AN ABSTRACT OF THE THESIS OF

Theresa T. Nguyen for the degree of Honors Baccalaureate of Science in Biology and Honors Baccalaureate of Arts in International Studies in Biology presented on May 23, 2011. Title: Molecular Analysis of Tomato Seed Germination and Tomato Production in Almería, Spain.

Abstract Approved: _____

Hiroyuki Nonogaki

Tomato is considered to be one of the most important vegetables for its value as a source of nutrients and antioxidants, and as a model crop for biological research, including seed biology. In this thesis, tissue-specific gene expression and its regulation in tomato seeds were analyzed to understand the mechanisms underlying tomato seed germination, which is key to producing a healthy tomato plant. Endosperm cap-specific genes were identified using GeneChip® analysis. Tomato *ETHYLENE RESPONSE FACTOR1* (*TERF1*), a hormone-signaling gene was found to be a potential upstream regulator of multiple endosperm cap-specific genes. In addition to the basic research on tomato seed germination, a case study of tomato production and distribution in Almería, Spain, one of the most important tomato exporting sectors in the world, was performed as part of international degree research. In this case study, the methods and efficiency of the tomato production systems in hundreds of greenhouses, so called '*mar del plástico*' (sea of plastic) and the tomato distribution systems were analyzed.

Key Words: tomato seed germination; tomato production; Almería, Spain; osmotin; ethylene-signaling pathway

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Molecular Analysis of Tomato Seed Germination and Tomato Production in Almería, Spain

by

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APPROVED:

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Director, International Degree

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Theresa T. Nguyen, Author

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LIST OF ABBREVIATIONS

ABA	abscisic acid
bp	base-pairs
C	cotyledon-half embryo
d	day(s)
DIG	digoxigenin
EC	endosperm cap
GA	gibberellin
GFP	green fluorescent protein
GUS	β-glucuronidase
h	hour(s)
HCl	hydrochloric acid
KAN	kanamycin
LE	lateral endosperm
$MgCl_2$	magnesium chloride
min	minute(s)
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
R	radicle-half embryo
RT	reverse transcription
s	second(s)
SDS	sodium dodecyl sulphate
SSC	sodium chloride sodium citrate buffer
UV	ultraviolet

DEDICATION

I would like to dedicate this thesis to my sister, Maria B. Nguyen, for her constant support and motivation throughout the writing process and to my parents, Joseph T. Nguyen and Rose H. Nguyen, for their love and support through all of my years. I would also like to dedicate this thesis to my mentor, Dr. Hiro Nonogaki, because without him, this project would not be possible, indefinitely. Thank you for all your hard work and patience with me as I learn to exceed expectations no matter what.

Molecular Analysis of Tomato Seed Germination and Tomato Production in Almería, Spain

Chapter 1

General Introduction

Theresa T. Nguyen

Tomato: A major vegetable crop

Tomato (*Solanum lycopersicum*) is a major vegetable crop that is widely produced and consumed globally for both the fresh vegetable market and processed food industries (Heuvelink, 2005). Tomatoes can be eaten fresh or in a variety of processed forms including tomato preserves (*e.g.,* juice and paste), dried tomatoes (*e.g.,* powder and flakes), and other types (*e.g.,* tomato sauce and ketchup) (Heuvelink, 2005). Tomato is processed in so many different types of food that its presence is often overlooked. Ripe tomato provides a substantial amount of essential nutrients including vitamins A and C, potassium, and various others. Mostly importantly, the red pigment in tomato, lycopene, is a powerful antioxidant shown to have anti-cancer properties (Jones, 2008).

Tomato belongs to the large Solanaceae family and is closely related to potato (*Solanum tuberosum*), pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), and tobacco (*Nicotiana tabacum*) (Nevins, 1987). Therefore, knowledge obtained from tomato research can be easily applied to these commercially important crops, which makes tomato a major model crop in plant genetics. Tomato is easily cultivated and produces thousands of seeds per plant. A single tomato plant contains 5 to 25 clusters of fruits, with each cluster containing 10 fruits, and each fruit containing more than 100 seeds (Heuvelink, 2005). Tomato seeds carry the genetic information important for agricultural production. At the same time, tomato seeds have become a model system and are used to study the physiology and biochemistry of seed development, germination, and dormancy. It is important to understand the physiological and molecular mechanisms of seed germination, because they are contributing factors to successful plant development

and maintenance. With vast literature available for tomato, it has and will continue to receive great attention in the fields of basic science, biotechnology, and horticulture.

Tomato seed germination

It is evident throughout history that seeds have always had an intimate connection with human life. Throughout the time of the hunter-gatherer society, the origin of agriculture, and the present time, manipulation of seeds has formed the basis of our social and cultural development (Black, 2000). Humans rely on seeds as a dietary staple to feed the vast population worldwide; these include, but are not limited to, legumes (*e.g.,* beans and peas), cereals or grains (*e.g.,* rice and wheat), and nuts (*e.g.,* almonds and cashews). The most substantial form of energy yielded from seeds is carbohydrate from cereals, oil from oilseeds, and protein from legumes. Many seeds are important sources of antioxidants including vitamin E and provide raw materials potentially used for a wide range of non-food products including plastics, lubricants, paints, cosmetics, and therapeutic agents (Murphy, 2000).

In addition to providing energy, nutrients, and industrial raw materials, seeds are also the genetic resources or dispersal units, from which plants grow, offering their fruits, leaves, shoots, and roots as primary food sources to maintain the life forms of the ecosystems. Ecosystems are networks of complex inter-dependent interactions between plants and animals, and the physical-chemical environment (Schulze et al., 2005). Ecosystems and its inhabitants are the fundamental life supporting system of the planet, which ultimately impacts the well-being and the survival of humans (Corvalan et al.,

2005). For that reason, it is critical that the propagation of vegetation through seeds is sustained to ensure balanced ecosystems.

Tomato has become a favored model system for characterizing the mechanisms involved in seed germination due to the well-understood features of seed tissues, such as the testa, endosperm, and embryo (Figure 1). The embryo is the living part of the seed

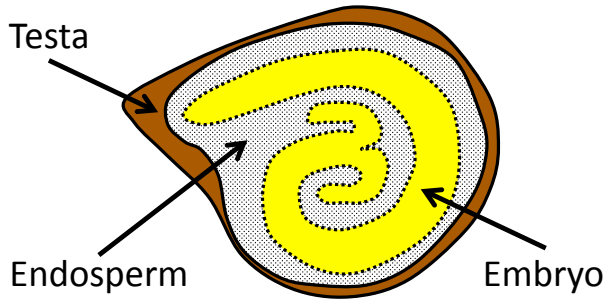

Figure 1. Three major tissues of a tomato seed involved in germination control. The testa plays a protective role, the endosperm provides nutrition to the embryo, the embryo will develop into the plant.

that contains precursor tissues, which will form the leaves, roots, and stems of an adult plant. The embryo is comprised of the embryonic axis and the cotyledons, or embryonic leaves. Tomato embryo has two cotyledons, and therefore, tomato is a dicotyledonous plant. The embryonic axis consists of the radicle, which will penetrate through the endosperm and testa to develop into the main root of the plant, and of the hypocotyl, which will develop into the plant stem to transport water to the cotyledons after germination. The endosperm is a layer of living, non-embryonic, storage tissue that surrounds the embryo and functions as a nutrient reserve during embryogenesis (Bewley and Black, 1994). In the case of tomato, the living endosperm persists in a mature seed.

The micropylar region of the endosperm that encloses the radicle tip of the embryo is cone-shaped, and is called endosperm cap. This tissue is rigid and provides the mechanical barrier against embryo expansion (Groot and Karssen, 1987; Chen and Bradford, 2000). The outermost layer of a seed is the testa, which fully encloses the endosperm and embryo to provide a physical barrier protecting the internal seed tissues from environmental damages during seed dispersal, storage, and germination (Bewley and Black, 1994).

In a strict sense, seed germination is defined as physiological events occurring in hydrated seeds before protrusion of the embryo from the endosperm and testa (Nonogaki et al., 2007). Dry seeds break the desiccated state through the uptake of water (called imbibition). Imbibition occurs even in dead or dormant seeds. Seed dormancy is a phenomenon in which intact and viable seeds fail to germinate even under optimal conditions. Dormancy prevents the seeds from entering the last phase of germination, which is the completion of germination or radicle emergence (Bewley and Black, 1994). Although seed dormancy is generally viewed as an undesirable characteristic in agriculture, in terms of initiating agricultural production, it is an evolved adaptive trait that optimizes the distribution of germination within a population of seeds over time (Bewley, 1997). Dormancy is gradually terminated by a single factor or combinations of the following factors: anaerobic conditions, darkness, prolonged exposure to white light, prolonged exposure to far-red light, temperatures above maximum for germination, temperatures below minimum for germination, water stress, and afterripening or dry storage (Bewley and Black, 1994). In tomato, seed dormancy is released with a decrease in abscisic acid (ABA), a germination inhibitor hormone (Groot et al., 1992). After

breaking seed dormancy, germination is completed with the emergence of the radicle from the micropylar region of the endosperm.

