Final Scientific Report

Cover Page

BARD Project Number: IS-3738-05 R

Date of Submission of the report: 1 Oct 2007

Project Title:

Molecular Identification and Characterization of Heat-Stress-Responsive Microgametogenesis Genes in Tomato and Sorghum – A Feasibility Study

Investigators

Principal Investigator (PI): Nurit Firon

Co-Principal Investigator (Co-PI): Kenneth J Boote

Collaborating Investigators:

Prem Chourey

Etan Pressman

Hartwell Allen

Institutions

The Volcani Center, A. R. O.

University of Florida

USDA, ARS & University of Florida

The Volcani Center, A. R. O.

USDA, ARS & University of Florida

Keywords

abiotic stress; cDNA-AFLP; pollen; defence mechanisms

Abbreviations:

A-n – n days before anthesis; AFLP - Amplified Fragment-Length Polymorphism; AHS – Acute Heat Stress; cv. – cultivar; CWI – Cell-wall invertase; DAS – Days after sowing; HS – Heat Stress; HSR – Heat Shock Response; MCHS – Mild Chronic Heat Stress; TDFs - Transcript-Derived Fragments.

Budget: IS: \$ 50,000

US: \$ 50,000

Total: \$ 100,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged		1		1
Submitted, in review, in preparation			1	1
Invited review papers				
Book chapters				
Books				
Master theses			1	1
Ph.D. theses				
Abstracts		1		1
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: Dr. Mukesh Jain, post-doctoral associate with Dr. Boote (772-10-7202)

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings				
Longer Visits (Sabbaticals)				

Description Cooperation:

As far as we know, the number of laboratories working on the effect of heat-stress on pollen quality and fruit-set is limited. This system is unique and was demonstrated by us to exhibit similar as well as different characteristics in dicots (tomato) and monocots (sorghum). The cooperation between the Israeli and US labs is thus manifested by accumulated data in both crops indicating the high sensitivity of the microgametogenesis phase to HS, the necessity to establish threshold HS conditions as a basis for the characterization of HS-responsive microgametogenesis genes, the significance of sugar-to-starch metabolism and the respective genes involved in determining pollen quality under HS and potential involvement of common defense mechanisms. The cooperation enabled the establishment of cDNA-AFLP protocols, in both US & Israeli labs (established in the lab of N. Firon with help from the US labs), as a preferred method for the identification and isolation of HS-responsive microgametogenesis-expressed genes. The combined databank of these tomato and sorghum genes, achieved during the one-year feasibility study and detailed in this final report, indicate the involvement of a variety of factors in microspore HS-response and will serve in a continuation project for functional analyses - in order to come up with genes that could be used for improving pollen quality under HS.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed

Final Scientific Report

ABSTRACT

Molecular Identification and Characterization of Heat-Stress-Responsive Microgametogenesis Genes in Tomato and Sorghum – A Feasibility Study

Nurit Firon, Kenneth J Boote, Prem Chourey, Etan Pressman, Hartwell Allen

Exposure to higher than optimal temperatures - heat-stress (HS) - is becoming increasingly common to all crop plants worldwide. Heat stress coinciding with microgametogenesis, especially during the post-meiotic phase that is marked by starch biosynthesis, is often associated with starch-deficient pollen and male sterility and ultimately, greatly reduced crop yields. The molecular basis for the high sensitivity of developing pollen grains, on one hand, and factors involved in pollen heat-tolerance, on the other, is poorly understood. The long-term goal of this project is to provide a better understanding of the genes that control pollen quality under heat-stress conditions. The specific objectives of this project were: (1) Determination of the threshold heat stress temperature(s) that affects tomato and sorghum pollen quality whether: **a)** Chronic mild heat stress conditions (CMHS), or **b)** Acute heat stress (AHS). (2) Isolation of heat-responsive, microgametogenesis-specific sequences.

During our one-year feasibility project, we have accomplished the proposed objectives as follows: **Objective 1:** We have determined the threshold HS conditions in tomato and sorghum. This was essential for achieving the 2nd objective, since our accumulated experience (both Israeli and US labs) indicate that when temperature is raised too high above "threshold HS levels" it may cause massive death of the developing pollen grains. Above-threshold conditions have additional major disadvantages including the "noise" caused by induced expression of genes involved in cell death and masking of the differences between heat-sensitive and heat-tolerant pollen grains. Two different types of HS conditions were determined: a) Season-long CMHS conditions: 32/26°C day/night temperatures confirmed in tomato and 36/26°C day maximum/night minimum temperatures in sorghum. b) Short-term AHS: In tomato, 2 hour exposure to 42-45°C (at 7 to 3 days before anthesis) followed by transfer to 28/22±2°C day/night temperatures until flower opening and pollen maturation, caused 50% reduced germinating pollen in the heat-sensitive 3017 cv.. In sorghum, 36/26°C day/night temperatures 10 to 5 days prior to panicle emergence, occurring at 35 days after sowing (DAS) in cv. DeKalb28E, produced starch-deficient and sterile pollen.

Objective 2: We have established protocols for the high throughput transcriptomic approach, cDNA-AFLP, for identifying and isolating genes exhibiting differential expression in developing microspores exposed to either ambient or HS conditions and created a databank of HS-responsive microgametogenesis-expressed genes. A subset of differentially displayed Transcript-Derived Fragments (TDFs) that were cloned and sequenced (35 & 23 TDFs in tomato and sorghum, respectively) show close sequence similarities with metabolic genes, genes involved in regulation of carbohydrate metabolism, genes implicated in thermotolerance (heat shock proteins), genes involved in long chain fatty acids elongation, genes involved in proteolysis, in oxidation-reduction, vesicle-mediated transport, cell division and transcription factors.

