ISOLATION AND CHARACTERIZATION OF PISTIL PREDOMINANT GENES FROM TOMATO

By

CHOONG CHIEH WEAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chairman : Ho Chai Ling, Ph.D.

Faculty : Biotechnology and Biomolecular Sciences

The tomato is an economically and nutritionally important crop that has been extensively used as a research model system to study plant development. In this study, genes predominantly expressed in the pistil were identified and isolated in order to develop an understanding of pistil development and function. Since there have been several studies done in the past examining pistil development, many genes have been isolated and identified. As it became progressively more difficult to identify new genes, alternative approaches have to be employed. In this study, Suppression Subtractive Hybridization (SSH) was used to isolate and identify novel genes predominantly expressed in the pistil. From a pistil subtraction cDNA library, 550 clones were isolated and analyzed where 32.5 % of the genes were found to be predominantly expressed in the pistil through reverse northern screening. All the putative pistil predominant genes were sequenced and 75 independent sequences were found, 42 which had no homology to any known genes from the database and thus appear to encode novel proteins. A collection of genes related to metabolism,

photosynthesis and transcription suggest that during anthesis, tomato pistils are more biologically active than other floral organs. Some of these tomato pistil predominant genes included extensin-like protein, ribosomal protein L13 and L13E, sterol-C-methyltransferase and protein kinase C inhibitor-like protein. Two clones, namely LePiHAT and LePiXTH, were screened for their full-length coding sequences from a pistil cDNA library. LePiHAT was found to be 90 % similar to histone acetyltransferase (HAT) from *Arabidopsis thaliana* while LePiXTH was 88 % similar to xyloglucan endotransglycosylase/hydrolase (XTH) from *A. thaliana*. LePiHAT was predominantly expressed in pistils but was also expressed in young stamens, sepals, petals, floral meristems and leaves at lower levels. It was postulated that LePiHAT might be involved in active chromatin remodelling in the pistil. LePiXTH on the other hand was upregulated in young and mature pistils, young stamens and in floral meristems but much lower levels were also detected in the petals, sepals and leaves. Its expression level was highest in floral meristems, followed by vegetative meristems and mature pistils. Its predominant expression in floral meristems, coupled with its localized expression in flower buds suggests its role in floral growth and differentiation. The expression level of LePiXTH in stamens decreased before anthesis but in mature pistils, it was localized in the transmitting tissue that led into the ovary that acts as a channel for pollen tube growth during fertilization. This suggests its role in aiding fertilization through cell walls reconstruction (xyloglucans molecular grafting). The expression level of LePiXTH increased in young fruitlets after anthesis but was not detected in ripe fruit, indicating a possible function in fruit development but not in fruit ripening.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMENCILAN DNA PENCIRIAN GEN PISTIL PREDOMINAN DARIPADA TOMATO

Oleh

CHOONG CHIEH WEAN

Ogos 2004

Pengerusi : Ho Chai Ling, Ph.D.

Fakulti : Bioteknologi dan Sains Biomolekular

Tomato ialah tanaman yang penting dari segi ekonomi dan khasiat pemakanan. Tomato juga digunakan secara meluas sebagai sistem model kajian perkembangan tumbuhan. Di dalam projek ini, gen predominan di dalam pistil telah dipencilkan dan dikenalpasti untuk memahami proses perkembangan pistil. Oleh kerana kajian-kajian seumpama ini pernah dibuat untuk memahami perkembangan pistil, banyak gen telah pun dipencil dan dikenalpasti. Oleh kerana pengenalpastian gen-gen baru semakin sukar, kaedah 'Suppression Subtractive Hybridization' (SSH) digunakan untuk memencil dan mengenalpasti gen-gen baru yang predominan di dalam pistil. Daripada perpustakaan penolakan pistil yang dibina, 550 klon telah dianalisis dan 32.5 peratus daripada gen-gen didapati predominan di dalam pistil melalui penyaringan 'reverse northern'. Semua gen yang disangka predominan di dalam pistil telah dijujuk dan 75 jenis jujukan diperolehi, di mana 42 daripadanya tiada mempunyai perhubungan dengan gen-gen yang diketahui di dalam pengkalan data. Di dalam perpustakaan penolakan tersebut, terdapat koleksi gen-gen yang mana