Two opposing forces contribute to the completion of seed germination: the mechanical resistance of the endosperm cap (and testa) and the growth potential of the embryo (Figure 2). The mechanical resistance of the endosperm cap physically impedes radicle growth. Once the embryo receives signals to grow, such as gibberellin (GA), a germination promoting plant hormone, there is an increase in the growth potential of the

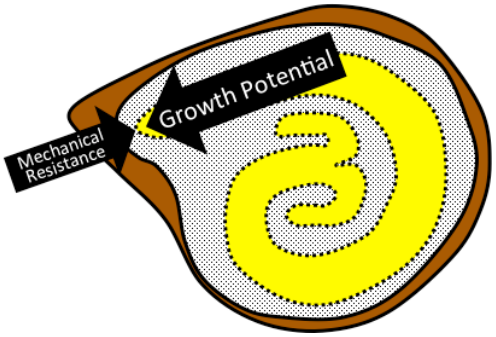

Figure 2. Opposing factors involved in tomato seed germination - the growth potential of the embryo and the mechanical resistance of the endosperm cap. In order for germination to occur, the growth potential of the embryo must exceed the mechanical resistance of the endosperm cap.

cells in the embryonic axis, primarily the radicle-hypocotyl region. The radicle continues to elongate and simultaneously the endosperm cap is weakened to allow for radicle penetration (Groot and Karssen, 1987). However, germination is completed only when the embryo growth potential exceeds the mechanical resistance of the endosperm cap. The endosperm cap cell wall is composed of 60% mannan polysaccharides in the form of galacto-, gluco-, or galactoglucomannans, which constitute to cell wall rigidity and also

serves as major carbohydrate reserves (Dahal et al., 1997). The cell wall of the endosperm cap is weakened by the degradation of the galactomannan chains, consequently lowering the mechanical resistance of the micropylar region to allow for radical protrusion. Three enzymes, α-galactosidase, β-mannosidase, and endo-β-mannanase, catalyze modifications of the galactomannan chains. The enzyme α-galactosidase is responsible for cleaving galactose from the mannan backbone, β-mannosidase cleaves mannose residues from the terminal end of the mannan backbone, and endo-β-mannanase hydrolyzes internal bonds within mannan chains (Bewley and Black, 1994). Therefore, characterizing a class of factors expressed in the endosperm cap, including cell wall-associated genes, is important for further understanding of the mechanisms of seed germination.

Purpose of study

In this thesis research, the mechanism of seed germination, which is important for tomato propagation, was investigated. The molecular and biochemical research focused on gene expression, with an emphasis on the regulation mechanisms of tissue-specific gene expression. In addition, an international case study of tomato production and distribution was also conducted, in fulfillment of an International Degree.

Molecular Analysis of Tomato Seed Germination and Tomato Production in Almería, Spain

Chapter 2

Identification of Endosperm Cap-Specific Genes

Theresa T. Nguyen

INTRODUCTION

Of the three enzymes known to degrade the cell wall of the endosperm cap of tomato seeds, endo-(1,4)-β-mannanase (EC 3.2.1.78) is most intensively investigated, because this endo-type enzyme is the most efficient in degrading the galactomannan polymers, and because it plays a critical role in endosperm weakening (Cantliffe et al., 1984; Groot et al., 1988). *Solanum lycopersicum endo-β-mannanase 2* (*SlMAN2*, previously *LeMAN2*) encodes an endo-β-mannanase in tomato (Nonogaki et al., 2000).

A technique called tissue-printing, in which mRNA localization is determined in tomato seed prints using hybridization with an RNA probe (in this case, *SlMAN2* probe), showed that *SlMAN2* was expressed exclusively in the endosperm cap prior to radicle emergence. Expression of *SlMAN2* is induced in the endosperm cap by biologically active GA (Nonogaki et al., 2000). GA plays a role in the control of seed germination, stem elongation, leaf expansion, and flower and seed development (Yamaguchi, 2008). *SlMAN2* is a key gene in endosperm cap cell wall degradation and tissue weakening, which are requirements for the completion of tomato seed germination. Upstream regulators of *SlMAN2* need to be identified in order to further understand the molecular mechanisms of *SlMAN2* regulation and the induction of germination.

High-throughput microarray analysis (GeneChip® analysis) was performed using RNA extracted from the micropylar region in order to determine upstream regulators of *SlMAN2* and other potential genes involved in germination. In addition, other seed tissues were also analyzed to compare endosperm- and embryo-specific gene expression. This chapter focuses on the identification of multiple endosperm cap-specific genes expressed during tomato seed germination and the characterization of those genes.

MATERIALS AND METHODS

Plant materials and germination

Tomato (*Solanum lycopersicum* Mill.) seeds (cv. Moneymaker) were used in this study. Tomato seeds (100-200) were plated on two layers of filter paper (No. 2 Whatman Inc., Clifton, NJ) moistened with 3.5 mL water in 9-cm Petri dishes, and were incubated at 25°C under dark conditions. For germination, counts were recorded periodically over a 100 h time period.

RNA isolation from tomato seed tissues

For RNA extraction, tomato seeds (18-h-imbibed) were dissected following the previously reported method (Nonogaki et al., 1992). Seeds were dissected into the micropylar tip and the remainder of the seed with intact testa. Embryonic tissues were removed from each part using forceps. The embryo-less micropylar tip and the embryo-less remainder of the seed were called the endosperm cap (EC) and lateral endosperm (LE), respectively. The extracted embryonic tissues were divided into two halves: the radicle-half embryo (R) and the cotyledon-half embryo (C). RNA was extracted from these four tissues using standard phenol-SDS extraction (Sambrook et al., 1989).

Microarray (GeneChip®) analysis

In collaboration with RIKEN Plant Science Center, Japan, the extracted RNA from four different tissues of tomato seed was hybridized with the GeneChip® Tomato Genome Array (Affimetrix) designed specifically to monitor gene expression in tomato. The comprehensive array consists of over 10,000 tomato probe sets to interrogate over 9,200

transcripts. Hybridization and other procedures were performed following the GeneChip® Expression Analysis Technical Manual (http://www.affymetrix.com/estore/browse/products.jsp?navMode=34000&productId=131513&navAction=jump&aId=productsNav#1_3). Raw data obtained from the hybridization was decoded and analyzed using the statistical software called 'R'. Gene annotation was analyzed using the tools at Tomato 10k Microarray Sequence ID (http://www.plexdb.org/modules/PD_probeset/annotation.php?GeneChip=Tomato10k).

Reverse transcription PCR and cloning

RNA extracted from 18-h-imbibed tomato seeds was used to amplify *NP24* cDNA by reverse transcription (RT) PCR, using primers 5' TTCAACAAACATGGGCTACTTG 3' and 5' TGAGATGTAACTCTTATTCCGGTCT 3'. The amplified fragment was cloned to pCR4.0 TOPO vector (Clonetech), and transcribed to make an antisense RNA probe for tissue printing.

Tissue printing and hybridization

Germinating (18-h-imbibed) tomato seeds were bisected longitudinally using a sharp, double-edged razor blade. Half seeds covered in plastic film were pressed with the cut surface down onto a positively charged membrane (Hybond-N^+, Amersham Pharmacia Biotech) for approximately 15 s to transfer a detectable amount of mRNA molecules to the membrane. Six layers of filter paper were placed underneath the membrane before pressing the seed to optimize depth and clarity of the print. After removal of seed tissue, the membrane was UV-cross-linked and pre-hybridized for 15 min at 60°C with

hybridization buffer containing 50% (v/v) deionized formamide, 4% (w/v) blocking reagent (Roche), 0.2% (w/v) SDS, and 5x SSC before being hybridized for 16-18 h at 60°C in the hybridization buffer with 100 ng mL^{-1} DIG-labeled, *NP24* RNA probe. Following incubation, the membrane was washed stringently and blocked with 5% nonfat milk. The DIG-labeled probes were detected using alkaline phosphatase (AP)-conjugated anti-DIG antibody and the signal was colorimetrically detected with 0.18 M Tris-HCl buffer, pH 8.8, containing 0.025 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate, 0.1 mg mL^{-1} nitroblue tetrazolium, and 2 mM $MgCl_2$ ((Nonogaki et al., 2000; Nonogaki and Bradford, 2003). Reaction time with AP varied from 1 h to overnight depending on the signal strength.