T-DNA-tagged Arabidopsis mutants for part of these genes were obtained to be used for their functional analysis. These studies are planned for a continuation project. Following functional analyses of these genes under HS – a valuable resource of genes, engaged in the HS-response of developing pollen grains, that could be modulated for the improvement of pollen quality under HS in both dicots and monocots and/or used to look for natural variability of such genes for selecting heat-tolerant germplasm - is expected.

Final Scientific Report

ACHIEVEMENTS

Exposure to higher than optimal temperatures - heat-stress (HS) - is becoming increasingly common to all crop plants worldwide. Heat stress coinciding with microgametogenesis, especially during the post-meiotic phase that is marked by starch biosynthesis, is often associated with starch-deficient pollen and male sterility and ultimately, greatly reduced crop yields. The molecular basis for the high sensitivity of developing pollen grains, on one hand, and pollen heat-tolerance, on the other, is poorly understood. The focus of our joint one-year feasibility project was to demonstrate the feasibility of use of the high throughput transcriptomic approach, cDNA-AFLP (Amplified-Fragment Length Polymorphism), for the isolation and characterization of HS-responsive pollen-expressed genes - for their eventual use as candidate genes in improving pollen quality under HS conditions. The specific objectives of our project were: (1) Determination of the threshold heat stress temperature(s) that affects tomato and sorghum pollen quality whether: **a)** Chronic mild heat stress conditions (CMHS), or **b)** Acute heat stress (AHS). (2) Isolation of heat-responsive, microgametogenesis-expressed sequences. During our one-year feasibility project, we have accomplished the proposed objectives as follows:

Objective 1. Determination of threshold heat stress levels. We have determined the threshold HS conditions in tomato and sorghum. Determination of threshold conditions was essential for achieving the 2nd objective, since our accumulated experience (both Israeli and US labs) indicate that when temperature is raised too high above "threshold HS levels" it may cause massive death of the developing pollen grains. Above-threshold conditions have additional major disadvantages including the "noise" caused by induced expression of genes involved in cell death and masking of the differences between heat-sensitive and heat-tolerant pollen grains. Two different types of HS conditions were determined: a) Season-long CMHS conditions: 32/26°C day/night temperatures confirmed in tomato [1-2] and 36/26°C day maximum/night minimum temperatures in sorghum [3-4]. b) Short-term AHS: In tomato, 2 hour exposure to 42-45°C (at 7 to 3 days before anthesis) followed by transfer to 28/22±2°C day/night temperatures until flower opening and pollen maturation, caused 50% reduced germinating pollen grains in the heat-sensitive 3017 cv. (Fig.1). In sorghum, 36/26°C day/night temperatures 10 to 5 days prior to panicle emergence (occurring at 35 days after sowing (DAS) in cv. DeKalb28E) produced starch-deficient and sterile pollen [4].

Objective 2. (a) Determination of differential transcript profiles of maturing microspores of tomato and sorghum, at ambient and heat stress conditions & isolation of

Final Scientific Report

HS-responsive sequences. In both tomato and sorghum, cDNA-AFLP has been established as a valuable tool for discovering HS-responsive microgametogenesis-expressed genes. The established protocols for tomato and sorghum are given in the Appendix. **In tomato**, we obtained transcript profiles of differentially expressed genes of microspores at two post-meiotic stages of development: A-5 and A-3 (5 and 3 days before anthesis, respectively). Microspores were derived from heat-sensitive and heat-tolerant cultivars, *cv.* 3017 and *cv.* 3042, respectively, exposed to either ambient or AHS conditions. For cDNA-AFLP analyses, 32 primer combinations were used for selective amplification. The results indicate that, at both stages of pollen development tested, 20-30% of the TDFs are altered upon exposure to HS (Fig. 2A). A subset of differentially displayed TDFs that were cloned and sequenced (35 TDFs; Table 1) show close sequence similarities with metabolic genes (UDP-glucose pyrophosphorylase (UDPGP), malate dehydrogenase, UDP-Gluconate 4-epimerase), regulation of carbohydrate metabolism (TFT2 & TFT9 members of the 14-3-3 family, AKIN gamma of the SnRK family), genes implicated in thermotolerance (HSP90, HSP81, HSP70), genes involved in proteolysis (a proteasome subunit, serine-type endopeptidase), in oxidation-reduction (adenylyl-sulfate reductase, SOD), vesicle-mediated transport (Vamp725), cell division (CDC48) and transcription factors (such as DBA-binding protein phosphatase 2C (DBP1), zinc finger, etc.; Fig. 2 & Table 1). **In sorghum**, cDNA-AFLP analyses using 32 selective primer combinations, revealed a large number of differentially expressed TDFs in microspores / young pollen from 'early' and 'late' stages of development under ambient and HS conditions (Fig. 2B).

