly affected by HTS in untransformed plants. The PSP promoter was shown to increase the level of *AtSUS1* mRNA in pollen under HTS and results obtained with line PSP-AtSUS1-8 may indicate that

overexpressing the *AtSUS1* transcript in pollen could have a positive effect on seed production, this may justify further investigation.

9. PSP-AtHXK1

9.1 Results

The PSP-AtHXK1 construct is intended to overexpress hexokinase 1, a pivotal enzyme for the initial step of sugar utilization in sink tissues, in the pollen of Arabidopsis. A total of 15 T_0 transformant lines (PSP-AtHXK1), 6 untransformed lines (WT) and 2 heat tolerant transformant lines (LeFRK) were heat stressed together and examined. Four transgenic lines were selected to verify transgene integration and expression.

9.1.1 Verification of Transgenic Lines

9.1.1.a Genomic PCR

The integration of PSP-AtHXK1 into the Arabidopsis genome was verified and confirmed in all transgenic lines except PSP-AtHXK1-1 (Figure 51).

9.1.1.b Transgene-specific *AtHXK1* RT-PCR

RT-PCR was performed in order to verify *AtHXK1* transgene expression. In accordance with the presence of the PSP-AtHXK1 genomic fragment, lines PSP-AtHXK1-2, PSP-AtHXK1-3 and PSP-AtHXK1-4 exhibited transgene expression (Figure 52).

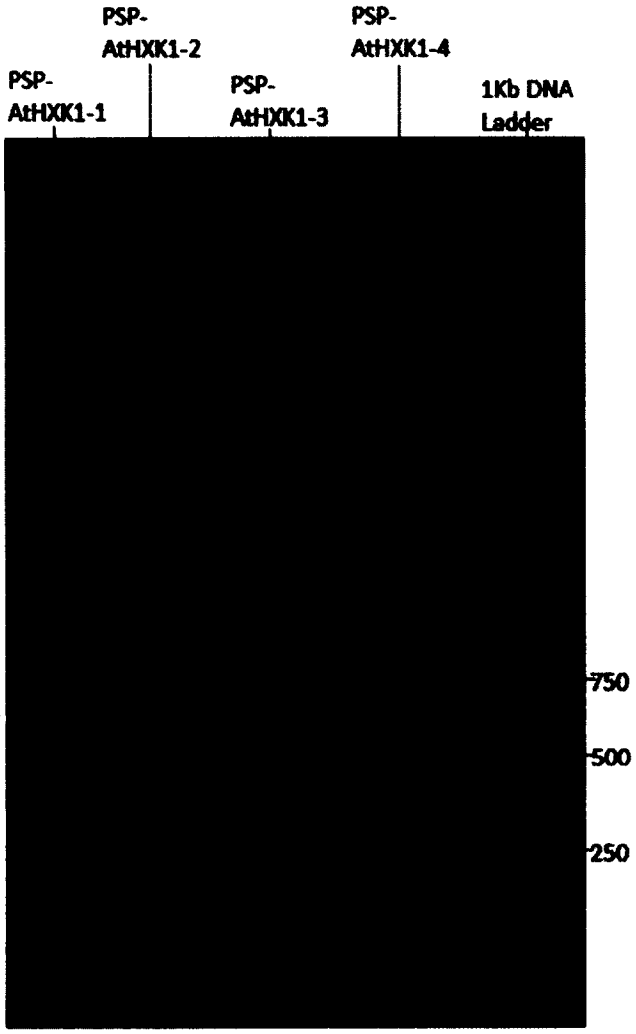

Figure 51. Agarose gel electrophoresis of genomic PCR fragment from individual PSP-AtHXK1 (PSP promoter-*Arabidopsis thaliana* Hexokinase 1, expected size: 557 bp) T_0 lines. The sizes of some standard DNA fragments are indicated in base pairs.

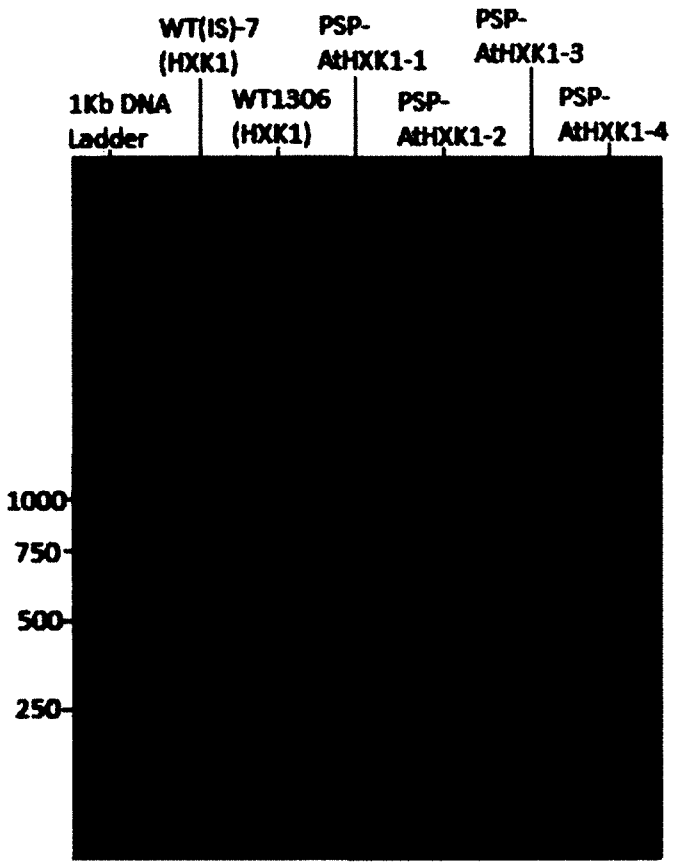

Figure 52. Agarose gel electrophoresis of transgene-specific RT-PCR fragments (Expected size: 557 bp) obtained with cDNA from heat stressed mature floral buds of individual PSP-AtHXK1 T_0 lines and WT lines. The sizes of some standard DNA fragments are indicated in base pairs.

9.1.1.c Semi-quantitative *AtHXK1* RT-PCR

The level of *AtHXK1* in WT was mostly unaffected by HTS and was lower than the combined level of endogenous and transgene *AtHXK1* expression observed in the transgenic lines (Figure 53).

9.1.2 Phenotypic Analysis of PSP-AtHXK1 Transgenic Lines (T_0 Generation)

Comparisons of the number and percentage of healthy looking siliques, as well as seed weight for PSP-AtHXK1 versus WT and LeFRK lines are shown in Figures 54, 55 and 56.

9.1.2.a Silique Number

Figure 54 shows the total number of healthy looking siliques for each individual WT and transgenic PSP-AtHXK1 and LeFRK lines after 14 days of heat treatment. All PSP-AtHXK1 T_0 lines yielded relatively low numbers of healthy looking siliques under heat stress, with the possible exception of PSP-AtHXH1-13 and PSP-AtHXK1-15.

Presenting the healthy silique data as a proportion of the total number of potential siliques yielded similar results (Figure 55).

9.1.2.b Seed Weight

When comparing the weight of healthy seeds as a function of plant weight, only one T_0 line, PSP-AtHXK1-4 (with confirmed *AtHXK1* overexpression), showed a higher value than WT lines, although it was surpassed by two LeFRK lines (Figure 56). In view

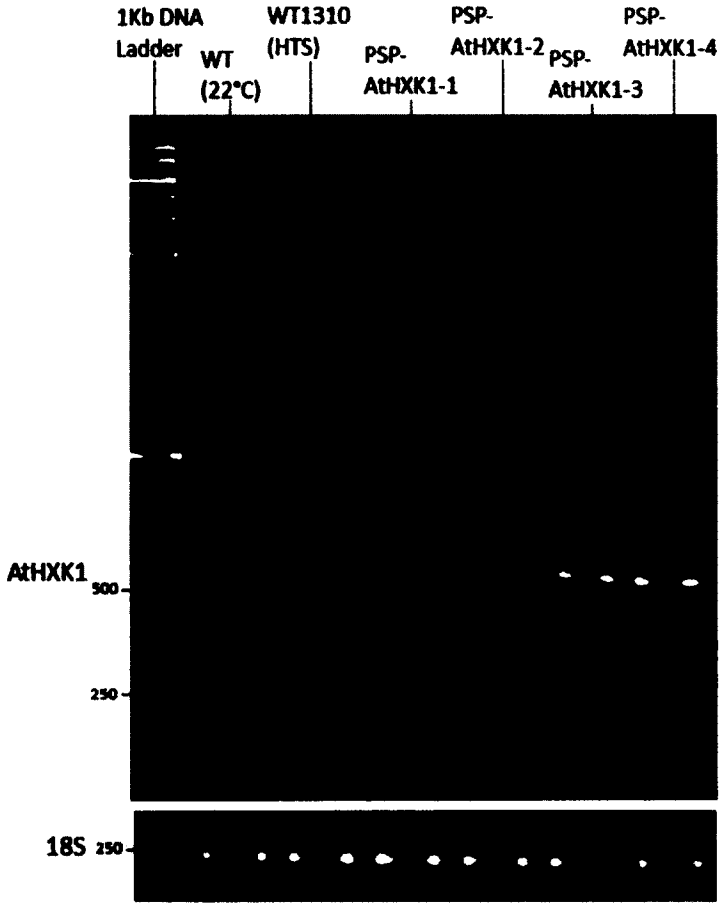

Figure 53. Agarose gel electrophoresis of semi-quantitative RT-PCR fragments (expected size: 530 bp) obtained with cDNA from mature floral buds of individual PSP-AtHXK1 (HTS) T_0 lines and untransformed (WT (22°C) and WT1310 (HTS)) lines. Ribosomal RNA (18S) was used as an internal control. The sizes of some standard DNA fragments are indicated in base pairs.