berfungsi dalam replikasi DNA, metabolisma, fotosintesis and trankripsi, menunjukkan pistil tomato adalah lebih aktif dari segi biologi daripada organ bunga yang lain. Beberapa gen-gen dikenalpasti predominan di dalam pistil tomato ialah extensin-like protein, ribosomal protein L13 and L13E, sterol-C-methyltransferase and protein kinase C inhibitor-like protein. Dua klon, iaitu LePiHAT dan LePiXTH, telah disaring untuk jujukan kod daripada perpustakaan cDNA pistil. LePiHAT adalah 90 % serupa dengan histone acetyltransferase (HAT) daripada *Arabidopsis thaliana* manakala LePiXTH pula 86 % serupa dengan xyloglucan endotransglycosylase /hydrolase (XTH) daripada *A. thaliana*. LePiHAT adalah predominan di dalam pistil tetapi juga dizahir di dalam stamen muda, sepal, petal, meristem bunga dan daun pada aras yang lebih rendah. Adalah disimpulkan bahawa LePiHAT mungkin terlibat secara aktif dalam senibentuk-semula kromatin di dalam pistil. LePiXTH pula dinaik-aturkan di dalam pistil muda dan matang, stamen muda dan meristem bunga dan juga dikesan di dalam petal, sepal dan daun pada aras yang jauh lebih rendah. Paras penzahirannya adalah paling tinggi di dalam meristem bunga, diikuti oleh meristem daun dan pistil matang. Sehubungan itu, penzahirannya yang predominan di dalam meristem bunga dan tumpuan penzahirannya di dalam pucuk bunga mencadangkan peranannya untuk pertumbuhan dan pembezaan bunga. Penzahiran LePiXTH di dalam stamen menurun sebelum antesis tetapi penzahirannya tertumpu pada lorong pertumbuhan tiub debunga semasa persenyawaan, lalu mencadangkan peranannya untuk menolong persenyawaan melalui pembinaan-semula dinding sel. Penzahiran LePiXTH meningkat di dalam buah muda selepas antesis tetapi tidak dapat dikesan di dalam buah masak menunjukkan fungsinya di dalam perkembangan buah tetapi tidak di dalam peranuman buah.

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Lastly, I would like to dedicate this thesis to my father and mother, for making this thesis a reality.

I certify that an Examination Committee met on 26 August 2004 to conduct the final examination of Choong Chieh Wean on his degree in Doctor of Philosophy thesis entitled "Isolation and Characterization of Pistil Predominant Genes from Tomato" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Norihan Mohd. Saleh, Ph.D.
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Ho Chai Ling, Ph.D.
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Suhaimi Napis, Ph.D.
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Rofina Yasmin Othman, Ph.D.
Associate Professor
Faculty of Science
Universiti of Malaya
(Member)

Roderick John Scott, Ph.D.
Professor
University of Bath
United Kingdom
(Independent Examiner)

_____
GULAM RUSUL RAHMAT ALI, Ph.D.
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Ho Chai Ling, Ph.D.
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Rofina Yasmin Othman, Ph.D.
Faculty of Science
University of Malaya
(Member)

K. Harikrishna, Ph.D.
Sime Darby Technology Center
(Member)

Suhaimi Napis, Ph.D.
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I hereby declare that the thesis is based on my original work except for equations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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CHOONG CHIEH WEAN

Date:

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AMV-RT	-	Avian Myeloblastosis Virus Reverse Transcriptase
BCIP	-	5-bromo-4-chloro-3-indolyl phosphate
bp	-	basepair
kbp	-	kilobasepair
BSA	-	bovine serum albumin
cfu	-	colony forming unit
CTAB	-	hexadecyl (or cetyl) trimethyl ammonium bromide
dATP	-	deoxyadenine triphosphate
dCTP	-	deoxycytosine triphosphate
dTTP	-	deoxythymine triphosphate
dGTP	-	deoxyguanine triphosphate
ATP	-	adenine triphosphate
CTP	-	cytosine triphosphate
UTP	-	uracil triphosphate
GTP	-	guanine triphosphate
DEPC	-	diethyl pyrocarbonate
DIG	-	Digoxigenin
DMSO	-	dimethyl sulphoxide
DNA	-	deoxyribonucleic acid
cDNA	-	complemetary deoxyribonucleic acid
DNase	-	nuclease
DTT	-	dithiothreitol
EDTA	-	ethylene diamine tetracetate
g	-	gram
mg	-	milligram
µg	-	microgram
HCl	-	hydrochloric acid
LB	-	Luria-Bertani
LiCl	-	lithium chloride
M	-	molar / molarity
mM	-	millimolar
µM	-	micromolar
$MgSO_4$	-	magnesium sulfate
ml	-	milliliter