Some of the TDFs were sequenced and used as probes for Northern Blot & semi-quantitative RT-PCR analyses for reconfirmation of differential transcriptional activity during microspore development under ambient and HS conditions in sorghum and tomato (Fig. 2C and D, respectively). In sorghum, TDFs 6.7, 6.11 and 6.16 showed close sequence similarities with small subunit of maize ADP glucose pyrophosphatase, fructokinase 2 and starch debranching enzyme (*Sul*) gene, respectively. TDF 6.20 displayed small but significant homology to *Arabidopsis thaliana* CER10/ECR (3-oxo-5-alpha-steroid 4-dehydrogenase / fatty acid elongase) gene implicated in very long chain fatty acids elongation reactions required for cuticular wax, storage lipid and sphingolipid metabolism. Likewise, TDFs 7.4, 7.6 and 7.9 were partially homologous to histone deacetylase 2 isoform C, ubiquinol-cytochrome C reductase and ribosomal S6 protein kinase genes respectively.

Final Scientific Report

(b) Transcriptional analysis of sugar-to-starch metabolic transition during tomato & sorghum microspore development: In tomato, we demonstrated earlier the potential involvement of sucrose-cleaving enzymes and the respective genes in determining pollen quality under HS [5]. During the feasibility study, we demonstrated HS-induced modulation of three microgametogenesis-expressed members of the 14-3-3 protein family (tft2, tft9 and tft12) as shown in Fig. 3. In sorghum, we [4] recently published a detailed account of significantly altered temporal expression profiles of cell-wall invertase (CWI), soluble invertase, sucrose synthases, hexose transporters, and starch biosynthesis genes, indicating that CWI-mediated sucrose cleavage, monosaccharide transport across symplastically discontinuous microspores and subsequent sucrose biosynthesis eventually leads to altered carbohydrate metabolism and pollen starch deficiency under HS growth conditions.

Significance of main scientific achievements & agricultural impacts of the research

findings: cDNA-AFLP was established, in both tomato and sorghum, for identifying and characterizing HS-responsive microgametogenesis-expressed genes, a databank of genes was generated and T-DNA-tagged Arabidopsis mutants for part of these genes were obtained (Table 2) to be used for their functional analysis. The latter is planned for a continuation project. Following functional analyses of these genes under HS – a valuable resource of genes, engaged in the HS-response of developing pollen grains, that could be modulated for the improvement of pollen quality under HS in both dicots and monocots and/or used to look for natural variability of such genes for selecting heat-tolerant germplasm - is expected.

Details of cooperation: Identifying potential HE-tolerance mechanisms in the most sensitive part of many plant species, the developing pollen, is a complex objective requiring the mutual efforts of more than one laboratory. Each of the laboratories involved contributed a specific expertise: Protocols for cDNA-AFLP based differential transcript profiling for sorghum microspore analyses have earlier been standardized in Prem Chourey's lab (US) and continuous exchange of ideas between the US and Israeli labs proved useful in standardizing the technique for tomato. Finally, mutual discussion and review of ongoing research has helped in integrating the cDNA-AFLP data from tomato (Firon's lab) and sorghum (Chourey's lab) for prioritizing concise agenda for functional characterization of heat stress induced transcriptional changes through transgenic approaches. The labs of Etan Pressman and Ken Boote & Hartwell Allen determined the threshold HS conditions in tomato and sorghum, respectively. The combined efforts thus enabled to accumulate data at both physiological & molecular levels.

Final Scientific Report

List of Publications

Jain M, Prasad PV, Boote KJ, Allen, LH Jr and PS Chourey , Effects of season-long high temperature growth conditions on sugar-to-starch metabolism in developing microspores of grain sorghum (*Sorghum bicolor* L. Moench). *Planta*, 2007, DOI 10.1007/s00425-007-0595-y

Final Scientific Report

APPENDIX

Table of contents

Unpublished data

Heat-stress conditions..... 9

Differential transcript profiles of maturing microspores of tomato and sorghum, at ambient and heat stress conditions & isolation of HS-responsive sequences..... 9

Detailed transcriptional analysis of sugar-to-starch metabolic transition during tomato & sorghum microspore development.....12

Functional analyses of selected HS-responsive microgametogenesis-expressed genes, by using T-DNA-tagged Arabidopsis mutants.....13

cDNA-AFLP protocols..... 14

Bibliography.....18

Published papers.....18

Final Scientific Report

Unpublished data

Heat-stress conditions determined

In tomato, threshold acute HS temperatures were found to be 2 hours exposure of the plants to 45 °C (Fig. 1). These conditions significantly affected the pollen germination capacity in all the heat-sensitive cultivars tested and had a small effect on pollen quality of the heat-tolerant cultivars.

Fig. 1. Germination capacity of pollen grains of heat-sensitive cv. 3017 and heat-tolerant cv. 3042 exposed to AHS (2 hours at 45 °C). C – control (26/22±2 °C day/night temperature); HS – exposure of the plants for 2 h at 45 °C. HS was applied at A-n; n=days before anthesis. Bars represent standard error.

Differential transcript profiles of maturing microspores of tomato and sorghum, at ambient and heat stress conditions & isolation of HS-responsive sequences

In both tomato and sorghum, cDNA-AFLP has been established as a valuable tool for discovering genes important to microspore and pollen development.

In tomato, 32 primer combinations were used for selective amplification. A subset of differentially displayed TDFs that were cloned and sequenced is presented in Fig. 2 and Table 1 below.