Promoter:reporter analysis

A promoter:reporter construct was created by fusing the 5' upstream sequence (1,903~+321) of the *NP24* gene and the coding sequences of a green fluorescent protein (GFP) and β-glucuronidase (GUS) (termed as *NP24:GFP-GUS*), and cloned to pGPTV-KAN vector. Tomato callus was transformed with *Agrobacterium tumefaciens* in collaboration with Prof. Ryszard Gorecki's laboratory at University Warmia and Mazury, Poland.

RESULTS

The first radicle emergence, which indicates the completion of germination, was observed approximately 40 h after the start of imbibition in the tomato seeds (Figure 3).

Cell wall-associated genes that are endosperm cap-specific are expressed at relatively late stages of germination (Nonogaki et al., 2000). Regulatory genes that are potentially

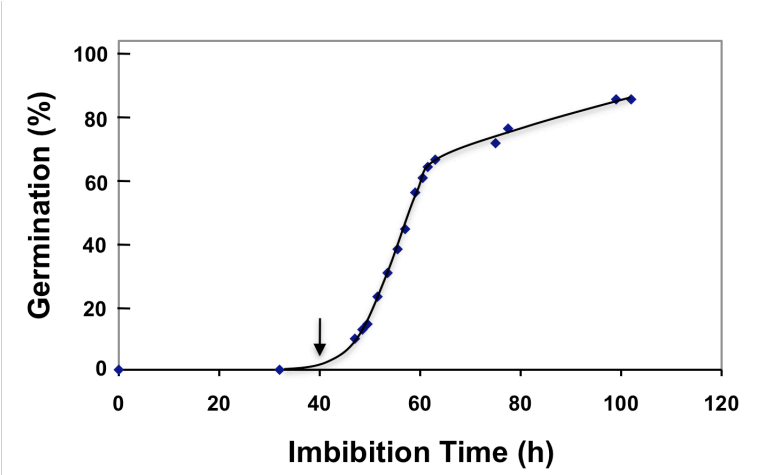

Figure 3. Germination time course of the tomato seeds used in this study. The first radicle emergence was observed approximately 40 h after the start of imbibition (indicated by an arrow).

involved in the induction of cell wall-associated genes or hormone metabolism and signal transduction-associated genes are expected to be expressed at relatively early stages of germination. To detect genes representative of both early and late stages of germination, an imbibition time point was selected between the range of 0 h to 40 h. The most appropriate and practical imbibition time of 18 h was used. Tomato seeds were imbibed for 18 h and then dissected into the micropylar part and the remaining part (termed as the "lateral" part). The embryonic tissues were removed from the micropylar and lateral parts and referred to as endosperm cap (EC) and lateral endosperm (LE), respectively (Figure 4). Although both parts still contain the testa, the term endosperm is used in this thesis because the testa is non-viable tissue in mature tomato seeds and does not alter

expression analysis. The embryo was also divided into radicle- and cotyledon- halves and referred to as radicle-half embryo (R) and cotyledon- half embryo (C), respectively (Figure 4). RNA extracted from these four tissues was used for GeneChip® analysis.

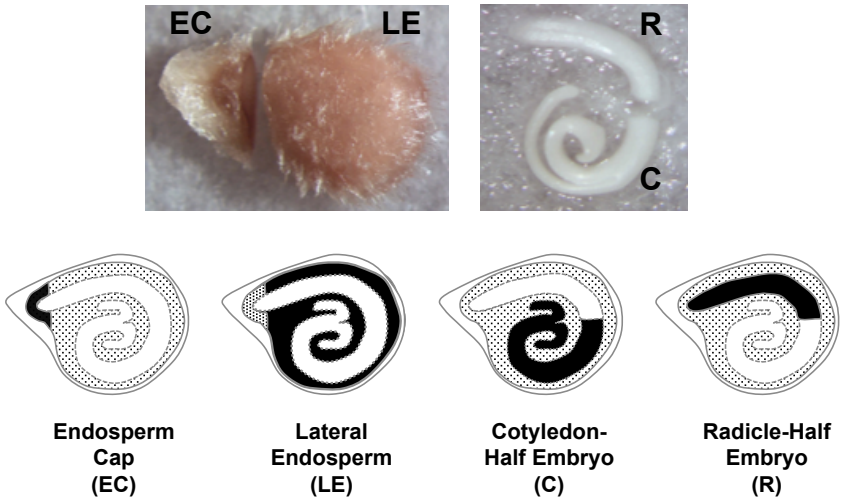

Figure 4. Photographs (top) and schematic representation (bottom) of tomato seed tissues. Seeds were imbibed for 18 h and then dissected into four different tissues. Intact seeds were first dissected into the micropylar and the rest (lateral part) of the seed. After removal of the embryonic tissue, micropylar with testa was designated as endosperm cap (EC) and the rest of seed with testa was designated as lateral endosperm (LE). The embryo was divided into the cotyledon-half embryo (C) and the radicle-half embryo (R).

Tissue-specific gene expression, particularly EC-specific gene expression, and mechanisms controlling the tissue-specific expression were focused on in this analysis. Initially, an emphasis was put on genes whose expression in one of the four tissues (EC, LC, R or C) was more than 5-fold (>5 fold) higher when compared to their expression in the other tissues. Subsequently, the analysis was expanded to the 2-fold-enriched genes as well. Thirty-four EC-enriched, four LE-enriched, one R-enriched, and five C-enriched genes were detected from the list of genes with >5-fold enrichment. The numbers of EC-,

LE-, R- and C-enriched genes increased to 150, 135, 72 and 29 when the analysis was expanded to genes with >2-fold enrichment (but smaller than 5 fold). There were genes expressed high in both EC and LE but low in R and C, i.e., endosperm-enriched genes, while other genes were enriched in both R and C, i.e., embryo-enriched genes. The >5-fold EC-enriched genes with available annotations were summarized and are ranked in order of relative expression levels (highest to lowest) in EC (Table 1). The >2-fold EC-enriched genes with available annotations were also summarized and ranked (Table 2). EC-enriched genes that have been characterized are shown in color: cell wall-associated genes are highlighted in green, pathogenesis-related genes are highlighted in orange, and hormone-associated genes are highlighted in green.

Table 1. Genes enriched (>5 fold changes) in the endosperm cap (EC) of 18-h-imbibed tomato seeds, for which information of gene annotation was obtained.

Gene Annotation for >5-fold changes	GenBank #	Relative Expression			
		EC	LE	R	C
NP24 protein	AF059488.1	1,063	180	7	0
expansin (LeEXP2)	AF096776.1	882	34	95	5
sucrose synthase	L19762.1	773	80	12	9
chitinase	BG629640	709	48	103	4
expansin 11 (exp11)	AJ560646.1	476	15	33	1
Pathogenesis-related PR5-like protein	AY257487.1	457	88	24	14
xyloglucan-specific fungal endoglucanase inhibitor (Xegip)	AY155579.1	297	9	4	1
(1-4)-beta-mannan endohydrolase (MAN2)	AF184238.1	279	15	0	0
endo-beta-1,4-D-glucanase	AF308936.1	269	5	18	0
weakly similar to peroxidase	CK720576	225	12	0	0
pathogenesis related P23	X70787.1	200	19	1	0
Similar to C3HC4-type RING finger	BT012911.1	188	26	21	17

expansin (EXPA6)	AF059490.1	117	5	16	4
GAST1	BG626882	108	10	1	0
similar to peroxidase	AI773309	95	16	2	1
similar to esterase	AW034398	90	13	2	1
aquaporin LePIP1	AY725511.1	69	5	4	11
asparagine synthetase	AW625684	66	11	9	8
similar to glycosyltransferase	BI421517	53	1	7	1
ethylene response factor (TERF1)	AY044236.1	47	5	1	0
similar to IAAs	AW034122	46	1	8	0
similar to myo-inositol 1-phosphate synthase (INS-1P)	BT013505.1	43	8	3	2
similar to elicitor induced gene	AI895341	38	2	1	0
similar to GH3, indole-3-acetic acid-amido synthetase	BT013446.1	37	2	2	1
aldehyde oxidase (AO1)	AF258808.1	33	5	0	1

*Colors are used to highlight cell wall (green)-, pathogenesis (orange)- and hormone (grey)-associated genes.

Table 2. Genes enriched (5>fold changes>2) in the endosperm cap (EC) of 18-h-imbibed tomato seeds, for which information of gene annotation was obtained.