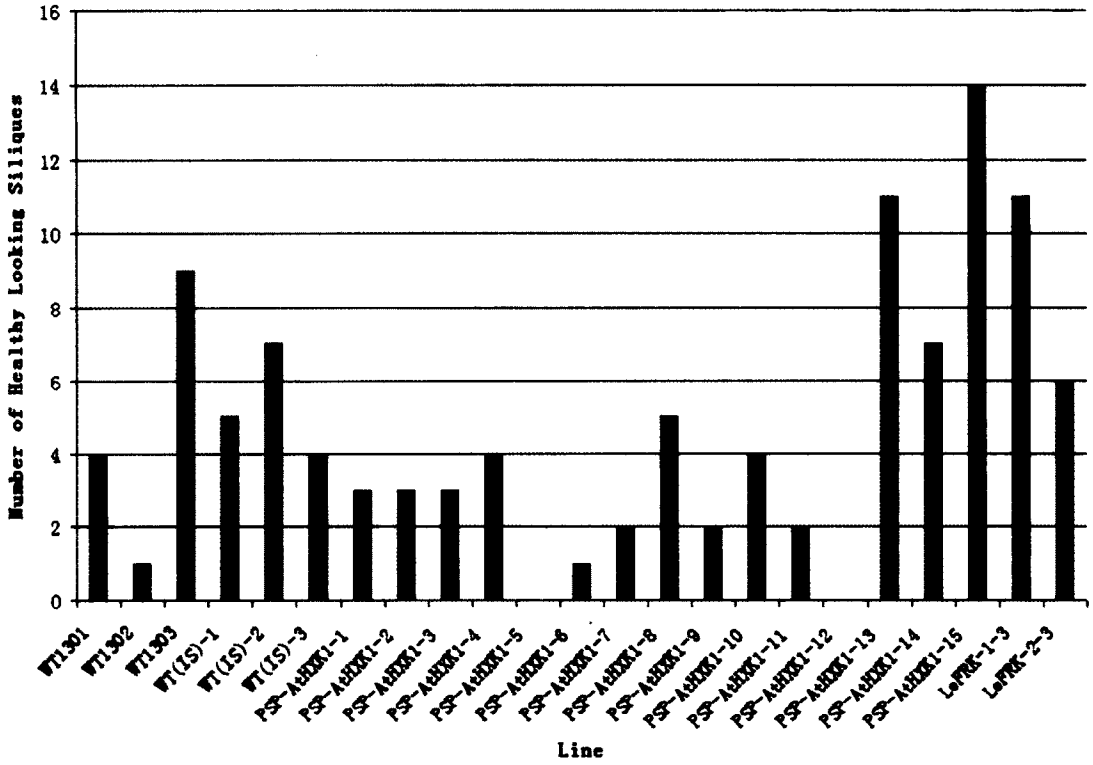

Figure 54. The number of healthy looking siliques for individual PSP-AtHXK1 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. The 15 PSP-AtHXK1, 6 LeFRK and 2 WT lines were located in the same flat.

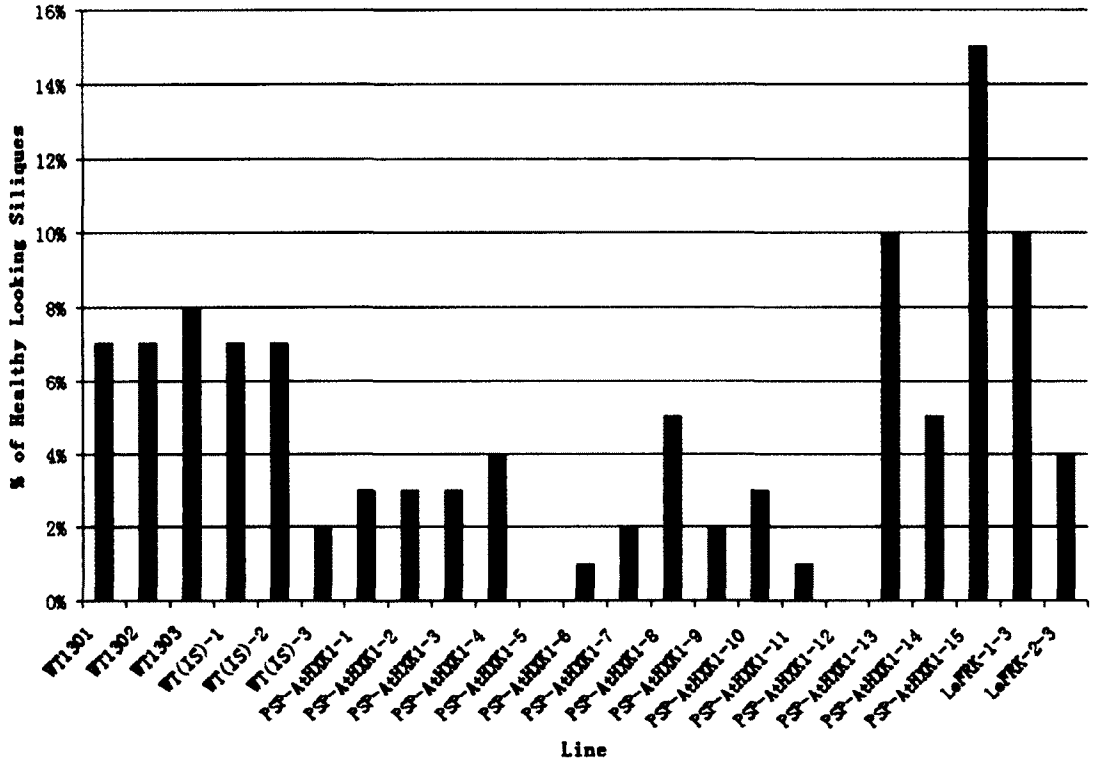

Figure 55.The percentage of healthy looking siliques for individual PSP-AtHXK1 T_0 transformants compared to untransformed plants (WT) and heat tolerant LeFRK lines after 14 days at 33°C. The 15 PSP-AtHXK1, 6 LeFRK and 2 WT lines were located in the same flat.

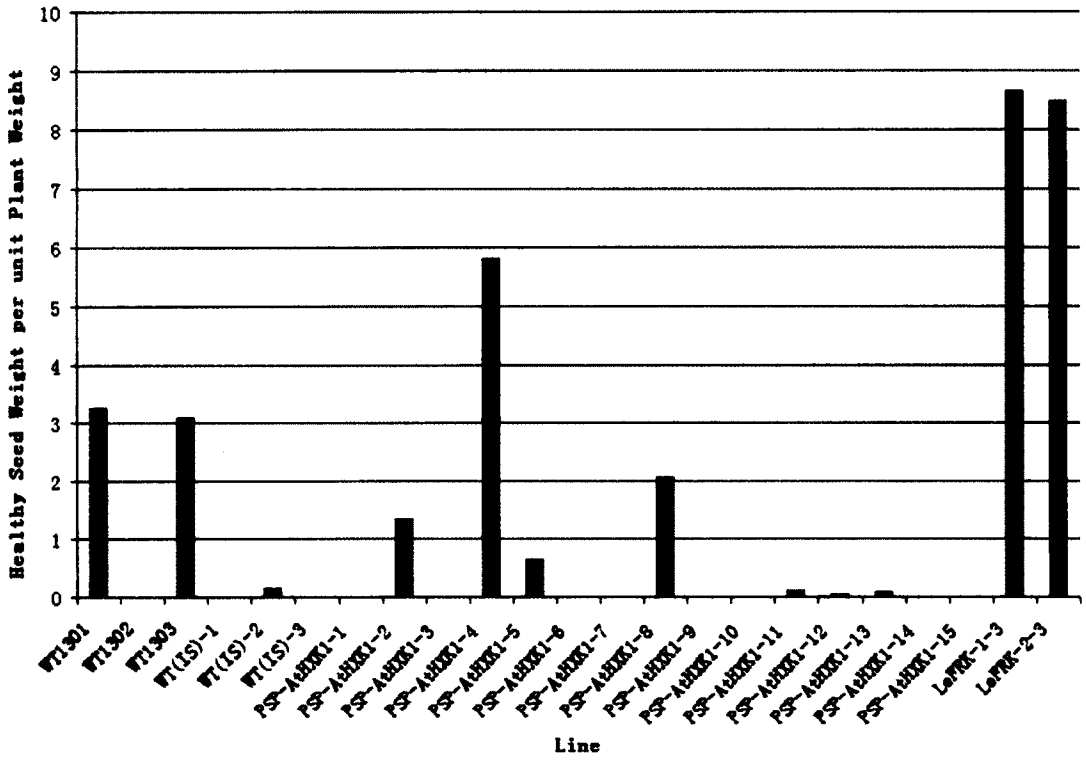

Figure 56. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) of each PSP-AtHXK1 T_0 line is compared to untransformed plants (WT) and heat tolerant LeFRK lines after 7 days at 32°C following 14 days heat treatment at 33°C. 15 PSP-AtHXK1, 6 LeFRK and 2 WT lines were located in the same flat.

of the fact that no PSP-AtHXK1 T0 line displayed a much improved performance under heat treatment when compared to the control plants, no lines were selected for further T_1 progeny analysis.

9.2 Discussion

Hexokinase is a pivotal enzyme for the initial step of sugar utilization in sink tissues. Sucrose is accumulated in developing pollen grains and will be hydrolysed together with imported sucrose by invertase and sucrose synthase at the onset of pollen germination. Before undergoing further metabolism, all liberated glucose and fructose from sucrose must be phosphorylated by hexokinase (HXK) to produce G6P or F6P (Karni & Aloni, 2002). In addition, HXK was found to control the rate of glycolysis, which not only provides substrates to fuel energy production and anabolic processes of living cells, but also is very important for adaptation to stress conditions (Xue *et al.*, 2008).