Final Scientific Report

Fig 2. cDNA-AFLP auto-radiographs showing TDFs patterns of (A,D) tomato and sorghum (B-C) microspores following HS. **In tomato, A** - the use of one set of primer combination is demonstrated (E-ACT/Mse-CAT). Lanes 1,2 and 3,4 represent duplicates of microspores of cv 3042, at A-3 and A-5 stages of development, respectively, exposed to either ambient (1,3) or AHS (lanes 2,4) conditions. Polymorphic bands between stages and temperature conditions are indicated by arrows. For example: #6-9 are up-regulated by HS at A-5 stage, #1-4 are down-regulated by HS at A-3. **D** – TDFs were eluted from the gel, sequenced and used for semi-quantitative RT-PCR analysis for reconfirmation of differential expression profiles. HS – exposure of the plants to 2h at 45°C. C- 28/22±2°C. HS was applied at A-n; n=days before anthesis. **In sorghum, B** -results of E-TG/M-CAG primer combination is shown. Various classes of differentially expressed TDFs readily seen include, e.g. #1: no change at ambient vs. high temperature, and early vs. late microspore development; #2: no effect of temperature, but down regulated at late developmental stages; #3: seen only in early stages of microspore development at ambient temperature; #4: up regulated by heat stress at both early and late, but more abundant in early stages of microspore development; #5: expressed late during pollen development and up regulated by heat stress; #6: down regulated during microspore development at ambient temperature, but up regulated by heat stress; and #7: up regulated by heat stress during early, and down regulated during late stages of pollen development. **C** – The TDFs were eluted from the gel, sequenced and used for RNA blot analyses for reconfirmation of differential expression profiles.

Table 1. Summary of HS-responsive (either induced or repressed) transcript derived fragments of maturing tomato pollen grains and their homologies to sequences in the databases.

cDNA-AFLP fragment	Length (bp)	Ind /Rep	Homology	Possible function	GenBank homolog	BLAST Score
1*	448	+	Lycopersicon esculentum adenylyl-sulfate reductase	Oxidation-reduction	AY568717	3.00E-66
2*	262	+	vesicle-associated membrane protein 725	Vesicle-mediated transport	O48850	2.00E-27
3	616	+	Arabidopsis cell division cycle protein 48 (CDC48)	Cell division	Q9SCN8	5.00E-109
4	570	+	putative zinc finger protein from Gossypium hirsutum	Transcription factor	AY919286	6.00E-27
5	361		putative alpha7 proteasome subunit from Nicotiana tabacum	Proteolysis-ubiquitin-dependent protein catabolic process	AJ291738	8.00E-58
6	396		UDP-glucose pyrophosphorylase from Solanum tuberosum	Sugar metabolism, callose deposition (pollen). Formation of UDP-glucose from glucose-1-phosphate and UTP.	D00667	5.00E-53
7	565	+	UDP-D-GLUCURONATE 4-EPIMERASE 5 (GAE5) from Arabidopsis	Nucleotide-sugar metabolic process	NM_117295	1.00E-77
8**	334	-	LAT56 gene for protein P56 from tomato	Probable pectate lyase P56 [Precursor]. Pectin degradation (during pollen development & tube growth?)	X15500	3.00E-57
9	480	+	AGO1-2 2 (ARGONAUTE 1) from Nicotiana benthamiana	Role in systemic silencing	DQ321489	8.00E-76
10*	146	-	CALCIUM-DEPENDENT PROTEIN KINASE 2 (ATCDPK2) from Arabidopsis	Protein amino acid phosphorylation. calcium-dependent protein kinase activity. [Signal transduction]	NM_103271	9.00E-09
11	92	+	citrate synthase from Nicotiana tabacum	Energy production and conversion. TCA cycle	X84226	3.3
12*	299	+	heat shock protein 90 (HSP90) from Lycopersicon esculentum	Protein conformation. Defense. Molecular chaperone, HSP90 family	AF123259	3.00E-13
13	323	+	mRNA for 14-3-3 protein (tft9 gene)	Regulatory factor. Key regulator of	X98865	7.00E-50