Gene Annotation for >2-fold changes	GenBank #	Relative Expression			
		EC	LE	R	C
expansin A4	AF059488.1	1303	34	170	34
xyloglucan endotransglycosylase XET4	AF186777.1	1022	111	206	8
S-adenosyl-L-methionine synthetase-like	BG628423	393	125	146	46
pectin methylesterase	U49330.1	244	51	86	26
endo-beta-1,4-D-glucanase Cel8	BT013727.1	153	8	35	7
IAA2 protein	AF022013.1	148	11	32	19
IAA1 protein	BI209735	142	33	71	57
pi1 protein	BT012973.1	106	23	23	6
beta-1,3-glucanase	M80608.1	74	21	1	1
IAA8 protein	BT014412.1	47	10	15	7
Lycopersicon esculentum clone 132101F, mRNA sequence	BT013446.1	38	3	3	1

*Colors are used to highlight cell wall (green)-, pathogenesis (orange)- and hormone (grey)-associated genes.

Cell wall-associated genes

The previously classified *SlMAN2* [AF184238.1] gene (Nonogaki et al., 2000) was detected with >18-fold enrichment in EC of tomato seeds along with other cell wall-associated genes (Table 1, highlighted in green**)**. Other known EC-specific genes, *expansin A4* (*EXPA4* or *LeEXP4* [AF059488.1]) (Chen and Bradford, 2000) and *xyloglucan endotransglycosylase* (*XET4* [AF186777.1]) (Chen et al., 2002) were detected with 4.2- and 4.9-fold enrichment in EC, respectively (Table 2). These genes served as an excellent internal control for EC-enrichment and verified the procedure for tissue isolation and the quality of GeneChip® analysis providing high confidence in our data and further analysis.

The >5-fold EC-enriched genes included other cell wall-associated genes, such as other *expansins* (*EXP2* [AF096776.1], *EXPA6* [AF059490]), *EXP11* [AJ560646.1]), and *endo-β-1,4-glucanase* [AF308936.1] (Table 1, highlighted in orange). Another *endo-β-1,4-glucanase* gene (*Cel8* [BT013727.1]) was enriched in EC more than 4-fold (Table 2.2). The >2-fold EC-enriched genes included *pectin methylesterase* (*PME*, [U49330.1]) (Table 2). *PME* has been suggested to be involved in the weakening of tomato endosperm cap during germination (Downie et al., 1998; Sitrit et al., 1999). A *glycosyltransferase* gene (BI421517) detected among >5-fold EC-enriched genes (Table 1) exhibits similarity to *Arabidopsis thaliana* and *Populus trichocarpa* enzymes involved in xylan synthesis (Kong et al., 2009). These results supported the idea that cell wall modification occurs exclusively in EC and that the change is a prerequisite for radicle emergence from tomato seeds (Nonogaki et al., 2007).

Pathogenesis-related genes

Another group of genes predominantly found in the >5-fold EC-enriched genes were pathogenesis related (PR)-genes (Table 1, highlighted in orange), such as *NP24* (or *osmotin*, [M21346.1]), which showed the highest expression level of all EC-enriched genes, *chitinase* (BG629640) (Danhash et al., 1993), *PR5-like* (AY257487.1), *P23* (X70787.1, SGN-U581103), a homologue to *NP24*, and an unnamed gene (AI895341) similar to elicitor-inducible genes. The >2-fold EC-enriched genes included *β-1,3-glucanase* (M80608.1) (Kan et al., 1992) and another pathogen- and wound-induced gene (BT012973.1). The expression of the *chitinase* (*Chi9*) and *β-1,3-glucanase* (*GluB*) in EC during tomato seed germination was previously shown (Wu et al., 2001). It is possible that *chitinase* and *β-1,3-glucanase* could contribute to cell wall modification through their ability to degrade their substrate polysaccharides, i.e., chitin and callose, respectively, however, there is no evidence to support their involvement in active degradation of endosperm cap cell wall (Wu et al., 2001). The characterization of other typical PR-genes, *NP24* (>5.9 fold), *PR5-like* (>5.1 fold) and *P23* (>10.5 fold) as EC-enriched genes (Table 1) suggests that there are some mechanisms similar to pathogen- or wounding response present in EC during tomato seed germination. In addition, *xyloglucan-specific fungal endoglucanase inhibitor protein precursor* gene (*Xegip*, [AY155579.1]), known to function in pathogen response in tomato (Qin et al., 2003), was also enriched in EC (Table 1). The >5-fold EC-enriched genes had two genes (CK720576 and AI773309) encoding proteins similar to peroxidase. Peroxidases have also been suggested for pathogen responses (Morohashi, 2002).

Hormone metabolism- or signal transduction-associated genes

Other EC-enriched genes were hormone metabolism- or signal transduction-associated genes (Table 1 and 2, highlighted in grey). *GAST* (BG626882) that is induced by exogenous GA in tomato (Shi et al., 1992) was enriched in EC more than 10-fold. Although there is little information about *GAST* involvement in the regulation of cell wall degradation, it is known that EC-enriched cell wall proteins are GA-inducible (Groot and Karssen, 1987; Chen and Bradford, 2000). The microarray analysis also identified ethylene-associated genes. *TERF1* (AY044236.1) (Huang et al., 2004), one of the ethylene response factors, was enriched >9 fold (Table 1). The >2-fold EC-enriched genes included a gene (BG628423) similar to *Nicotiana tabacum S-adenosylmethionine synthase* (*SAM*) (AF321140.1) (Table 2), which indicates EC-specific ethylene biosynthesis. These results are consistent with recent discoveries of the key regulatory role of ethylene in EC weakening in seeds of other species such as *Lepidium sativum* and *Arabidopsis thaliana* (Linkies et al., 2009).

A gene (AW034122) encoding a protein similar to auxin signal transduction proteins (IAAs) was detected among the >5-fold EC-enriched genes (Table 1). Tomato *IAA1* (BI209735), *IAA2* (AF022013.1), and *IAA8* (BT014412.1) were also enriched in EC (Table 2), suggesting a possible involvement of auxin in EC-specific events. Interestingly, a gene (BT013446.1) similar to *GH3*, which encodes an *indole-3-acetic acid-amido synthetase*, was also enriched in EC, suggesting EC-specific auxin conjugation. An *aldehyde oxidase* (*AO1*, AF258808.1), which has potential to catalyze the final steps of both auxin and ABA biosynthesis (Min et al., 2000), was detected among the >5-fold EC-enriched genes.

Characterization of novel endosperm cap-enriched genes

We characterized expression patterns of the newly identified EC-enriched to verify the data obtained from GeneChip® analysis. RT-PCR was performed using RNA extracted from 18-h-imbibed tomato seeds in order to amplify *NP24* (Table 1) cDNA, then cloned into a vector. After confirming the *NP24* identity by sequencing, an RNA probe was synthesized to *NP24* and then used for tissue printing to examine localization of mRNA expression. Strong signal was detected exclusively in EC of germinating tomato seeds verifying the specificity of *NP24* expression to EC and confirmed the quality of GeneChip® analysis (Figure 5A). Recall that *NP24* was detected as the strongest expressed EC-enriched gene by GeneChip® analysis. The 5' upstream sequence of *NP24* can be a strong enough promoter to drive EC-specific expression of foreign genes for development of technology to control seed germination. Therefore, we characterized the 5' upstream sequence (-1,903~+321) of *NP24* and took advantage of a vector containing dual reporters: green fluorescent protein (GFP) and β-glucuronidase (GUS) to create *NP24:GFP-GUS* construct (Figure 5B). Wild-type tomatoes were then transformed with *Agrobacterium* containing *NP24:GFP-GUS*. When transgenic seeds were harvested and examined for reporter gene expression, a strong EC-enriched signal was observed along with minor reporter signals from LE and the embryo tissues (Figures 5C and 5D).