HXK isoforms in different species have been shown to respond to abiotic stresses differently. In wheat, the expression level of *TaHxK8* and *TaHxK9* mRNA increased in the leaves during drought stress, whereas *TaHxK1, TaHxK3* and *TaHxK5* showed a slight reduction (Xue *et al.*, 2008). HXK was shown to play an essential role in anther dehiscence, pollen germination and grain filling in rice (Xu *et al.*, 2008). This study showed that suppressing *OsHXK10* expression in rice using RNAi reduced the level of *OsHXK10* transcript leading to an increased number of flowers with non-dehiscent anthers, significantly decreased pollen germination capacity and to a marked increase in

empty seeds (Xu *et al.*, 2008). Overexpression of *AtHXK1* has been studied in different species, such as tomato, arabidopsis (Dai *et al.*, 1999; Jang & Sheen, 1994; Pourtau *et al.*, 2006). In Arabidopsis, overexpressing *AtHXK1* not only inhibited photosynthesis and seedling development and growth, but also accelerated leaf senescence (Jang & Sheen, 1994; Pourtau *et al.*, 2006). Similar results were also reported in tomato plants with no visible effect in roots (Dai *et al.*, 1999). More importantly, tomato plants overexpressing *AtHXK1* were also shown to result in an increased number of pollen grains with significantly higher viability under HTS (Dai *et al.*, 1999).

In Arabidopsis, 4 HXK isozymes were identified and *AtHXK1* displayed the highest level of expression in the stamen and pollen grain (Claeyssen & Rivoal, 2007) with relatively high transcript levels in the bicellular and tricellular pollen, and moderate levels at the other stages (Honys & Twell, 2004). Therefore, given its expression pattern and the results in tomato pollen described above, *AtHXK1* was selected to be overexpressed by the PSP promoter which should enhance its expression level throughout pollen development.

In this study, the integration and expression of PSP-AtHXK1 were confirmed in 3 of 4 selected transgenic lines (Figures 51 and 52) and those three lines were shown to have higher levels of *AtHXK1* than WT lines (which showed no huge difference under HTS) (Figure 53). Generally, the PSP-AtHXK1 T_0 lines did not perform better than WT lines with only two lines yielding a higher number or percentage of healthy looking siliques and a single line having a greater seed production. These results were unexpected in view

of the positive effect of overexpressing *AtHXK1* in tomato pollen (Dai *et al.*, 1999). It is possible that the high level of expression obtained with the PSP promoter is detrimental to the pollen although additional investigations will be necessary to test this hypothesis.

9.3 Conclusion

The level of *AtHXK1* in WT Arabidopsis did not appear to be greatly altered by HTS. The majority of the PSP-AtHXK1 plants performed poorly under HTS with many not producing seeds at all. This may indicate that beyond a certain level of expression AtHXK1 could have a detrimental effect on pollen development and function.

10. PSP-AtcwINV2i

10.1 Results

The PSP-AtcwINV2i construct should overexpress in pollen the Arabidopsis cell wall invertase 2. The cell wall invertase has been shown to be critical to carbohydrate metabolism in pollen. A total of 17 T_0 transformant lines (PSP-AtcwINV2i) were heat stressed and evaluated. Four transgenic lines were chosen for verification of transgene integration and expression.

10.1.1 Verification of Transgenic Lines

10.1.1.a Genomic PCR

Genomic PCR was performed to verify the integration of PSP-AtcwINV2i into transgenic Arabidopsis and the expected fusion-specific fragment was clearly observed in all 4 lines (Figure 57).

10.1.1.b Transgene-specific *AtcwINV2* RT-PCR

AtcwINV2 transgene expression was verified using construct-specific RT-PCR and the transgene-specific fragment was observed in 3 of 4 transgenic lines (Figure 58). Given that all four lines possessed the transgene, the lack of expression in line PSP-AtcwINV2i-9 could be due to a gene rearrangement beyond the genomic region amplified above or may reflect an integration event in a poorly transcribed region of the genome.

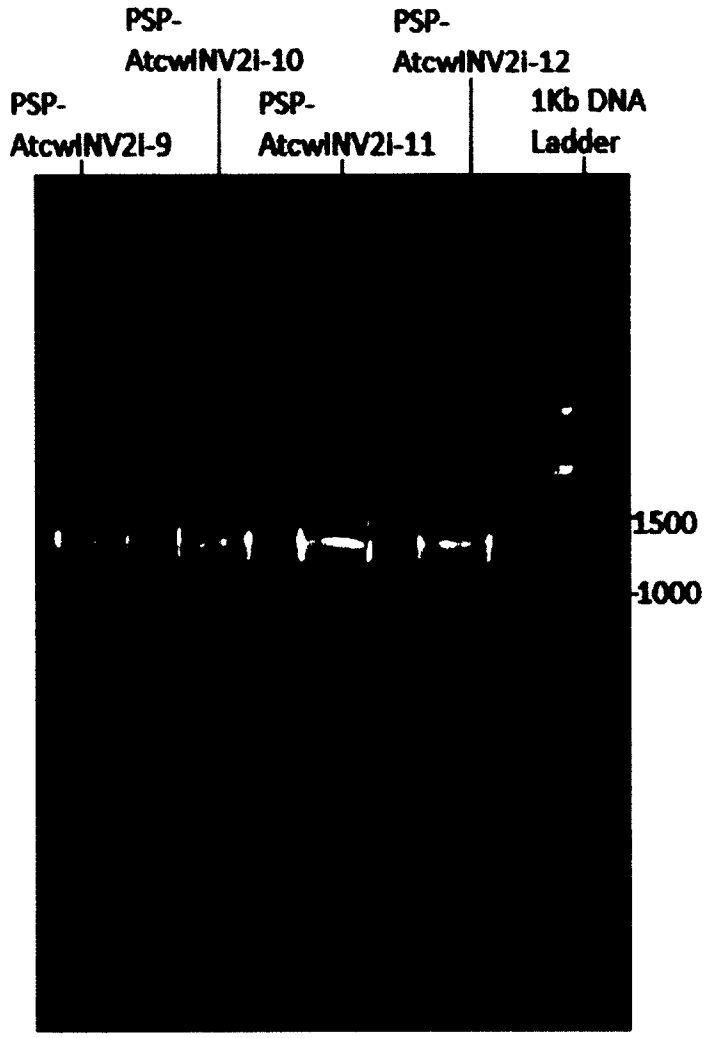

Figure 57. Agarose gel electrophoresis of genomic PCR fragment from individual PSP-AtcwINV2i (PSP promoter-*Arabidopsis thaliana* cell wall Invertase 2 + intron, expected size: 1188 bp) T_0 lines. The sizes of some standard DNA fragments are indicated in base pairs.

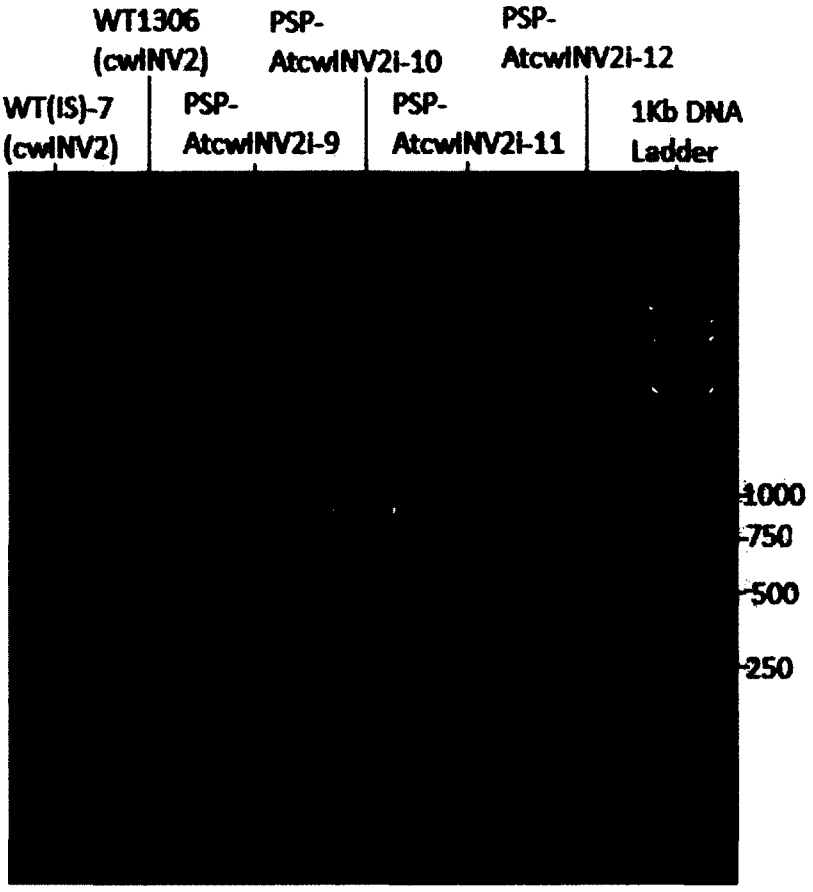

Figure 58. Agarose gel electrophoresis of transgene-specific RT-PCR fragments (Expected size: 946 bp) obtained with cDNA from heat stressed mature floral buds of individual PSP-AtcwINV2i T_0 lines and WT lines. The sizes of some standard DNA fragments are indicated in base pairs.

10.1.1.c Semi-quantitative *AtcwINV2* RT-PCR

To verify the variation in expression level of *AtcwINV2*, semi-quantitative RT-PCR (measuring simultaneously the expression of the endogenous gene and the transgene) was performed (Figure 59). Interestingly, the level of endogenous *AtcwINV2* was drastically reduced in WT under heat stress. The transgenic lines (PSP-AtcwINV2i-10, PSP-AtcwINV2i-11 and PSP-AtcwINV2i-12) appeared to express more *AtcwINV2* than WT under HTS condition, but less than WT under normal temperature.