Final Scientific Report

			from <i>Lycopersicon esculentum</i>	primary metabolism and membrane transport		
14*	377	+	heptahelical protein 4 (HHP4) from <i>Arabidopsis thaliana</i>	Heptahelical transmembrane protein HHP4. Involved in response to sucrose stimulus. Involved in response to hormone stimulus. Receptor activity. Integral to membrane.	NM_119931	1.00E-22
15*	301	+	rhomboid family protein from <i>Arabidopsis thaliana</i>	serine-type endopeptidase, similar to ATRBL2	NM_120807	2.00E-10
16	122	+	DNA-binding protein phosphatase 2C (DBP1) from <i>Nicotiana tabacum</i>	transcription factor involved in plant defense endowed with protein phosphatase activity	AF520810	2.00E-04
17	179	-	GRAM domain-containing protein / ABA-responsive protein-related from <i>Arabidopsis thaliana</i>	Domain in glucosyltransferases, myotubularins and other putative membrane-associated proteins	NM_127813	7.00E-14
18	309	+	Protein amino acid binding		NM_103573	0.24
19	159	+	Unidentified			
20	101	+	Unidentified			
21*	320	+	HSP81-1	Protein conformation. Defense. Molecular chaperone, HSP90 family	NM_124642	3E-48
22	135	+	Malate dehydrogenase precursor	Malate metabolism (a component of both the TCA cycle and the glyoxylate cycle)	Z23023	4E-05
23**	67	+	Receptor-like protein kinase (LePRK1)	Potential signal transduction component	U58474	0.19
24	286	+	Unidentified			
25	131	+	Superoxide dismutase-4A (Sod4A gene).	Reactive oxygen species scavenging	Maize TC338885.	59% identity
26	95	+	Unidentified			
27	390	+	HSP81-1 gene for 81kDa heat-shock protein	Protein conformation. Defense. Molecular chaperone, HSP90 family	D00710	5E-44
28	267	+	Endomembrane protein 70 (EMP70), putative	Transporter activity. [Golgi apparatus] Integral to membrane	NM_122907	2E-11
29	192	+	Unidentified			
30	173	+	<i>Lycopersicon esculentum</i> clone 135129R. Homologous (7E-175) to ubiquitin-associated (UBA)/TS-N domain-containing protein	Ubiquitin Associated domain (commonly occurring sequence motif in some members of the ubiquitination pathway, UV excision repair proteins, and certain protein kinases). Putative protein-protein interaction site	BT013338	1E-19
31*	523	+	Adenylyl-sulfate reductase [PDIA family, 5'-Adenylylsulfate (APS) reductase subfamily; composed of plant-type APS reductases containing a C-terminal redox active TRX domain and an N-terminal reductase domain which is part of a superfamily that includes N type ATP PPases]	Response to oxidative stress. The reduction of sulfate and ATP to adenosine-phosphosulfate (APS) and pyrophosphate. assimilatory sulfate reduction. Chloroplast.	AY568717	3E-92
32	116	+	AKIN gamma	Part of the SnRK1 complex. Protein kinase, AMP-activated, gamma 3 non-catalytic subunit. [SNF1-related protein kinase regulatory subunit gamma 1 (AKIN gamma1) (AKING1).]	AY247269	8.6E-09
33	811	+	Zinc finger (GATA type) family protein	Transcription factor activity. In plants, GATA DNA motifs have been implicated in light-dependent and nitrate-dependent control of	NM_122484	8E-46

Final Scientific Report

				transcription.		
34	208	+	Unidentified			
35	300	+	Unidentified			

* The Arabidopsis ortholog is highly expressed in pollen

** Pollen-specific expression

Ind/Rep – Induced (+)/Repressed(-) in at least one tested pollen developmental stage

In sorghum, cDNA-AFLP analyses using 32 selective primer combinations, revealed a large number of differentially expressed TDFs in microspores / young pollen from ‘early’ and ‘late’ stages of development under ambient and HS conditions (Fig. 2B). Some of the TDFs were sequenced and used as probes for Northern Blot & semi-quantitative RT-PCR analyses for reconfirmation of differential transcriptional activity during microspore development under ambient and HS conditions in sorghum and tomato (Fig. 2C, D, respectively). TDFs 6.7, 6.11 and 6.16 showed close sequence similarities with small subunit of maize ADP glucose pyrophosphatase, fructokinase 2 and starch debranching enzyme (*Su1*) gene, respectively. TDF 6.20 displayed small but significant homology to *Arabidopsis thaliana* CER10/ECR (3-oxo-5-alpha-steroid 4-dehydrogenase / fatty acid elongase) gene implicated in very long chain fatty acids elongation reactions required for cuticular wax, storage lipid and sphingolipid metabolism. Likewise, TDFs 7.4, 7.6 and 7.9 were partially homologous to histone deacetylase 2 isoform C, ubiquinol-cytochrome C reductase and ribosomal S6 protein kinase genes respectively.

Detailed transcriptional analysis of sugar-to-starch metabolic transition during tomato & sorghum microspore development

In tomato, in the frame of the feasibility study, we demonstrated HS-induced modulation of three microgametogenesis-expressed members of the 14-3-3 regulatory protein family (tft2, tft9 & tft12) as shown in Fig. 3.

Fig. 3. Expression profiles of members of the 14-3-3 family, in maturing microspores of two tomato cultivars, exposed to either control or HS conditions.

TDFs were eluted from the gel, sequenced and used for semi-quantitative RT-PCR analysis for reconfirmation of differential expression profiles. HS – exposure of the plants to 2h at 45°C. C- 28/22±2°C. HS was applied at A-n; n=days before anthesis.

Final Scientific Report

In sorghum, we [4] recently published a detailed account of significantly altered temporal expression profiles of CWI, soluble invertase, sucrose synthases, hexose transporters, and starch biosynthesis genes indicating that CWI-mediated sucrose cleavage, monosaccharide transport across symplastically discontinuous microspores and subsequent sucrose biosynthesis eventually leads to altered carbohydrate metabolism and pollen starch deficiency under HS growth conditions.

In addition, Northern blot analyses also showed high temperature-induced changes in the expression profiles of various genes involved in the regulatory and metabolic pathways of sugar-to-starch metabolic transition e.g. *SnRK1b*, *Grf1*; and in the transcriptional activity of developmentally regulated genes (*MADS1* and 2).

Finally, in an ensuing study of effects of high temperature stress on sorghum, we found that chronic heat stress (44/34 °C, day-time maximum / night-time minimum) caused complete developmental arrest of sorghum plants in a robust vegetative phase and inhibited initiation of reproductive primordia. This extreme stunted phenotype was associated with significant changes in stress-related hormone levels ABA and jasmonic acid in the internodes. Preliminary data indicated an involvement of GA biosynthesis and degradation, and DELLA protein signaling pathways integrating floral development under environmental stress conditions.