µl	-	microliter
N	-	Normality
NaCl	-	sodium chloride
NaOH	-	sodium hydroxide
NBT	-	nitroblue tetrazolium chloride
ng	-	nanogram
NTE	-	**N**aCl-**T**ris-**E**DTA
OD	-	optical density
PBS	-	phosphate buffer saline
PCR	-	Polymerase Chain Reaction
pfu	-	plaque forming unit
pmole	-	picomole
RNA	-	ribonucleic acid
mRNA	-	messenger ribonucleic acid
rRNA	-	ribosomal ribonucleic acid
RNase	-	ribonuclease
rpm	-	revolution per minute
RT	-	Reverse Transcriptase
SAAP	-	streptavidin-alkaline phosphatase conjugate
SDS	-	sodium dodecyl sulfate / sodium lauryl sulfate
SSC	-	standard saline citrate
TBS	-	tris buffer saline
TCA	-	trichloroacetic acid
TE	-	**T**ris-**E**DTA
Tris	-	tris[hydroxymethyl]aminomethane
Tris-HCl	-	tris hydrochloride
U	-	unit
UV	-	ultraviolet
V	-	volt
v/v	-	volume per volume
w/v	-	weight per volume
X	-	times

CHAPTER 1

INTRODUCTION

The tomato is a very well known vegetable crop worldwide where it is extensively used in cooking, for flavoring, as a garnishing and for use with pasta. Tomato adds flavor to food and also supplies important nutrition for our everyday needs. Since tomatoes are popular fruit and in some cases are treated as vegetables, they provide a large boost to several industries. This is evident especially in the food industries and bakeries worldwide. Examples of the product offered by these industries are ketchup sauce, pizza sauce, spaghetti sauce and canned tomato puree. It also helps to promote other products by serving as a flavoring or a garnishing in food and bakery products. In 1999, the processed tomato production in the United States of America was more than 9 million metric tons (USA/USDA/Economic Research Service, 1999).

In recent nutritional and medical studies, tomato was found to be able to significantly reduce prostate cancer risks in men if taken daily (Gann *et al*., 1999; Arnold and Eckstein, 2001; Chen *et al*., 2001; Giovannucci, 2002; Giovannucci *et al*., 2002; Hadley *et al*., 2002). Tomato contains one of the most potent antioxidants among the carotenoids known as lycopene. This gives the tomato its attractive reddish or orangish appearance and is found in higher amounts in tomato than in any other fruits. Since humans cannot produce lycopene, it is obtained strictly from the diet and stored in the internal organs such as the liver, lung, skin, colon, prostrate gland, etc., at higher concentrations than any other known carotenoid. Besides its

ability at reducing prostate cancer and other types of cancer, lycopene was demonstrated to reduce cardiovascular diseases and strengthen the immune system. Lycopene has been made available in pills as a supplement and this in turn benefits the pharmaceutical industry. Lycopene is more concentrated in tomato sauce and represents the free form of lycopene that is easier to be absorbed by the body compared to the bounded form from fresh tomato fruits.

Tomato has been used extensively as a research model system as it is easy to grow, has a short life cycle and is easy to work with. Due to its short life cycle, tomato is a suitable subject for studying plant development. It is easy to germinate and fast to flower, which makes tomato a suitable study system for studying both vegetative and floral development. The tomato flowers are of a reasonable size and are easy to work with. However despite this, relatively little work has been done on flower development in tomato.