GeneChip® analysis had indicated that expression of *TERF1* in EC was relatively low compared to expression of other EC-specific genes. However, its enrichment specifically in EC was more that 9-fold (Table 1). Therefore, we performed RT-PCR

using RNA extracted from EC, LE, R and C to test the tissue- and stage-specific expression of *TERF1*. The spatial patterns of *TERF1* expression were consistent with the

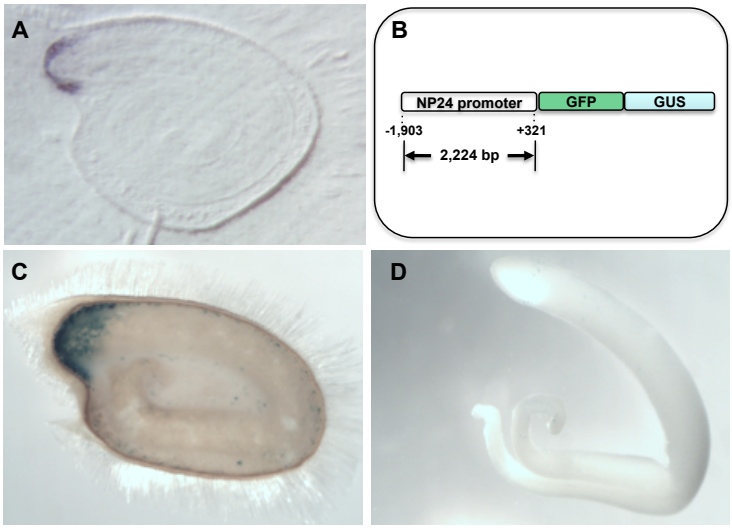

Figure 5. Endosperm cap (EC)-enriched expression of *NP24* in tomato seeds. (A) Tissue printing of 18-h-imbibed tomato seed, probed with a digoxigenin (DIG)-labeled, antisense *NP24* probe. The purple signal, which resulted from colorimetric reaction by an alkaline phosphatase conjugated with anti-DIG antibody used for probe detection, represents the localization of *NP24* mRNA. (B) Schematic representation of the reporter gene construct used for the characterization of 5' upstream sequence of *NP24*. The promoter region (-1,903~+321) was placed upstream of the green fluorescent protein (GFP) and β-glucuronidase (GUS) coding regions (named *NP24:GFP-GUS*). (C) GUS staining of the endosperm (plus testa) of transgenic seed expressing *NP24:GFP-GUS*. (D) GUS staining of the embryo of transgenic seed expressing *NP24:GFP-GUS*.

GeneChip® data and EC enrichment of *TERF1* was clear in the RT-PCR data (Figure 6A)**.** By analyzing temporal patterns of *TERF1* expression in imbibed tomato seeds, low *TERF1* accumulation was detected in dry seeds. *TERF1* mRNA accumulation was observed when imbibed seeds were pre-chilled at 4°C for 3 d (Figure 6B)**.** When pre-

chilled seeds were transferred to 25°C for germination, there was a temporal reduction in *TERF1* accumulation (~6 h), followed by a progressive increase toward radicle emergence **(**Figure 6B**),** suggesting the involvement of this gene in germination events.

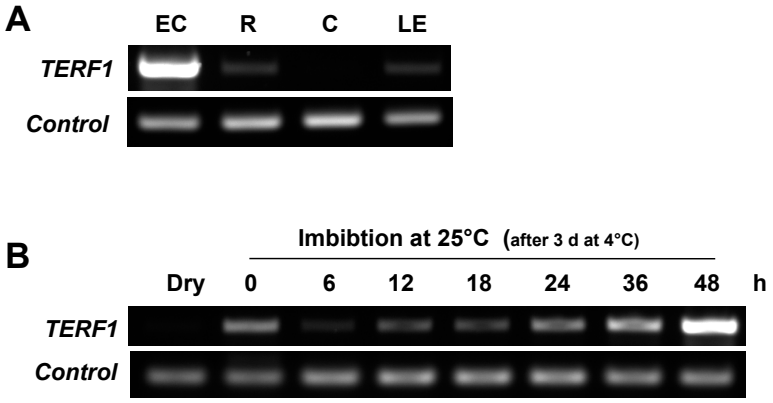

Figure 6. Spatial and temporal expression of *TERF1* in tomato seeds. (A) RT-PCR for *TERF1* mRNA accumulation in the endosperm cap (EC), lateral endosperm (LE), radicle-half embryo (R) and cotyledon-half embryo (C). An expressed, house-keeping unigene SGN-U346908 was used as control for RT-PCR. (B) RT-PCR for *TERF1* mRNA accumulation in dry tomato seeds (Dry), seeds imbibed at 4°C for 3 d (0 h) and seeds imbibed further at 25°C for indicated period of time (6-48 h). An expressed, house-keeping unigene SGN-U346908 was used as control for RT-PCR.

DISCUSSION

It is known that *SlMAN2* encodes for the endosperm cell wall modifying enzyme endo-β-mannanase and is induced by GA (Nonogaki et al., 2000); however, upstream regulators of *SlMAN2* remain unknown. Taking advantage of the tomato GeneChip®, a high-throughput microarray analysis was performed to identify genes expressed in the endosperm cap during tomato seed germination. *SlMAN2* was highly enriched in the

endosperm cap, which confirmed the quality of GeneChip® analysis and its endosperm cap-specific expression. Other endosperm cap-enriched genes included cell wall-associated genes, pathogenesis related-genes, and hormone metabolism- or signal transduction-associated genes (Table 1).

It is physiologically advantageous for seeds to use PR-genes for both cell wall degradation and defense against microorganisms during germination. During seed development, the endosperm accumulates food reserves including carbohydrates (mannan polysaccharides), oils, and proteins. These organic materials are stored in seeds for reserve mobilization, which support seedling growth following germination (Bewley and Black, 1994). However, these storage reserves are also an attractive food source for microorganisms. During the transition from germination to post-germination, the rupture of the endosperm cap leaves seeds vulnerable to microorganisms. Interestingly, *NP24*, a PR-gene, was found to have the strongest EC-enriched expression during this transition stage, indicating that it is involved in EC-specific events in addition to other known EC-specific PR-genes, *Chi9* and *GluB* (Wu et al., 2001).

Plant hormones like ABA and GA are important signaling molecules that impose changes in the environment to seeds during germination (Yamaguchi et al., 2007). The detection of *AO1*, which could potentially catalyze auxin and ABA biosynthesis, suggests that EC-specific auxin and/or ABA biosynthesis occurs during tomato seed germination. As mentioned above, *TERF1* expression is highly enriched in the EC during tomato seed germination. In tomato leaf tissue, *TERF1* is known to bind to multiple, repetitive DNA-binding motifs (AGCCGCC) present in upstream and coding regions of the endosperm cap-enriched PR-gene, *NP24* (Huang et al., 2004) (Figure 7A and 7B). The same motif

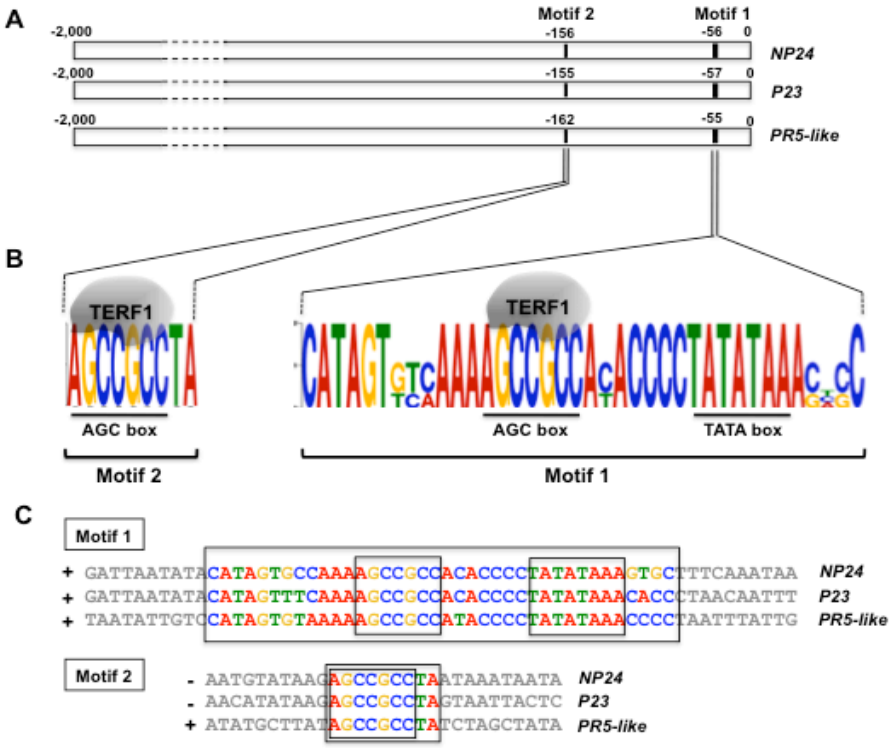

Figure 7. Consensus DNA motifs found in the 5' upstream sequences of EC-enriched *NP24*, *P23,* and *PR5-like*. (A) Schematic representation of the 5' upstream sequences of NP24, P23, and PR5-like to indicate the positions of two consensus DNA motifs commonly found in the three genes. Negative numbers indicate the positions of the motif starting sites relative to the predicted transcription initiation sites (0). (B) Conserved DNA motifs (termed Motifs 1 and 2) found in the 5' upstream sequences of *NP24*, *P23* and *PR5-like*, using the Multiple Em for Motif Elicitation (MEME) Suite (http://meme.sdsc.edu/meme4_4_0/intro.html). The TATA box contained in Motif 1 and AGC boxes contained in Motifs 1 and 2 are highlighted with underlines. The schematic shows TERF1 binding to AGC box. (C) Alignment of Motif 1- and Motif 2-containing regions of *NP24*, *P23* and *PR5-like*. The symbols (+) and (-) shown to the left of the sequences indicate direction of the motifs relative to the promoter sequences (plus- and minus strand).

exists in the promoter regions of *P23* and *PR5-like* that were found to be specific to EC (Figure 7C). It is likely that *TERF1* is a master regulator of multiple PR-genes in tomato seeds. However, this scheme is yet to be examined. Ethylene, a hormone that regulates stress response in plants, has been shown to induce *TERF1* expression (Huang et al., 2004). The EC-enriched expression of *TERF1* suggests that the ethylene-signaling pathway is involved in the regulation of tomato seed germination.