10.1.2 Phenotypic Analysis of PSP-AtcwINV2i Transgenic Lines (T_0 Generation)

There were no control plants for the T_0 screening due to contamination of all WT and LeFRK plants during the selection process preceding the heat treatment. The number and percentage of healthy looking siliques, as well as seed weight could only be compared among the PSP-AtcwINV2i transgenic lines themselves. Results are shown in Figures 60, 61, 62.

10.1.2.a Silique Number

The total number of healthy looking siliques for each individual transgenic PSP-AtcwINV2i T_0 line following 14 days of heat stress at 32°C is shown in Figure 60. PSP-AtcwINV2i-13 possessed the highest number of healthy looking siliques of all lines examined. However, when the percentage of healthy looking siliques versus the total number of potential siliques was calculated (Figure 61), PSP-AtcwINV2i-13 had a lower value than most of tested lines and lines PSP-AtcwINV2i-1 and PSP-AtcwINV2i-16 had

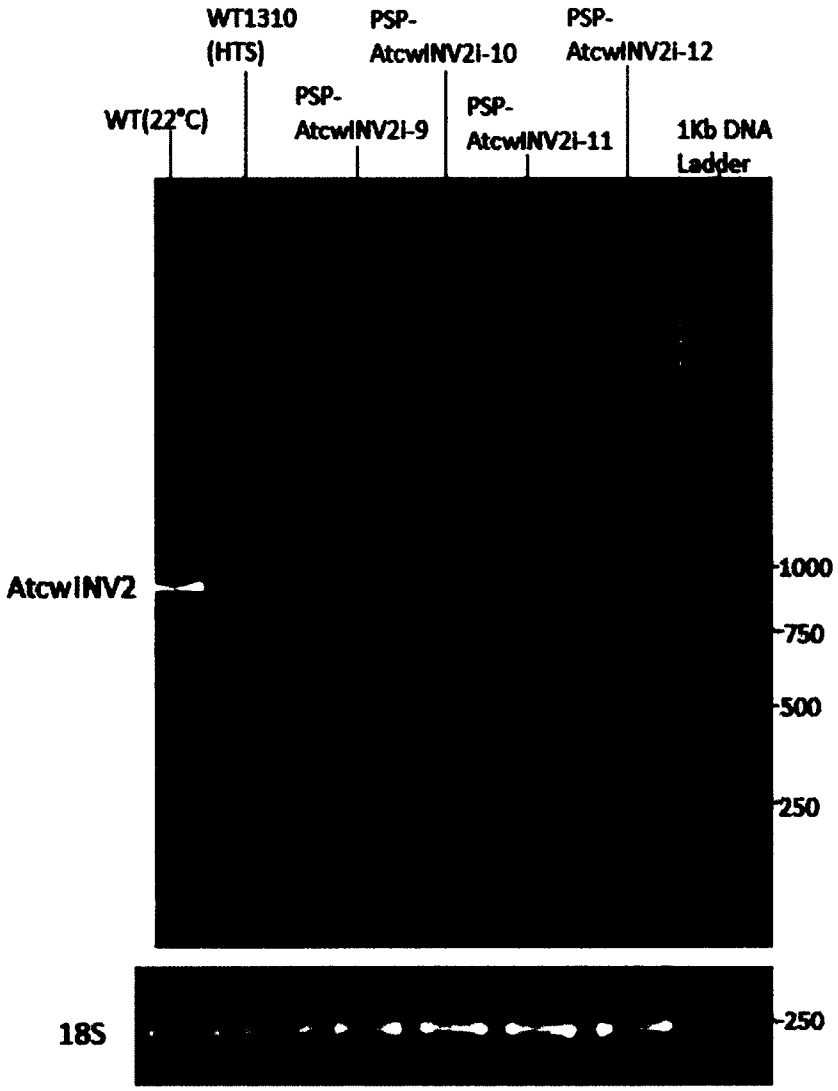

Figure 59. Agarose gel electrophoresis of semi-quantitative RT-PCR fragments (expected size: 919 bp) obtained with cDNA from mature floral buds of individual PSP-AtcwINV2i (HTS) T_0 lines and untransformed (WT (22°C) and WT1310 (HTS)) lines. Ribosomal RNA (18S) was used as an internal control. The sizes of some standard DNA fragments are indicated in base pairs.

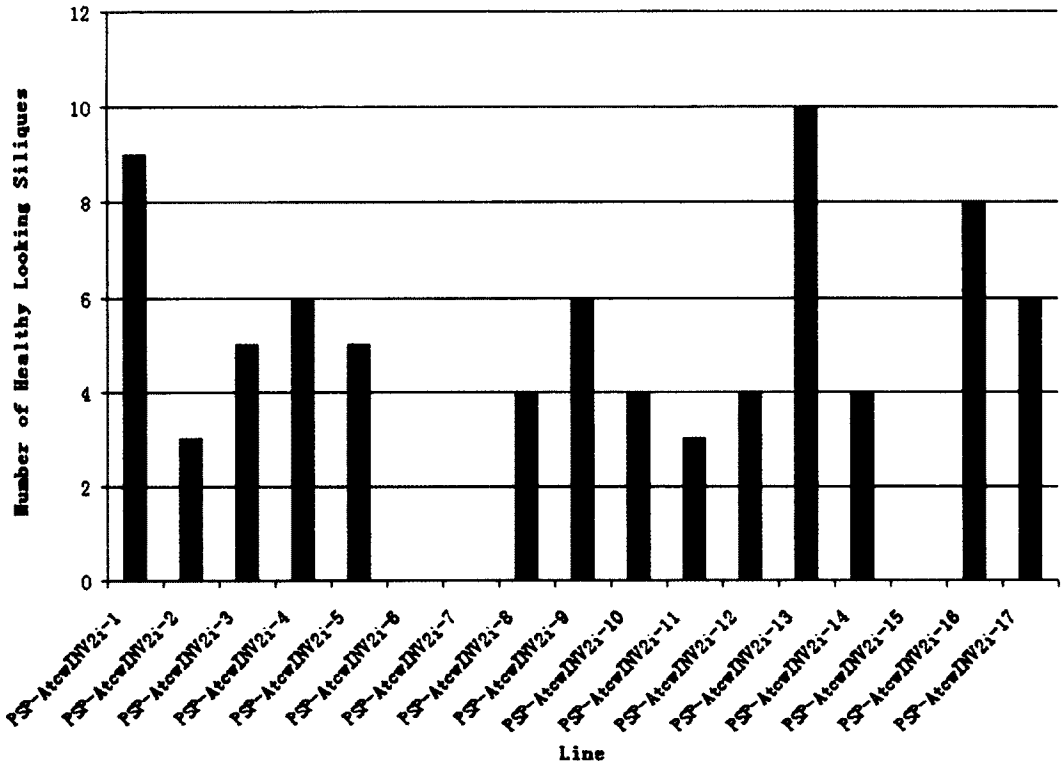

Figure 60. The number of healthy looking siliques for individual PSP-AtcwINV2i T_0 transformants after 14 days at 32°C.

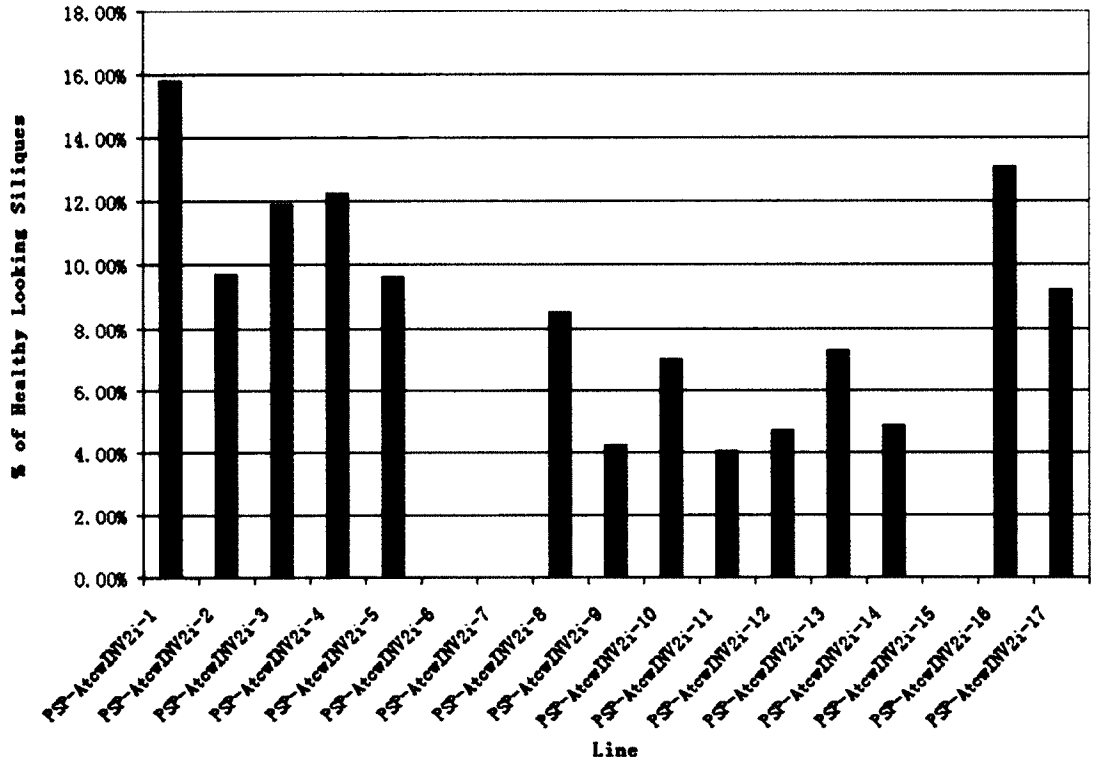

Figure 61. The percentage of healthy looking siliques for individual PSP-AtcwINV2i T_0 transformants after 14 days at 32°C.

the highest values.