Floral development in plants is an exciting subject to study because of the unique developmental processes that occur in flowers. Flowering includes a combination of developmental processes such as vegetative development, organogenesis and embryogenesis. Organogenesis of floral organs involving several homeotic genes has been studied extensively for the past decade. We understand today the mechanism of floral organs formation in general that involves a family of transcription factors known as MADS box genes. However there is still a need to study floral determination from vegetative tissue as it is complicated and may vary from one plant species to another.

There has also been little research on molecular biology of zygotic embryogenesis despite its importance as a tissue that will eventually develop into fruit and seeds of economical importance. Several types of fruits are much sought after that are seedless. Some of these seedless fruits are watermelon, papaya, guava, banana, pomelo, etc. However seedless tomato is not sought after because the seeds and the pulp surrounding the seeds provide good taste and texture both in food and tomato puree. Artificial pollination is performed deliberately to increase seed numbers and for pulp formation, thus increasing the size of the fruit. Therefore it is crucial to study very early events in fruit development leading to seed development such as just prior to and after pollination. By unraveling the complex interaction between proteins in the pistil, we hope to shed light on the process of fertilization and fruit formation in this important fruit.

The objectives of this study are to isolate and characterize pistil predominant genes from tomato to understand the function of the tomato pistil. In order to carry out the objectives, a pistil cDNA subtraction library was constructed and screened for pistil predominant genes by reverse northern, followed by sequence analysis and other molecular characterization techniques such as Southern, northern and *in situ* hybridization.

CHAPTER 2

LITERATURE REVIEW

2.1 Tomato

The tomato is a dicotyledonous plant and its scientific name is *Lycopersicon esculentum*. It belongs to a family known as Solanaceae that includes other plants from other genus such as tobacco, chili, potato, petunia and eggplant. Tomato has a diploid chromosomal number of 24 (2n = 24). Today, tomato is planted worldwide for human consumption. Tomato is generally used as a flavoring and as a garnishing for home cooking and food industries.

Lycopene is a carotenoid that is present in tomatoes, processed tomato products and other fruits. It is one of the most potent antioxidants among dietary carotenoids. Antioxidants are protective agents that inactivate reactive oxygen damage. Antioxidants such as superoxide dismutase, catalase and glutathione peroxidase are naturally present within human cells. In addition, antioxidants such as vitamin E, vitamin C, polyphenols and carotenoids are available from food (Agarwal and Rao, 2000). In tomato, lycopene is the most prominent (abundant) carotenoid followed by beta-carotene, gamma-carotene and phytoene as well as several minor carotenoids. The antioxidant activity of lycopene as well as several other carotenoids and their abundance in tomatoes makes these foods rich sources of antioxidant activity (Beecher, 1998).

There are over 600 carotenoids found in nature where one, lycopene, is synthesized by plants and microorganisms but not by animals (Paiva and Russell, 1999; Agarwal and Rao, 2000). It is an acyclic isomer of beta-carotene and is highly unsaturated containing 11 conjugated and 2 unconjugated double bonds (Agarwal and Rao, 2000). In humans, the bioavailability of lycopene is greater from tomato paste than from fresh tomato (Gartner *et al*., 1997). Lycopene appears to be equally bioavailable from tomato juice and from supplements used in one study (Paetau *et al*., 1998).

In general vegetables, fruits and cooked tomatoes, together with olive oil, appear to be the nutritional traditions that account for the lower risk of several important chronic diseases, including coronary heart disease and a number of types of cancer associated with nutritional traditions, such as breast, colon and prostate cancer (Weisburger, 2002). In addition, prolonged tomato juice consumption increased plasma lycopene concentrations without significantly affecting cell-mediated immunity in well-nourished elderly subjects in a study (Watzl *et al*., 2000).

Stahl *et al*. (2001) demonstrated that it is feasible to achieve protection against UV light-induced erythema by ingestion of a commonly consumed dietary source of lycopene. Other than that, there was a study which indicated that consumption of commercial tomato juice increases plasma lycopene levels and the intrinsic resistance of LDL to oxidation almost as effectively as supplementation with a high dose of vitamin E, which also decreases plasma levels of C-RP, a risk factor for myocardial infarction, in patients with diabetes. These findings may be