GA is thought to be synthesized in the elongation zone of the radicle and diffuse to the EC to induce EC-specific genes (Groot and Karssen, 1987). GA is a highly diffusible hormone, however, GA-inducible genes are expressed specifically in EC tissue. The mechanisms of the activation of genes by GA exclusively in EC, without affecting other parts of the endosperm, need to be explained. It is possible that GA receptors localize exclusively in EC tissue, GA response occurs only in EC, and EC-specific genes are directly induced by GA (Figure 8A). Another possible explanation is that GA is important primarily for the embryo, for example, the generation of embryo growth potential, which expands cells and creates pressure against EC by the radicle tip. In this scheme, the radicle pressure is perceived as a type of wounding to EC (mechanosensing) (Monshausen and Gilroy, 2009). Wounding stimulates ethylene production as a stress response (Bradford and Yang, 1980), thus inducing *TERF1* expression to turn on downstream PR genes (Figure 8B).

From the analysis in this thesis research, a new hypothesis was generated to explain the mechanisms of EC-specific gene induction. Examination of this hypothesis will advance our understanding of the mechanisms of seed germination and the biology of seeds.

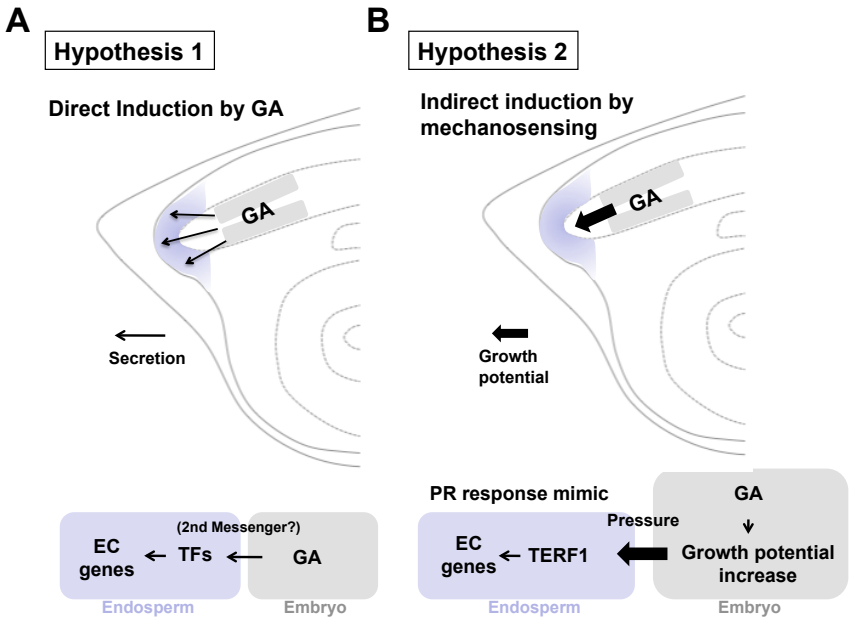

Figure 8. Schematic representation of two hypotheses of EC gene induction. (A) Hypothesis 1: Direct induction EC genes by GA. GA that is produced in the embryonic axis (Groot and Karssen, 1987), is secreted to the endosperm cap and induces EC-specific transcription factors (TFs), which directly or indirectly induce EC- genes. The diffusion of GA only to EC without affecting the other part of endosperm needs to be explained. It is possible that GA receptors are present exclusively in EC and only EC can respond to diffused GA. Alternatively, non-diffusible secondary messengers can be produced and migrated to the endosperm. (B) Hypothesis 2: Indirect induction of EC genes by mechanosensing. GA does not stimulate EC directly, but does induce EC gene expression through its effects on cell expansion in the embryonic axis. In this hypothesis, the growth potential of the embryo generated through GA biosynthesis provides pressure to EC. This triggers mechanosensing by EC, which mimics wounding or pathogenesis response, a major consequence of which is ethylene response including the activation of *TERF1*. While *TERF1* involvement in EC gene induction was verified, evidence for mechanosensing remains to be shown. Note that the major role of ethylene signal transduction in EC does not conflict with well-known GA inducibility of EC genes.

Molecular Analysis of Tomato Seed Germination and Tomato Production in Almería, Spain

Chapter 3

Tomato Production – A Case Study: Almería, Spain

Theresa T. Nguyen

INTRODUCTION

Tomato is native to South America, particularly Peru and the Galapagos Islands, and was introduced into Europe by Spanish and Portuguese explorers in the early 16th century. It is thought that tomato domestication first occurred in Mexico and the seeds were brought over to Europe later on (Jones, 2008). The production and consumption of tomato had not always been so widespread as it is today since it was thought to be poisonous. Interest in tomato expanded rapidly after its introduction from southern Europe to countries such as China, Japan, and the United States (Heuvelink, 2005). The global production of tomatoes (fresh and processed) has increased by 516%, from 55 billion pounds of tomato produced in 1961 to over 285 billion pounds within the past five decades, making the tomato industry one of the most advanced and globalized horticultural industries in the world (USDA).

Tomatoes are an essential staple food in Spain and the selected food choice for *'La Tomatina'*, an annual food fight festival, in the town of Buñol, Valencia. As the second largest producer of tomatoes in Europe, a large portion of tomato production in Spain is supplied to the fresh vegetable market (Heuvelink, 2005). Over the last two decades, Spain has consistently ranked among the top three countries for tomato export volume in billion pounds, ranking first of 176 world countries in 1997 and in 1999 through 2005 (USDA, 2010). In 2007, tomato export volume from Spain contributed to 13% of the total world export volume with over 1.9 billion pounds and an export value of approximately 1,171 million USD (USDA) (Figure 9). Among the top ten tomato-producing countries, 24% of tomatoes produced in Spain (and 34% of tomatoes produced in Mexico) are for exportation, while the percentage of tomatoes exported from the other

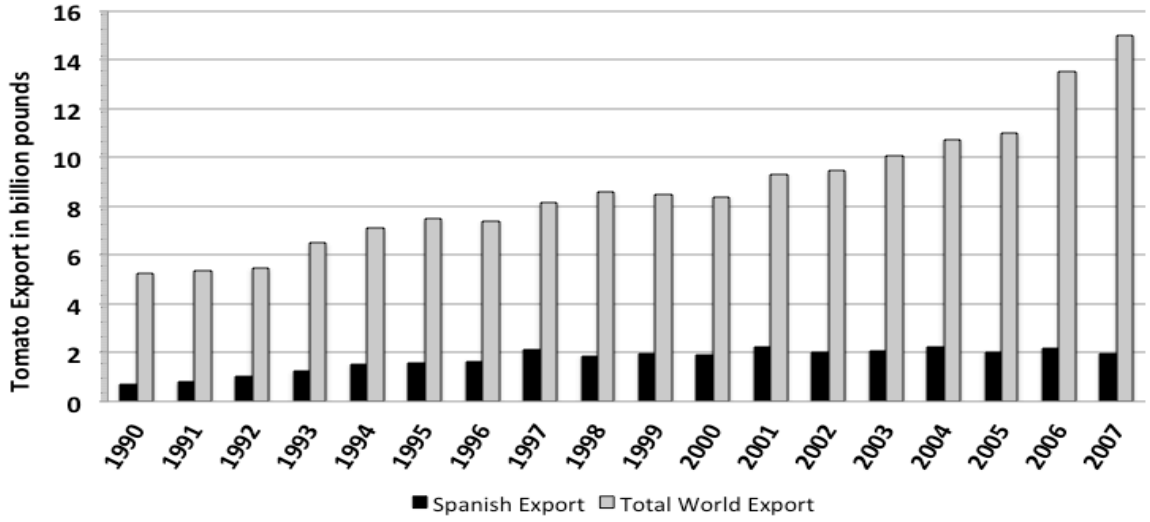

Figure 9. Tomato export volume in billion pounds from Spain as compared to the total world tomato export volume of 173 countries including Spain. Spain consistently ranks in the top three tomato-exporting countries, ranking first in 1997, and in 1999 through 2005, with a high export volume of 2.11 billion pounds in 1997 constituting to 26% of the total world value. *Source: United Nations, Food and Agriculture Organization, FAOStat (07/2010)*

Table 3. The top ten tomato-producing countries in the world in 2007 with export volume and export percentages in relation to production. Spain is a leader in tomato export with an annual value of 1,171 million USD, second only to Mexico.