10.1.2.b Seed Weight

In spite of the fact that PSP-AtcwINV2i-12 did not produce a relatively high number of healthy looking siliques, it largely surpassed all other PSP-AtcwINV2i lines in healthy seed weight per unit plant weight (Figure 62). Most PSP-AtcwINV2i T_0 lines did not produce any seeds and this was also true of other lines present in the same growth cabinet (results not shown). Accordingly, the results obtained with PSP-AtcwINV2i may not truly reflect transgene overexpression in the pollen.

10.2 Discussion

There are three INV isoforms in plants: vacuolar invertase (vINV), cytosolic invertase (cINV) and cell wall invertase (cwINV). The activity of cwINV was shown to determine if apoplastic sucrose or hexoses is transferred into the cell (Sherson *et al.*, 2003). The transported sucrose must be cleaved into glucose and fructose by a cwINV before undergoing further metabolism in pollen grains. A study showed that suppressing a tapetum- and pollen-specific *cwINV* gene in tobacco reduced the starch level in the developing pollen grain resulting in pollen sterility (Goetz *et al.*, 2001).

The effect of abiotic stresses on INV during reproduction has been well investigated. For example, the activity of cwINV was shown to be inhibited in maize ovaries under drought stress (Zinselmeier *et al.*, 1995). Similarly, wheat anther-specific cwINV and

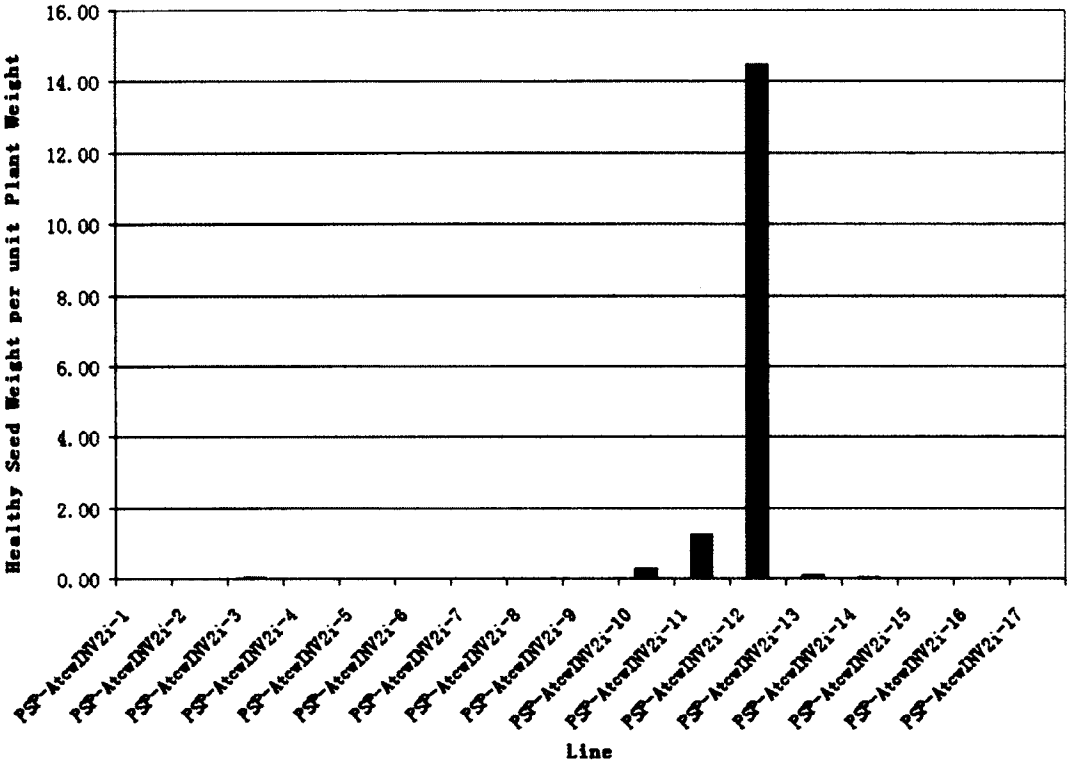

Figure 62. The healthy seed weight per unit plant weight (seed weight/plant weight x 1000) of each PSP-AtcwINV2i T_0 transformant after 14 days at 32°C.

vINV activity was reduced under drought stress leading to pollen sterility and low seed production (Koonjul *et al.*, 2005). Several studies demonstrated that cold stress reduces the expression level of *cwINV* and *vINV* mRNA in rice, causing male sterility and grain abortion (Oliver *et al.*, 2007). For example, a study in rice showed that cold stress inhibited the transcription of the tapetum-specific invertase gene *OsINV4,* hindering the sugar supply to the tapetum and pollen grains and leading to reduced grain set (Oliver *et al.*, 2005). Heat stress was also shown to repress INV activity, disturbing carbohydrate metabolism in the developing pollen grains and leading to male sterility and fruit abortion in pepper and tomato (Aloni *et al.*, 2001; Pressman *et al.*, 2002). Another study pointed out that the anther-specific cwINV in sorghum didn't function properly under heat stress, inhibiting sucrose hydrolysis and causing starch deficiency in developing pollen grains (Jain *et al.*, 2007). Ji *et al.* (2010) proposed that maintaining the sucrose and hexose supply is critical to male fertility and seed production under all abiotic stresses. Lastly, the comparison between heat tolerant and heat sensitive tomato lines under HTS revealed that the heat tolerant lines had a higher level of transcript of a cwINV gene, *Lin7,* leading to higher sucrose level in young fruit (Li *et al.*, 2012). These data suggested that higher activity of cwINV could enhance the heat tolerance of plants.

To my knowledge, overexpression of AtcwINV2 during pollen development under HTS has never been studied. However, overexpressing invertases in other plant tissues has been attempted. For example, a study showed that overexpression of cwINV under a root-specific promoter in Arabidopsis plants enhanced the apoplastic cwINV activity,

leading to more developed secondary roots, higher plant biomass and early flowering (Schweinichen & Büttner, 2005). Overexpressing extracellular INV under the SAG12 promoter increased the INV activity in the leaves prior to yellowing, thus delaying leaf senescence (Balibrea-Lara *et al.*, 2004). Another study demonstrated that elevating activity of cwINV by inhibiting INVINH1 (an invertase inhibitor) led to delayed leaf senescence and enhanced seed weight and fruit hexose level (Jin *et al.*, 2009).

In Arabidopsis, 6 *cwINV* genes have been identified and *AtcwINV2* is the only anther-specific cwINV showing high expression in the tricellular and mature pollen (Honys & Twell, 2004; Tymowska-Lalanne & Kreis, 1998). Based on the literature, abiotic stresses can reduce cwINV expression, it is therefore expected that the PSP promoter will serve to maintain *AtcwINV2* expression during the late stages of pollen development.

In this study, the level of the pollen-specific *AtcwINV2* transcript in untransformed Arabidopsis plants was shown to be drastically reduced under HTS (Figure 59). These results are consistent with other studies (Aloni *et al.*, 2001; Jain *et al.*, 2007; Pressman *et al.*, 2002). Transgenic lines PSP-AtcwINV2i-10, PSP-AtcwINV2i-11 and PSP-AtcwINV2i-12 showed higher expression of *AtcwINV2* transcript than untransformed lines under HTS, but not quite to the level observed in WT under normal growth conditions (Figure 59). Although silique production did not appear to differ greatly from that obtained for transgenic lines containing the other candidate gene

constructs, the seed yield for PSP-AtcwINV2i and other lines present in the same cabinet was generally very low due to an unknown reason.

10.3 Conclusion

The level of *AtcwINV2* in untransformed Arabidopsis was dramatically reduced under heat stress. Overexpression of this gene with the PSP promoter appeared to recover much of the transcript's level. However, in the absence of the WT and LeFRK control plants and given the unexplained poor performance of other plants sharing the PSP-AtcwINV2i cabinet, the results for the overexpression of *AtcwINV2* in pollen are inconclusive and will require additional investigations.

11. General Discussion

The purpose of this study was to investigate the effect of increasing or maintaining the level of expression of key carbohydrate metabolism genes on pollen heat tolerance. Candidate genes were chosen based on (1) their expression pattern in developing pollen, (2) the importance of their role in pollen carbohydrate metabolism and (3) the available information regarding their behaviour during heat stress. In most cases, this was the first time such genes had ever been overexpressed in pollen. More than 140 T_0 and 120 T_1 plants were analyzed individually following a heat treatment. Generally, no genetic construct conveyed an evident increase in heat tolerance to the transgenic plants. A number of possibilities that might explain these results and some future research directions are discussed below.

Firstly, the heat treatment conditions might not have been optimal. Transgenic Arabidopsis plants overexpressing *LeFRK1* (*L. esculentum fructokinase 1*) under the control of the relative weak LeFRK4 pollen-specific promoter were recently shown to produce siliques at 30°C whereas they were absent in untransformed plants (Granot, personal communication). Although much effort was devoted to the optimization of the heat treatment conditions, these results could not be faithfully reproduced with the LeFRK plants. Furthermore, the LeFRK seeds provided by Dr. D. Granot were homozygous which should have reduced plant to plant variation during heat treatment experiments. However, the performance of the LeFRK transgenic lines was found to vary considerably from flat to flat and within the same flat as measured by seed production.

This may indicate that certain aspects of the HTS treatment, such as temperature, humidity or plant developmental stage, could be further optimized.