Functional analyses of selected HS-responsive microgametogenesis-expressed genes, by using T-DNA-tagged Arabidopsis mutants

Based upon the database of genes, established by us in both tomato and sorghum, during the feasibility study we have already ordered T-DNA-tagged Arabidopsis mutants for starting functional analysis. The list of genes and mutants are listed in Table 2 and include genes that were identified in both tomato and sorghum as well as genes identified in tomato. We expect to conduct the functional analyses in the frame of a continued BARD project.

Table 2.

List of T-DNA-tagged mutants - for functional analyses of pollen quality under HS conditions

Gene	Arabidopsis	Clone
Cytosolic HSP101	At1g74310	SALK_099583.52.45 SALK_036423.35.70
Cytosolic HSP90 (HSP81-1)	At5g52640	SALK_130861.54.00.n SALK_039626.44.75.x
GRF12 (14-3-3 protein)	At1g26480	SALK_110798.42.10.x SALK_099441.30.10.x

Final Scientific Report

Vamp725	At2g32670	GABI_751A12 SALK_003926.54.10.x
Adenylyl-sulfate reductase	At4g04610	GABI_460B02
Cytosolic SOD	At1g08830	SALK_109389.43.90.x
Cytosolic APX1	At1g07890	SALK_000249.38.45.x SALK_143111.44.10.x
SnRK1	At4g16360	SALK_037416.54.75.x

cDNA-AFLP protocols

In both tomato & sorghum the protocol follows that of Breyne et al [7] with some modifications.

Tomato

Preparation of poly (A)⁺ RNA and cDNA synthesis

Microspores and pollen grains were ground to a fine powder under liquid nitrogen and total RNA was extracted using Tri reagent (Sigma-Aldrich). 75 µg total RNA was used for poly (A)⁺ RNA preparation. Poly (A)⁺ RNA was prepared using a Dynal (Dynal, Oslo, Norway) purification kit according to the manufacturer's instructions. First strand cDNA was synthesized as described by Breyne et al. [7] with some modifications by using SuperScript™ First-strand Synthesis System for RT-PCR (Invitrogen, CA, U.S.A) by mixing 100 ng Poly (A)⁺ RNA, 500 ng oligo(dT)₁₂₋₁₈ (Promega, Madison, WI, USA) and 500µM dNTPs to a final volume of 10µl and incubation at 65°C for 5 min. 10 x first strand RT Buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 5mM MgCl₂, 10 mM DTT and 40U RNaseOUT™ Ribonuclease Inhibitor (Invitrogen, CA, U.S.A) were added and the mixture was incubated at 42°C for 2 min, followed by addition of 50 units of Superscript II reverse transcriptase (Invitrogen, CA, U.S.A) and incubation at 42°C 50 min. The reaction was terminated by inactivation at 70°C for 15 min. Second strand synthesis was performed in second strand synthesis buffer (20 mM Tris HCl pH 7.5, 100 mM KCl, 10 mM NH₄SO₄) by adding 5 mM MgCl₂, 0.15 mM β-NAD⁺, 0.5 mg/ml BSA, 0.2 mM dNTPs, 10µl of template ss cDNA, 6 U DNA polymerase I, 0.25 U RNase-H and 1.2 U *E.coli* DNA ligase (New England BioLabs, USA). The reaction was incubated at 16°C for 2 h, after which 6 U T4 DNA Polymerase (New England BioLabs, USA) were added and the reaction was incubated for additional 30 min at 16°C. The reaction was terminated by adding 10 mM EDTA. Glycogen (1mg/ml) was added and the resulting double-stranded cDNA was phenol-extracted by adding phenol: chloroform: isoamil-alcohol (25:24:1) and using Phase Lock Gel (Eppendorf, Hamburg, Germany).

Template preparation, AFLP reactions and PAGE analysis of products

Templates for AFLP were prepared by performing cDNA digestion and adaptor ligation in the same reaction mixture as follows: 500 ng cDNA were added to a reaction mixture which included 50 mM NaCl, 1mg/ml BSA, *E.coli* DNA ligase buffer (3 mM Tris-HCl pH 8, 0.4 mM MgCl₂, 100 µM DTT, 2.6 µM NAD⁺, 5µg/ml BSA), EcoR I and Mse I (2.5 U each), EcoR I and Mse I adaptors (GibcoBRL, now Invitrogen™, Life Technologies, CA, USA; see the

Final Scientific Report

sequences below) and 1 U T4 DNA ligase (New England BioLabs, USA). The reaction was incubated at 37°C for 2 h in a final volume of 10 µl. The resultant fragments were subjected to pre-amplification as described in GibcoBRL (now Invitrogen™, Life Technologies) instruction manual using pre-amplification primers (see sequences below), except that 1 U Ex Taq DNA polymerase (TAKARA, Otsu, Shiga, Japan) was used. The obtained amplification mixture was diluted 50-fold and 3 µl was used for selective amplification. End-labeling of one of the selective primers and selective amplifications were carried out basically as described in GibcoBRL (now Invitrogen™, Life Technologies) instruction manual. The EcoR I primer was end-labeled with 2 µCi [γ -³³P]ATP per reaction using T4 polynucleotide kinase (New England BioLabs, USA). Selective amplification was performed using 0.5 pmol labeled primer, EcoR I or Mse I, and 0.5 pmol Mse I or EcoR I primer respectively, 0.1 mM dNTPs and 1 U Ex Taq polymerase (TAKARA, Otsu, Shiga, Japan) in a 20 µl reaction volume.