Country	Production (thousand pounds)	Export Volume (thousand pounds)	Export Percentage (%)	Export Value (million USD)
China	74,068,354	197,924	0.3	28
USA	31,272,932	540,827	1.7	311
Turkey	21,925,034	820,327	3.7	219
India	22,166,572	297,282	1.3	37
Italy	14,396,521	241,532	1.7	241
Iran	11,023,100	6,506	0.1	3
Egypt	19,045,756	43,852	0.2	4
Brazil	7,564,558	21,451	0.3	3
Spain	8,077,948	1,941,457	24.0	1,171
Mexico	6,945,325	2,364,780	34.0	1,220

*Source: United Nations, Food and Agriculture Organization, FAOStat (07/2010).

countries is less than 10%, combined (Table 3). Global production of tomato for fresh-market and processing is predicted to further increase, and Spain will continue to be a leading supplier to the European markets (Heuvelink, 2005).

In particular, the province of Almería in southeastern Spain has emerged as an important and competitive area for the production and exportation of fresh greenhouse tomatoes (Heuvelink, 2005). They have taken advantage of their climate and their *'mar del plastíco'* (sea of plastic, *i.e.,* many greenhouses) near the coast of Almería to supply fresh tomatoes and other crops year round. In recent years, Almería has been named the principal exporting sector in all of Spain (Valenciano and Perez Mesa, 2008). This chapter will focus on tomato production, distribution, and the current economical situation of tomato farming in Almería, Spain.

Greenhouse tomato production

In Almería, it is typical for growers to use plastic greenhouses called *parral* or *Almería-type* greenhouses in place of traditional glasshouses for tomato production (Figure 10A). These greenhouses are covered entirely with polyethylene plastic film, which lasts for approximately three years before it is recycled, and held up mostly by braids, cords, and wires attached to metal poles (Figure 10B). Although, these structures are not as modern as glass-greenhouses, they are convenient for adjustment to the land and are cost-effective in terms of productivity. Evidently, these plastic parral greenhouses used for crop production constitute a large percentage of the province spanning 64,247 acres in 2007 along the coast, making it the largest and widest concentration of greenhouses in the world (Campra et al, 2008). This area is so outstanding that it can be seen aerially and by satellite (Figure 11).

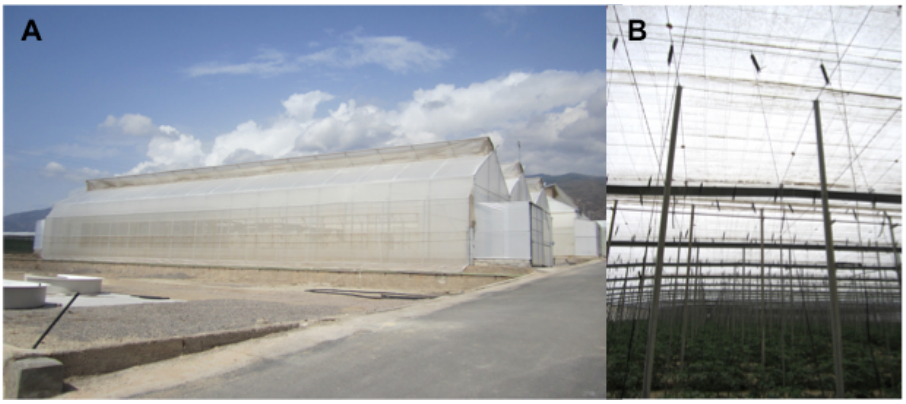

Figure 10. Typical *parral* or *Almería-type* greenhouses used for tomato production. (A) These greenhouse structures are covered with polyethylene plastic film (*Photograph taken 09/20/2010 at Las Palmerillas – Cajamar Experimental Station, Almería*). (B) The polyethylene material is held up by braids, cords, and wires attached to metal poles to create the house-shaped structure (*Photograph taken by Theresa T. Nguyen on 9/20/2010 at Cooperativa Agrícola San Isidro (CASI), Almería*).

The plastic material is white, which reflects sunrays back into the atmosphere and consequently slows heating of the surface. While temperatures in the rest of Spain have been increasing, temperatures in the province of Almería have been decreasing by 0.3 degrees per decade (Campra et al, 2008). The cooling of surface temperatures occurring alongside the expansion of the greenhouse industry in Almería suggests that these plastic structures are offsetting the rising temperatures associated with global warming.

Greenhouse tomato production is more expensive than open-field production in terms of structure, equipment, labor, energy, plant material, substrate, and fertilizer costs (Heuevelink, 2005). However, tomatoes produced in greenhouses are less subject to environmental damage and pests thus yielding higher quality and quantity of fruits. Growers in Almería use controlled methods of pest protection, including insects to fight

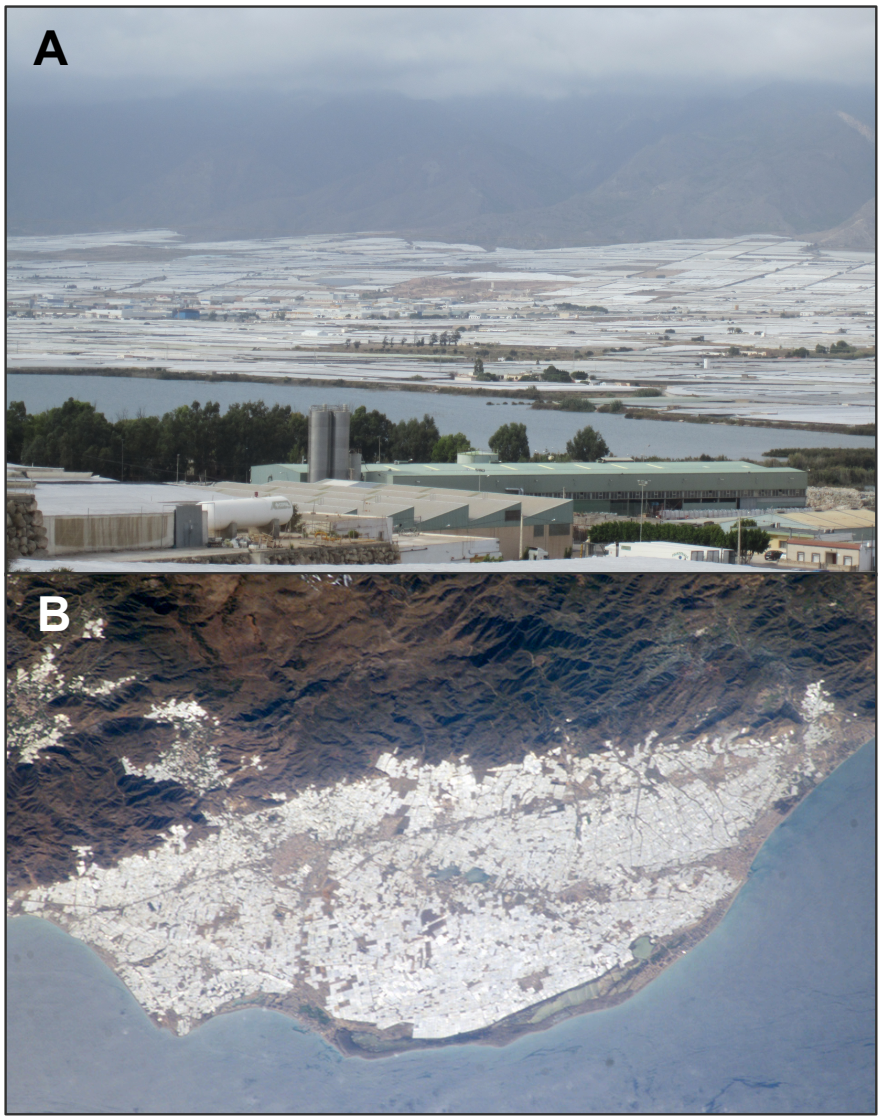

Figure 11. The largest concentration of greenhouses in the world is located off the coast of Almería, Spain. (A) The concentration of plastic greenhouses can be seen from almost any high point in the province (*Photograph taken by Theresa T. Nguyen on 9/20/2010 at Ramiro Arnedo Semillas, Almería*). (B) A photograph taken with a Kodak DCS760 digital camera equipped with an 400-mm lens from the International Space Station. Notice the 64,247 acres of white, plastic greenhouses extending from the shoreline all the way to the base of the mountains is outstanding, thus obtaining the name '*mar del plástico*' (sea of plastic) (*Photo courtesy of Earth Observations Laboratory, Johnson Space Center, taken on February 7, 2004*).