Secondly, silique and seed production may not have been the best assays. The impact of HTS on the reproductive process has often been demonstrated using seed production (e.g. Firon *et al.*, 2006; Zinn *et al.*, 2010) and this approach was certainly appropriate in the experiments with the LeFRK plants described above. However, this assay may be more suitable when the transgene has a large impact on pollen function and viability under HTS. Pollen is usually produced in vast excess with respect to the number of ovules and this may limit the ability to detect more subtle differences brought about by the transgenes. Attempts were made to evaluate the effect of the candidate transgenes directly on pollen germination but it is difficult to obtain consistently high *in vitro* pollen germination with Arabidopsis and more work will be required to optimize this assay. Performing these assays on transgenic plants in which transgene expression has been previously quantified would also be useful. Quantitative PCR (qPCR) experiments were attempted to measure the quantity of transgene transcript but in most cases the expression level was too low to obtain consistent data. This is one of the challenges of working with a limited supply of biological material such as pollen. Nonetheless, further optimization of the qPCR assay may be warranted. Selecting homozygous plants may also help increase transgene expression levels and reduce plant to plant variation.

Thirdly, the timing and level of expression driven by the PSP promoter may not have been optimal for all candidate genes. Overexpression is a very common approach used to

study the role of a gene of interest in plant development. However, in this case it is possible that overexpressing a candidate gene may have had an effect on carbohydrate metabolism that was detrimental to pollen development and this may explain the poor performance of some transgenic lines. Pollen viability and germination tests following the heat treatment might address this concern. The PSP promoter might also overexpress a gene at a time when it is no longer needed (e.g. *AtAPS1* and *AtSUS1*). Alternatively, the PSP promoter might not have made a sufficient difference to the endogenous gene's expression level. For example, it is well established that the early stages of pollen development are the most susceptible to HTS (Sakata *et al.*, 2010) and these are the stages where the PSP promoter is the weakest (Table 1). Accordingly, the PSP promoter could have had a limited impact on the level of expression of *AtAPS1* or *AtHXK1* at the UNM stage. This may explain why no evident improvement was observed with the PSP-AtHXK1 construct while *AtHXK1* overexpression in tomato pollen improved heat tolerance (Dai *et al.*, 1999).

The semi-quantitative RT-PCR data showing the differences in expression level among untransformed and transgenic plants did not always match the microarray expression data predictions (Table 1). Generally, the combined level of the endogenous gene and transgene appeared lower than expected given the strength of the PSP promoter. It is possible that a longer HTS treatment had a negative effect on the activity of the PSP promoter and this could be tested using the PSP-GFP fusion or by monitoring the *PSP* transcript itself. Alternatively, transgene expression variation might have been caused by

different copy numbers, post-transcriptional gene silencing or epigenetic position effects (Butaye *et al.*, 2005).

Fourthly, some of the genes selected might not be greatly affected by HTS. Consistent with earlier findings on sugar transporters and cell wall invertases (Aloni *et al.*, 2001; Jain *et al.*, 2007; Parish *et al.*, 2012; Pressman *et al.*, 2002), the expression level of *AtSTP6* and *AtcwINV2* was shown to be greatly reduced in WT plants following the HTS treatment. However, little impact was observed on the expression level of *AtAPS1, AtPFK3, AtSUS1* and *AtHXK1*. It is therefore possible that the latter 4 genes are not limiting under HTS and further increasing their expression level has a minimal effect on their contribution to pollen carbohydrate metabolism. However, semi-quantitative PCR was only performed on mature buds and it is also possible that the activity of the genes that express primarily early in pollen development (e.g. *AtAPS1* and *AtSUS1*) might have been affected by HTS. A thorough quantitative developmental analysis of the expression level of all the genes used in this study under HTS should address this question.

Fifthly, it is possible that overexpressing a single gene in pollen is insufficient to have an impact on the pollen's overall carbohydrate metabolism. For example, overexpressing *AtHXK1* should lead to more glucose being converted to G6P which could then be used as a starting material for starch synthesis. However, the low level of *AtcwINV2* under HTS may greatly repress glucose production and this in turn may become limiting for starch production in spite of high AtHXK1 activity. A similar

limiting scenario could be envisaged if sugar transport was reduced due to the HTS effect on *AtSTP6* expression. Therefore, simultaneously overexpressing more than one carbohydrate metabolism gene in pollen may have a better chance to improve plant performance under HTS.

Lastly, in line with much of the published data reporting the effect of abiotic stress on carbohydrate metabolism, molecular analyses in this study were only performed at the mRNA level and it is possible that the quantity and activity of the candidate proteins are regulated under HTS in a manner which is not influenced by increased gene expression.

12. Conclusion

In conclusion, six key carbohydrate metabolism genes were overexpressed in pollen to investigate whether they could bring about an improvement in seed production under high temperature stress. For most of the candidate genes, this study not only confirmed earlier findings or provided new insight into the role of these genes in anther/pollen development and the effect of heat stress on their expression level, but also contributed novel information on the effect of overexpressing certain carbohydrate metabolism genes on pollen heat tolerance. The effect of overexpressing candidate genes AtAPS1, AtPFK3, AtSTP6, AtcwINV2 and AtSUS1 in pollen grains under heat stress had never been studied. Moreover, ADP-glucose pyrophosphorylase small subunit 1 and phosphofructokinase 3 had never been overexpressed in any species. This study also set the stage for future experiments. For example, based on the results obtained, increasing sucrose synthase expression in Arabidopsis pollen may deserve further attention. Similarly, given the demonstrated involvement of cwINV in the stress response of male reproductive tissues and the fact that *AtcwINV2* pollen expression was mostly restored by the PSP promoter, it may be worthwhile repeating the PSP-AtcwINV2i experiment that failed. Lastly, overexpressing more than one candidate gene simultaneously, for example by crossing plants with good *AtHXK1* and *AtcwINV2* transgene expression may also be a venue worth pursuing.

13. References

Aloni, B., Peet, M., Pharr, M., Karni, L. (2001) The effect of high temperature and high atmospheric CO_2 on carbohydrate changes in bell pepper (*Capsicum annuum*) pollen in relation to its germination. *Physiol. Plantar.* 112: 505-512

Bahaji, A., Li, J., Ovecka, M., Ezquer, I., Munoz, F.J., Baroja-Fernandez, E., Romero, J.M., Almagro, G., Montero, M., Hidalgo, M., Sesma, M.T., Pozueta-Romero, J. (2011) *Arabidopsis thaliana* mutants lacking ADP-glucose pyrophosphorylase accumulate starch and wild-type ADP-glucose content: further evidence for the occurrence of important sources, other than ADP-glucose pyrophosphorylase, of ADP-glucose linked to leaf starch biosynthesis. *Plant Cell Physiol.* 52: 1162-1176

Balasubramanian, S., Sureshkumar, S., Lempe, J., Weigel, D. (2006) Potent induction of *Arabidopsis thaliana* flowering by elevated growth temperature. *PloS Genet.* 2: e106

Balibrea-Lara, M.E., Garcia, M.C., Gonzalez, F.T., Ehness, R., Lee, T.K., Proels, R., Tanner, W., Roitsch, T. (2004) Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. *Plant Cell.* 16: 1276-1287

Barratt, D.H.P., Derbyshire, P., Findlay, K., Pike, M., Wellner, N., Lunn, J., Feil, R., Simpson, C., Maule, A.J., Smith, A.M. (2009) Normal growth of Arabidopsis requires cytosolic invertase but not sucrose synthase. *PNAS.* 106: 13124-13129

Baud, S., Vaultier, M., Rochat, C. (2004) Structure and expression profile of the sucrose synthase multigene family in Arabidopsis. *J. Exp. Bot.* 55: 397-409

Bennett, M., Leitch, I., Price, H., Johnston, J. (2003). Comparisons with *Caenorhabditis* (~100 Mb) and Drosophila (~175 Mb) using flow cytometry show genome size in Arabidopsis to be ~157 Mb and thus ~25% larger than the Arabidopsis genome initiative estimate of ~125 Mb. *Ann. Bot.* 91: 547-557

Berger, J.D., Ali, M., Basu, P.S., Chaudhary, B.D., Chaturvedi, S.K., Deshmukh, P.S., Dharmaraj, P.S., Dwivedi, S.K., Gangadhar, G.C., Gaur, P.M., Kumar, J., Pannu, R.K., Siddique, K.H.M., Singh, D.N., Singh, D.P., Singh, S.J., Turner, N.C., Yadava, H.S., Yadav, S.S. (2006) Genotype by environment studies demonstrate the critical role of phenology in adaptation of chickpea (*Cicer arietinum* L.) to high and low yielding environments of India. *Field Crop Res.* 98: 230-244

Bieniawska, Z., Barratt, D.H.P., Garlick, A.P., Thole, V., Kruger, N.J., Martin, C., Zrenner, R., Smith, A.M. (2007) Analysis of the sucrose synthase gene family in Arabidopsis. *Plant J.* 49: 810–828

Butaye, K.M.J., Cammue, B.P.A., Delaure, S.L., Bolle, M.F.C.D. (2005) Approaches to minimize variation of transgene expression in plants. *Mol. Breed.* 16: 79-91

Büttner, M. (2007) The monosaccharide transporter(-like) gene family in Arabidopsis. *FEBS Letters.* 581: 2318-2324

Cavell, A., Lydiate, D., Parkin, I., Dean, C., Trick, M. (1998) Collinearity between a 30-centimorgan segment of *Arabidopsis thaliana* chromosome 4 and duplicated regions within the *Brassica napus* genome. *Genome.* 41: 62-69

Chengappa, S., Guilleroux, M., Phillips, W., Shields, R. (1999) Transgenic tomato plants with decreased sucrose synthase are unaltered in starch and sugar accumulation in the fruit. *Plant Mol. Biol.* 40: 213-221

Churchill, T.A., Cheetham, K.M., Fuller, B.J. (1994) Glycolysis and energy metabolism in rat liver during warm and cold ischemia: evidence of an activation of the regulatory enzyme phosphofructokinase. *Cryobiol.* 31: 441-452

Claeyssen, E., Rivoal, J. (2007) Isozymes of plant hexokinase: Occurrence, properties and functions. *Phytochem.* 68: 709-731

Clough, S., Bent, A. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16: 735-743