Adapter sequences:

EcoRI ad1: CTCGTAGACTGCGTACC
EcoRI ad2: AATTGGTACGCAGTCTAC

Mse I ad1: TACTCAGGACTCAT
Mse I ad2: GACGATGAGTCCTGAG

Pre-amplification primer sequences:

EcoRI :GACTGCGTACCAATTCA
Mse I :GATGAGTCCTGAGTAAC

The following primer sequences were used for selective amplification:

EcoR I primers:

GACTGCGTACCAATTCACT;	GACTGCGTACCAATTCACA;
GACTGCGTACCAATTC AAC;	GACTGCGTACCAATTCACC;
GACTGCGTACCAATTCAGC;	GACTGCGTACCAATTC AAG;
GACTGCGTACCAATTCAGG;	GACTGCGTACCAATTC ACG.

Mse I primers:

CATGAGTCCTGAGTAAC TG;	CATGAGTCCTGAGTAAC TT;
CATGAGTCCTGAGTAACAG;	CATGAGTCCTGAGTAACAT;
CATGAGTCCTGAGTAAC TA;	CATGAGTCCTGAGTAAC TC;
CATGAGTCCTGAGTAACAA;	CATGAGTCCTGAGTAACAC.

Amplification was done using a touchdown program for 11 cycles (the first cycle being 94°C for 60 s; 65°C for 60 s and 72°C for 90 s. Annealing temperature was then lowered by 1°C each cycle, during 11 cycles) followed by additional 23 regular program cycles (94°C for 30 s; 56°C

Final Scientific Report

for 30 s and 72°C for 60 s). Amplifications were performed in a ATC401 thermal cycler (CLP, San Diego, U.S.A).

Isolation and sequencing of transcript-derived fragments

Samples for electrophoresis were mixed with formamide dye (98% formamide, 10 mM EDTA, 0.01gr bromophenol blue, 0.01gr xylen cyanole), heated for 2 min at 90°C and placed on ice. Electrophoresis was performed in an Amersham Biosciences SQ3 Sequencer Apparatus (Amersham, CA, USA). Reactions were run on 30 x 40 cm gels using 6% acrylamide (19:1 acrylamide:bis; 7.5 M urea, 1 x TBE buffer). Gels were dried in a HOEFER SLAB gel dryer Model GD2000 (Amersham, CA, USA) and exposed to x-ray film (Kodak Biomax MR-1 Film) at -80°C for 3 days.

Bands corresponding to differentially expressed transcripts were eluted from the gel (incubated in 50 µl of TE buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0) for 30 min. at 65°C. AFLP fragments were recovered by PCR under the same conditions that were used for the pre-amplification. Resultant amplified fragments were cloned into pGEM-T easy (Promega, Madison, WI, USA), transformed into *E.coli* DH5- α and individual clones were sequenced (Danyel Biotech, Rehovot, Israel). Usually 3-5 clones were sequenced from each transformation. Nucleotide sequences and translated sequences were compared with nucleotide and translated sequences of GeneBank databases and sequences of expressed sequence tag databases including the tomato database of The Institute of Genomic Research (TIGR), by using the BLAST sequence alignment program [6].

Validation of the cDNA-AFLP results using semi-quantitative RT-PCR analyses

cDNA was synthesized using 1µg total RNA and Superscript II (Invitrogen, CA, U.S.A) reverse transcriptase, according to the manufacturer's instructions. 1µl of first-strand cDNA was used for semi-quantitative RT-PCR that was performed in three biological replicates. Transcript accumulation of genes under investigation was evaluated using primers designed according to the AFLP fragment sequences (Hy-labs, Rehovot, Israel), in a final volume of 20 µL using 0.05 U of Ex Taq (TAKARA), 0.1mM dNTP's, in a ATC401 thermal cycler (CLP, San Diego, U.S.A). For each reaction, a set of a different number of cycles ranging between 23 and 35 was tested to choose those corresponding to the exponential phase. Each cycle consisted of 30 s denaturation at 94 °C, 30 s annealing at T_m temperature and 30 s extension at 72 °C; cycles were followed by a final 5 min extension at 72 °C. The PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and images obtained via ultraviolet (UV) exposition elaborated using the KODAK 1D 3.6 software (Scientific Imaging Systems-Eastman Kodak Company, Rochester, NY, USA). The expression level was evaluated, normalized with the 18S gene (accession X51576) and expressed in arbitrary units.

Sorghum

Preparation of poly (A)+ RNA and cDNA synthesis

Pre-emergent panicles were staged for microspore developmental stages and sectioned into the “early” lower and “late” upper portions for microspore isolation. Microspores RNA was

Final Scientific Report

extracted using Trizol reagent (Invitrogen). Poly(A)⁺ RNA was prepared using a Dynal mRNA purification kit (Dynal, Oslo, Norway). First strand cDNA was synthesized by mixing ca. 1 µg poly(A)⁺ RNA, ca. 700 ng biotinylated oligo(dT)23 (Promega), 5× first strand buffer, 10 mM DTT, 500 µM dNTPs, and 200 U Superscript II (Invitrogen) reverse transcriptase. The reaction was incubated for 2 h at 42°C. Second strand synthesis was conducted by adding an equal volume of second strand buffer including dNTPs, DTT, *E. coli* ligase, *E. coli* polymerase I, and RNase-H. The reaction was incubated 1 h at 12°C and 1 h at 22°C prior to extraction with phenol/chloroform.