Coleman, H.D., Ellis, D.D., Gilbert, M., Mansfield, S.D. (2006) Upregulation of sucrose synthase and UDP-glucose pyrophosphorylase impacts plant growth and metabolism. *Plant Biotechnol. J.* 4: 87-101

Coleman, H.D., Yan, J., Mansfield, S.D. (2009) Sucrose synthase affects carbon partitioning to increase cellulose production and altered cell wall ultrastructure. *PNAS.* 106: 13118-13123

Craufurd, P.Q., Flower, D.J., Peacock, J. M. (1993). Effect of heat and drought stress on sorghum (*Sorghum bicolor*). 1. Panicle development and leaf appearance. *Exp. Agric.* 29: 61-76

Crevillen, P., Ventriglia, T., Pinto, F., Orea, A., Merida, A., Romero, J. (2005) Differential pattern of expression and sugar regulation of *Arabidopsis thaliana* ADP-glucose pyrophosphorylase-encoding genes. *The J. Biol. Chem.* 280: 8143-8149

D'Aoust, M., Yelle, S., Nguyen-Quoc, B. (1999) Antisense inhibition of tomato fruit sucrose synthase decreases fruit setting and the sucrose unloading capacity of young fruit. *Plant Cell.* 11: 2407-2418

Dai, N., Schaffer, A., Petreikov, M., Shahak, Y., Giller, Y., Ratner, K., Levine, A., Granot, D, (1999) Overexpression of Arabidopsis hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. *Plant Cell.* 11: 1253-1266

Damari-Weissler, H., Ginzburg, A., Gidoni, D., Mett, A., Krassovskaya, I., Weber, A.P., Belausov, E., Granot, D. (2007) Spinach SoHXK1 is a mitochondria-associated hexokinase. *Planta.* 226: 1053-1058

Dejardin, A., Sokolov, L., Kleczkowski, L. (1999) Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in *Arabidopsis*. *Biochem. J.* 344: 503-50

Denyer, K., Foster, J., Smith, A.M. (1995) The contributions of adenosine 5'-diphosphoglucose pyrophosphorylase and starch-branching enzyme to the control of starch synthesis in developing pea embryos. *Planta.* 197: 57-62

Dorion, S., Lalonde, S., Saini, H.S. (1996) Induction of male sterility in wheat by meiotic-stage water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. *Plant Physiol.* 111: 137-145

Firon, N., Shaked, R., Peet, M., Pharr, D., Zamski, E., Rosenfeld, K., Althan, L., Pressman, E. (2006) Pollen grains of heat tolerant tomato cultivars retain higher carbohydrate concentration under heat stress conditions. *Scientia Hort.* 109: 212-217

Fischer, G., Shah, M., Tubiello, F.N., Velhuizen, H.V. (2005) Socioeconomic and climate change impacts on agriculture: an integrated assessment, 1990–2080. *Phil. Trans. R. Soc. B.* 360: 2067-2083

Franchi, G.G., Bellani, L., Nepi, M., Pacini, E. (1996) Types of carbohydrate reserves in pollen: Localization, systematic distribution and ecophysiological significance. *Flora.* 191: 143-159

Goetz, M., Godt, M.E., Guivarc'h, A., Kahmann, U., Chriqui, D., Roitsch, T. (2001) Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. *PNAS.* 98: 6522-6527

Granot, D. (2008) Sugar metabolism and signaling in plants. *In* A Transgenic Approach in Plant Biochemistry and Physiology. Research Signpost, Kerala, India. pp 51-72

Holsters, M., Waele, D., Depicker, A., Messens, E., Montagu, M., Schell, J. (1978) Transfection and transformation of *Agrobacterium tumefaciens. Mol. Gen. Genet.* 163: 181-187

Honys, D., Twell, D. (2004) Transcriptome analysis of haploid male gametophyte development in *Arabidopsis. Genome Biol.* 5: R85

Houghton, J.T., Ding, Y., Griggs, D.J., Noguer, M., Linden, P.J., Dai, X., Maskell, K., Johnson, C.A. (2001) Climate change 2001: the scientific basis. Cambridge University Press, Cambridge, UK.

Hua, S., Yu, H., Zhang, Y., Lin, B., Ding, H., Zhang, D., Ren, Y. (2012) High temperature induced fertility transition and anther carbohydrate metabolism modification in a canola recessive genic male sterile line. *African J. Agr. Res.* 7: 2475-2489

Jain, M., Prasad, P.V.V., Boote, K.J., Hartwell, A.L., Chourey, P.S. (2007) Effects of season-long high temperature growth conditions on sugar-to-starch metabolism in developing microspores of grain sorghum (*Sorghum bicolor* L. Moench). *Planta.* 227: 67-79

Jang, J.C., Sheen, J. (1994) Sugar sensing in higher plants. *Plant Cell.* 6: 1665-1679

Ji, X., Shiran, B., Wan, J., Lewis, D.C., Jenkins, C.L.D., Condon, A.G, Richards, R.A., Dolferus, R. (2010) Importance of pre-anthesis anther sink strength for maintenance of grain number during reproductive stage water stress in wheat. *Plant Cell Environ.* 33: 926-942

Jin, Y., Ni, D.A., Ruan, Y.L. (2009) Posttranslational elevation of cell wall Invertase activity by silencing its inhibitor in tomato delays leaf senescence and increases seed weight and fruit hexose level. *Plant Cell.* 21: 2072-2089

Karni, L., Aloni, B. (2002) Fructokinase and hexokinase from pollen grains of Bell pepper (*Capsicum annuum L.*): Possible role in pollen germination under conditions of high temperature and CO_2 enrichment. *Ann. Bot.* 90: 607-612

Khalil, M.F.M., Kajiura, H., Fujiyama, K., Koike, K., Ishida, N., Tanaka, N. (2010) The impact of the overexpression of human UDP-galactose transporter gene *hUGT*1 in tobacco plants. *J. Biosci. Bioengineer.* 109: 159-169

Kim, S.Y., Hong, C.B., Lee, I. (2001) Heat shock stress causes stage-specific male sterility in *Arabidopsis thaliana*. *J. Plant Res.* 114: 301-307

Kleines, M., Elster, R.C., Rodrigo, M.J., Blervacq, A.S., Salamini, F., Bartels, D. (1999) Isolation and expression analysis of two stress-responsive sucrose-synthase genes from the resurrection plant *Craterostigma plantagineum* (Hochst.) *Planta* 209: 13-24

Koonjul, P.K., Minhas, J.S., Nunes, C., Sheoran, I.S., Saini, H.S. (2005) Selective transcriptional down-regulation of anther invertases precedes the failure of pollen development in water-stressed wheat. *J. Exp. Bot.* 56: 179-190

Kurek, I., Chang, T.K., Bertain, S.M., Madrigal, A., Liu, L., Lassner, M.W., Zhu, G. (2007) Enhanced thermostability of *Arabidopsis* rubisco activase improves photosynthesis and growth rates under moderate heat stress. *Plant Cell.* 19: 3230-3241

Lardon, A., Triboi-Blondel, A.M. (1994) Freezing injury to ovules, pollen and seed in winter rape. *J. Exp. Bot.* 45: 1177-1181

Larkindale, J., Knight, M.R. (2002) Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. *Plant Physiol.* 128: 682-695

Lee, J.Y., Lee, D.H. (2003) Use of serial analysis of gene expression technology to reveal changes in gene expression in Arabidopsis pollen undergoing cold stress. *Plant Physiol.* 132: 517–529

Li, Z., Palmer, W.M., Martin, A.P., Wang, R., Rainsford, F., Jin, Y., Patrick, J.W., Yang, Y., Ruan, Y.L. (2012) High invertase activity in tomato reproductive organs correlates with enhanced sucrose import into, and heat tolerance of, young fruit. *J. Exp. Bot.* 63: 1155-1166

Meinke, D.W., Sussex, I.M. (1979) Embryo-lethal mutants of *Arabidopsis thaliana*: A model system for genetic analysis of plant embryo development. *Dev. Biol.* 72: 50-61

Mittler, R. (2006) Abiotic stress, the field environment and stress combination. *Trends in Plant Sci.* 11: 15-19

Mustroph, A., Sonnewald, U., Biemelt, S. (2007) Characterisation of the ATP-dependent phosphofructokinase gene family from *Arabidopsis thaliana*. *FEBS Letters*. 581: 2401-2410

Neuhaus, H.E., Stitt, M. (1990) Control analysis of photosynthate partitioning: Impact of reduced activity of ADP-glucose pyrophosphorylase or plastid phosphoglucomutase on the fluxes to starch and sucrose in *Arabidopsis thaliana* (L.) Heynh. *Planta*. 182: 445–454

Oliver, S.N., Dennis, E.S., Dolferus, R. (2007) ABA regulates apoplastic sugar transport and is a potential signal for cold-induced pollen sterility in rice. *Plant Cell Physiol*. 48: 1319-1330

Oliver, S.N., Van Dongen, J.T., Alfred, S.C., Mamun, E.A., Zhao, X., Saini, H.S., Fernandes, S.F., Blanchard, C.L., Sutton, B.G, Geigenberger, P., Dennis, E.S., Dolferus, R. (2005) Cold-induced repression of the rice anther-specific cell wall invertase gene *OSINV4* is correlated with sucrose accumulation and pollen sterility. *Plant Cell Environ*. 28: 1534-1551

Olsson, T., Thelander, M., Ronne, H. (2003) A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylating enzyme in the moss *Physcomitrella patens*. *J. Biol. Chem*. 278: 44439-44447

Owen, H., Makaroff, C. (1995) Ultrastructure of microsporogenesis and microgametogenesis in *Arabidopsis thaliana* (L.) Heynh. ecotype Wassilewskija (Brassicaceae). *Protoplasma*. 185: 7-21

Panchuk, I.I., Volkov, R.A., Schoffl, F., (2002) Heat stress- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in Arabidopsis. *Plant Physiol*. 129: 838-853

Parish, R.W., Phan, H.A., Iacuone, S., Li, S.F. (2012) Tapetal development and abiotic stress: a centre of vulnerability. *Funct. Plant Biol*. 39: 553-559

Plaxton, W.C. (1996) The organization and regulation of plant glycolysis. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 47: 185-214

Pourtau, N., Jennings, R., Pelzer, E., Pallas, J., Wingler, A. (2006) Effect of sugar-induced senescence on gene expression and implications for the regulation of senescence in Arabidopsis. *Planta*. 224: 556-558

Prasad, P.V.V., Pisipati, S.R., Momcilovic, I., Ristic, Z. (2011) Independent and combined effects of high temperature and drought stress during grain filling on plant yield and chloroplast EF-Tu expression in spring wheat. *J. Agron. Crop Sci.* 197: 430-441

Pressman, E., Harel, D., Zamski, E., Shaked, R., Althan, L., Rosenfeld, K., Firon, N. (2006) The effect of high temperatures on the expression and activity of sucrose-cleaving enzymes during tomato (*Lycopersicon esculentum*) anther development. *J. Horticult. Sci. Biotechnol.* 81: 341-348

Pressman, E., Peet, M.M., Pharr, D.M. (2002) The effect of heat stress on tomato pollen characteristics is associated with changes in carbohydrate concentration in the developing anthers. *Ann. Bot.* 90: 631-636

Raven, P.H., Evert, R.F., Eichhorn, S.E. (2005). Chapter 19. *In* Biology of Plants, 7th Edition. New York: W. H. Freeman

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., Mittler, R. (2004) When defense pathways collide. The response of Arabidopsis to a combination of drought and heat Stress. *Plant Physiol.* 134: 1683-1696

Ruan, Y.L., Llewellyn, D.J., Furbank, R.T. (2003) Suppression of sucrose synthase gene expression represses cotton fiber cell initiation, elongation, and seed development. *Plant Cell.* 15: 952-964

Saini, H.S., Westgate, M.E. (2000) Reproductive development in grain crops during drought. *Adv. Agron.* 68: 59-96

Sakata, T., Oshino, T., Miura, S., Tomabechi, M., Tsunaga, Y., Higashitani, N., Miyazawa, Y., Takahashi, H., Watanabe, M., Higashitani, A. (2010) Auxins reverse plant male sterility caused by high temperatures. *PNAS.* 107: 8569-8574

Sakata, T., Takahashi, H., Nishiyama, I., Higashitani, A. (2000) Effects of high temperature on the development of pollen mother cells and microspores in barley *Hordeum vulgare* L. *J. Plant Res.* 113: 395-402

Salamone, P., Greene, T., Kavakli, I., Okita, T. (2000) Isolation and characterization of a higher plant ADP-glucose pyrophosphorylase small subunit homotetramer. *FEBS Letters.* 482: 113-118

Sanjaya, S., Durrett, T.P., Weise, S.E., Benning, C. (2011) Increasing the energy density of vegetative tissues by diverting carbon from starch to oil biosynthesis in transgenic Arabidopsis. *Plant Biotechnol. J.* 9: 874-883

Sato, S., Peet, M.M., Thomas, J.F. (2002) Determining critical pre- and post-anthesis periods and physiological processes in *Lycopersicon esculentum* Mill. exposed to moderately elevated temperatures. *J. Exp. Bot.* 53: 1187-1195

Scheffler, J., Sharpe, A., Schmidt, H., Sperling, P., Parkin, I., Lydiate, D., Heinz, E. (1997) Desaturase multigene families of *Brassica napus* arose through genome duplication. *Theor. Appl. Genet.* 94: 583-591

Schofield, R.A., Bi, Y.M., Kant, S., Rothstein, S.J. (2009) Over-expression of *STP13*, a hexose transporter, improves plant growth and nitrogen use in *Arabidopsis thaliana* seedlings. *Plant, Cell Environ.* 32: 271-285

Scholz-Starke, J., Büttner, M., Sauer, N. (2003) AtSTP6, a New Pollen-Specific H^+-Monosaccharide Symporter from Arabidopsis. *Plant Physiol.* 131: 70-77

Schweinichen, C.V., Büttner, M. (2005) Expression of a plant cell wall invertase in roots of *Arabidopsis* leads to early flowering and an increase in whole plant biomass. *Plant Biol.* 7: 469-475

Shenton, D., Grant, C.M. (2003) Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Biochem. J.* 374: 513-519

Sherson, S., Alford, H., Forbes, S., Wallace, G., Smith, S. (2003) Roles of cell-wall invertases and monosaccharide transporters in the growth and development of *Arabidopsis*. *J. Exp. Bot.* 54: 525-531

Sherwood, S.C., Huber, M. (2010) An adaptability limit to climate change due to heat stress. *PNAS.* 107: 9552-9555

Sun, A., Dai, Y., Zhang, X., Li, C., Meng, K., Xu, H., Wei, X., Xiao, G, Ouwerkerk, P.B.F., Wang, M., Zhu, Z. (2011) A transgenic study on affecting potato tuber yield by expressing the rice sucrose transporter genes *OsSUT5Z* and *OsSUT2M*. *J. Integr. Plant Biol.* 53: 586-595

Takeoka, Y., Mamun, A.A., Wada, T., Kaufman, P.B. (1992) Reproductive adaptation of rice to environmental stress. Japan Scientific Societies Press/Elsevier, Tokyo.

Tang, G., Luscher, M., Sturm, A. (1999) Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. *Plant Cell.* 11: 177-189

Thakura, P., Kumara, S., Malika, J.A., Bergerb, J.D., Nayyara, H. (2010) Cold stress effects on reproductive development in grain crops: an overview. *Environ. Exp. Bot.* 67: 429-443

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*. 408: 796-815

Tiessen, A., Hendriks, J.H.M., Stitt, M., Branscheid, A., Gibon, Y., Farré, E.M., Geigenberger, P. (2002) Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase: a novel regulatory mechanism linking starch synthesis to the sucrose supply. *Plant Cell.* 14: 2191-2213

Tonsor, S.J., Scott, C., Boumaza, I., Liss, T.R., Brodsky, J.L., Vierling, E. (2008) Heat shock protein 101 effects in *A. thaliana*: genetic variation, fitness and pleiotropy in controlled temperature conditions. *Mol. Ecol.* 17: 1614-1626

Truernit, E., Stadler, R., Baier, K., Sauer, N. (1999) A male gametophyte-specific monosaccharide transporter in *Arabidopsis. Plant J.* 17: 191-201

Turner, J., Turner, D. (1975) The regulation of carbohydrate metabolism. *Ann. Rev. Plant Physiol.* 26: 159-186

Tymowska-Lalanne, Z., Kreis, M. (1998) Expression of the *Arabidopsis thaliana* invertase gene family. *Planta.* 207: 259-265

Wahid, A., Farooq, M., Hussain, I., Rasheed, R., Galani, S. (2012) Responses and management of heat stress in plants. *In* Environmental adaptations and stress tolerance of plants in the era of climate change. Springer Science and Business Media, LLC. pp 135-157

Wang, Z., Huang, B. (2004) Physiological recovery of Kentuchy bluegrass from simultaneous drought and heat stress. *Crop Sci.* 44: 1729-1736

Weichert, N., Saalbach, I., Weichert, H., Kohl, S., Erban, A., Kopka, J., Hause, B., Varshney, A., Sreenivasulu, N., Strickert, M., Kumlehn, J., Weschke, W., Weber, H. (2010) Increasing sucrose uptake capacity of wheat grains stimulates storage protein synthesis. *Plant Physiol.* 152: 698-710

Whittle, C.A., Otto, S.P., Johnston, M.O., Krochko, J.E. (2009) Adaptive epigenetic memory of ancestral temperature regime in *Arabidopsis thaliana. Bot.* 87: 650-657

Williams, L.E., Lemoine, R., Sauer, N. (2000) Sugar transporters in higher plants – a diversity of roles and complex regulation. *Trend in Plant Science.* 5: 283-290

Winkler, C., Delvos, B., Martin, W., Henze, K. (2007) Purification, microsequencing and cloning of spinach ATP-dependent phosphofructokinase link sequence and function for the plant enzyme. *FEBS J.* 274: 429-438

Xu, S.M., Brill, E., Llewellyn, D.J., Furbank, R.T., Ruan, Y.L. (2012) Overexpression of a potato sucrose synthase gene in cotton accelerates leaf expansion, reduces seed abortion, and enhances fiber production. *Mol. Plant.* 5: 430–441

Xu, F.Q., Li, X.R., Ruan, Y.L. (2008) RNAi-mediated suppression of hexokinase gene *OsHXK10* in rice leads to non-dehiscent anther and reduction of pollen germination. *Plant Sci.* 175: 674–684

Xu, F.Q, Joshi, C.P. (2010) Overexpression of aspen sucrose synthase gene promotes growth and development of transgenic *Arabidopsis* plants. *Adv, Biosci. Biotechnol.* 1: 426-438

Xue, G.P., McIntyre, C.L., Glassop, D., Shorter, R. (2008) Use of expression analysis to dissect alterations in carbohydrate metabolism in wheat leaves during drought stress. *Plant Mol Biol.* 67: 197-214

Young, L.W., Wilen, R.W., Bonham-Smith, P.C. (2004) High temperature stress of *Brassica napus* during flowering reduces micro- and megagametophyte fertility, induces fruit abortion, and disrupts seed production. *J. Exp. Bot.* 55: 485-495

Zinn, K.E., Tunc-Ozdemir, M., Harper, J.F. (2010) Temperature stress and plant sexual reproduction: uncovering the weakest links. *J. Exp. Bot.* 61: 1959-1968

Zinselmeier, C., Westgate, M.E., Schussler, J.R., Jones, R.J. (1995) Low water potential disrupts carbohydrate metabolism in maize (*Zea mays* L.) ovaries. *Plant Physiol.* 107: 385-391