AFLP procedures:

The restriction enzymes *Bst*YI and *Mse*I were utilized for AFLP as described. Templates for AFLP were prepared by digesting ca. 200–500 ng cDNA with *Bst*YI (New England Biolabs) for 2 h at 60°C, followed by immobilization of 3' terminal fragments on streptavidin Dynabeads M-280 (Dynal) by virtue of the 3' poly d(T)⁺ biotinylated primer. In a final volume of 120 µl, 10 µl Dynabeads were mixed with cDNA, incubated for 30 min and the beads were collected with a Dynal MPC-S magnet. Non-biotinylated fragments were removed by withdrawing the non-bound fraction, and the beads were washed a total of five times in this manner. The beads were resuspended and incubated with *Mse*I (New England Biolabs) for 2 h at 37°C in a volume of 40 µl. The *Mse*I-3'-terminal fragment immobilized on the Dynabeads was then separated from the *Bst*YI-*Mse*I fragments by capture of the former with the magnet. The non-retained fragments were utilized as AFLP template.

The resultant fragments were subjected to adaptor ligation, preamplification, amplification as described, including primers used for each step[7]. The liberated fragments were annealed and ligated to *Bst*YI and *Mse*I adaptors and preamplification was performed with a *Bst*YI primer terminating in either T or C. Selective amplification was carried out with a *Bst*YI TC primer and *Mse*I +3 primers for comparisons of early and late samples under both temperature conditions. The *Bst*YI primer was labeled with [γ -³³P] ATP using T4 polynucleotide kinase. Selective amplification was performed with 5 ng labelled *Bst*YI primer, 30 ng *Mse*I primer, 0.2 µl 10 mM dNTPs, and 0.5 U Taq polymerase (Sigma) in a 10 µl reaction volume. Amplification was preceded by 10 min at 94°C prior to one cycle of 30 s at 94°C, 30 s at 65°C, and 60 s at 72°C. A touchdown PCR program was then used wherein the annealing temperature was lowered 0.7°C/cycle for 12 cycles, followed by 23 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C. The reaction mix was layered with oil prior to amplification in 200 µl thin-walled tubes in 96-well formatted MJ Research PTC-100 thermocycler.

Gel electrophoresis and autoradiography

Samples for electrophoresis were mixed with stop solution/loading dye, and heated at 95°C for 4 min prior to electrophoresis in Bio-Rad Sequi-Gen GT units, using 24-well combs. Typically one-third of the reaction was loaded on gels. Reactions were run on 21×50 cm gels using 5% Long Ranger (Cambrex) acrylamide. Overnight exposure with Kodak BioMax MR-1 film was adequate for most interpretations, and exposures of up to 5 days were made (if required) for detection of weak bands. Once identified, representative cDNA-amplified fragments were gel-purified, cloned (PCR TOPO, Invitrogen), sequenced and used as probes for Northern blot analyses.

Final Scientific Report

Bibliography

1. Pressman E, Peet MM and MD Pharr, The effect of heat-stress on tomato pollen characteristics is associated with changes in carbohydrate concentration in the developing anthers. *Ann Bot*, 2002. **90**: 631-636.
2. Firon N, Peet MM, Pharr DM, Zamski E, Rosenfeld K, Althan L and E Pressman, Pollen grains of heat tolerant tomato cultivars retain higher carbohydrate concentration under heat stress conditions. *Scient Hort*, 2006. **109**: 212-217.
3. Mukesh J, Andrew F, Prasad PVV, Allen LH, Boote KJ and PS Chourey, Effects of elevated high temperature growing conditions on sugar-to-starch metabolism in developing microspores in sorghum {*Sorghum bicolor* L. (Moench)}.in: American Society of Plant Biologists meeting 5-9 Aug 2006, Boston, MA, USA.
4. Jain M, Prasad PV, Boote KJ, Allen, LH Jr and PS Chourey , Effects of season-long high temperature growth conditions on sugar-to-starch metabolism in developing microspores of grain sorghum (*Sorghum bicolor* L. Moench). *Planta*, 2007, DOI 10.1007/s00425-007-0595-y
5. Pressman E, Harel D, Zamski E, Shaked R, Althan L, Rosenfeld K and N Firon, The effect of high temperatures on the expression and activity of sucrose cleaving enzymes during tomato (*Lycopersicon esculentum*) anther development. *J Hort Sci Biotechnol*, 2006. **81**: 341-348.
6. Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W and DJ Lipman, Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res*, 1997. **25**: 3389 – 3402.
7. Breyne P, Dreesen R, Vandepoele K, Veylder LD, Breusegem FV, Callewaert L, Rombauts S, Raes J, Cannoot B, Engler G, Inze D and M Zabeau, Transcriptome analysis during cell division in plants. *Proc Natl Acad Sci*, 2002. **99**: 14825 – 14830.

Published papers

Jain, M, Prasad PV, Boote KJ, Allen, LH Jr and PS Chourey , Effects of season-long high temperature growth conditions on sugar-to-starch metabolism in developing microspores of grain sorghum (*Sorghum bicolor* L. Moench). *Planta*, 2007, DOI 10.1007/s00425-007-0595-y

PDF file is attached