other insects and environmental-friendly chemicals such as sodium perchloride and 50% hydrogen peroxide. Although it is difficult to control the internal temperature inside these *parral* greenhouses, growers in the semi-arid climate of Almería manage temperatures ranging from 25°C (68°F) during the year up to 50° (122°F) during the summertime. Growers typically use perlite, a soil-less substrate, in place of traditional tomato production directly in soil or sand. Perlite is formed when volcanic lava cools very rapidly creating glass with small quantities of water trapped within it (Heuvelink, 2005). This glass is then crushed and heated to vaporize the trapped gas forming foam-like perlite pellets, which are then sealed into polyethylene bags (Heuvelink, 2005). Tomato seeds are typically germinated in rockwool blocks, and seedlings are grown until true leaves emerge. Then, the plant is placed into an opening of the perlite bag (the perlite substrate lasts for the lifetime of the plant) (Jones, 2008). These growers line long bags filled with perlite substrate with multiple tomato plants and attach them to a drip irrigation growing system (Figure 12). The collective term used for soil-less tomato production is *'hydroponics'*, and this type of cultivation significantly reduces water and energy usage for higher productivity (Jones, 2008). With the drip irrigation system, water is delivered to the base of the plant continuously via water supply pipes; the water supply is injected with aliquots of fertilizer to provide nutrient solution to the plants (Jones, 2008). Growers use approximately 7-8 kg of fertilizer throughout the lifetime of one tomato plant depending on the variety. In September, the tomato plants were approximately 2-feet tall and are expected to grow up to 9-feet tall at the time of harvest.

Figure 12. 'Hydroponics' used by tomato growers. Tomato plants are grown in a soil-less perlite substrate, which is a more efficient growing system than traditional soil. The plants are connected to a drip irrigation growing system (inset) in which water and nutrients are constantly fed to the base of the plant via water supply pipes (*Photograph taken by Theresa T. Nguyen on September 20, 2011 at Las Palmerillas – Cajamar Experimental Station, Almería*).

Tomato distribution (cooperativas)

'Cooperativas' are the main distributors of tomatoes in the province of Almería. They act as a grower's society in which growers come to auction off their tomatoes with a small auctioning service fee. Cooperativa Agrícola San Isidro (CASI) is one of the largest, private cooperatives that specialize in tomato production and trading, although they also trade melons, watermelons, peppers, aubergines (*i.e.*, eggplants), cucumbers,

beans, and courgettes (*i.e.,* zucchini). Created more than 60 years ago, CASI is the largest marketing outlet for tomatoes in the world.

The tomato season starts in late October or early November. In Spain, the tomato season continues through January and February, supplying fresh tomatoes to the European markets throughout the winter season. Depending on the season, a large warehouse can hold and transport up to 2 million kilograms of tomato per day with 400 – 500 workers caring for and inspecting the tomatoes. Although the warehouse was empty during the time of visit (September), workers were still preparing for the tomato season, dividing up the entire warehouse using signs to indicate the variety of red and green tomato (Figure 13). During the tomato season, growers bring their produce to the CASI warehouse for storage in large freezers, packaging, and further inspection for quality until they are auctioned off. Tomatoes at CASI are packaged into cardboard crates, cardboard trays, plastic baskets, omni-packs, or mesh bags depending on the variety and customer

Figure 13. A tomato warehouse at one of the largest cooperativas in Almería, Cooperativa Agrícola San Isidro (CASI). Tomatoes are transported to the *cooperativa* warehouse from growers to be inspected, packaged, and stored until they are auctioned off (left). Although empty at the time, workers had already started to prepare the warehouse for tomato storage and transportation dividing the warehouse with tags indicated the different types of varieties (right). (*Photograph taken by Theresa T. Nguyen on September 20, 2011 at CASI, Almería*).

preference. Tomatoes are standardized based on a variety of guidelines for size and other characteristics, which have to be met before they are auctioned off (Table 4).

Table 4. Standardization of the tomato products at CASI. Minimum characteristics, quality, homogeneity, and size standards are summarized for round tomato, plum tomato, green tomato, and tomato on the vine.

Round tomato	
Minimum characteristics	Regular shape fruit, clean, with a fresh and good aspect (no rottenness). No rare taste and smell.
I Quality	Good quality, no green color, it may have light defects of size, color, surface, growth and skin.
II Quality	Must be firm without open cracks, may have scarred cracks maximum of 3 cm long, may have some defects in form, surface, development, coloring, and slight bruises
Homogeneity	Each package should contain fruits of the same origin, quality, variety, and size. The visible part of each package must be representative of color and ripeness.
Size	9 → 82 – 102 mm; 8 → 67 – 82 mm; 7 → 57 – 67 mm; 6 → 47 – 57 mm
Plum Tomato	
Minimum characteristics	Regular shape fruit, clean, with a fresh and good aspect (no rottenness). No rare taste and smell.
I Quality	Good quality, no green color, it may have light defects of size, color, surface, growth and skin.
II Quality	Must be firm without open cracks, may have scarred cracks maximum of 3 cm long, may have some defects in form, surface, development, coloring, and slight bruises
Homogeneity	Each package should contain fruits of the same origin, quality, variety, and size. The visible part of each package must be representative of color and ripeness.
Size	8→ 67 – 82 mm; 7 → 57 – 67 mm; 6 → 47 – 57 mm; 5 → 40 – 47 mm
Green Tomato	
Minimum characteristics	Regular shape fruit, clean, with a fresh and good aspect (no rottenness). No rare taste and smell or exterior abnormal humidity.
I Quality	Good quality, it may have light defects of size, color, surface, growth and skin.
II Quality	Must be firm without open cracks, may have scarred cracks maximum of 3 cm long, may have some defects in form, surface, development, coloring, and slight bruises
Homogeneity	Each package should contain fruits of the same origin, quality, variety, and size. The visible part of each package must be representative of color and ripeness.
Size	10 → 102 mm +; 9 → 82 – 102 mm; 8 → 67 – 82 mm; 7 → 57 – 67 mm
Tomato on the Vine	
Minimum characteristics	Regular shape fruit, clean, with a fresh and good aspect (no rottenness). No rare taste and smell.
I Quality	Good quality, no green color, it may have light defects of size, color, surface, growth and skin, the truss must have at lease 4 units meeting quality I
II Quality	Must be firm without open cracks, may have scarred cracks maximum of 3 cm long, may have some defects in form, surface, development, coloring, and slight bruises, truss of 2 or 3 units and those with 4 or more units of different sizes are allowed
Homogeneity	Each package should contain fruits of the same origin, quality, variety, and size. The visible part of each package must be representative of color and ripeness.
Size	8→ 67 – 82 mm; → 57 – 67 mm; 6 → 47 – 57 mm

Tomatoes at CASI are auctioned using a stop-out price selling system, which averages the beginning auction price with the lowest dollar value price of sold produce, allowing for a fluid and transparent marketing system. The auction room at CASI seats around 100 individual local buyers, with phone lines available for international buyers to phone into during the formal auction (Figure 14). At the end of the auction, growers pay a small auctioning fee of 7% to CASI for their services. Interestingly, about 15% of tomatoes from CASI are sold to the United States.

Figure 14. The tomato auction room at Cooperativa Agrícola San Isidro (CASI). Tomatoes varieties are auctioned off in one of these rooms, which seat up to 100 individual local buyers with phone lines available for international buyers. Seated individuals bid anonymously using a button underneath their table. (*Photograph taken by Theresa T. Nguyen on September 20, 2011 at Cooperativa Agrícola San Isidro (CASI), Almería*).

There is current research being conducted at Las Palmerillas – Cajamar Experimental Station in Almería in order to optimize tomato production in plastic greenhouses. The government and Cajamar bank are funding these experiments. A major effort is made to reduce fruit cracking during production, which is a serious problem for growers. Researchers are finding that increased shading reduces fruit cracking. Other experiments that are being conducted at the station include those on the effects of increasing natural ventilation inside the plastic greenhouses, of flatter ceilings coverings for removing condensation, and of the production in soil versus soil-less substrates (perlite).

With the increasing global tomato production, the province of Almería has maintained its share in tomato exportation. Although there are other competitors in tomato exportation, such as the Netherlands and Morocco, Spanish export (primarily from Almería) to the European Union is not greatly influenced by other tomato exporters (Valenciano and Perez Mesa, 2008). However, this situation can change depending on production costs and consumption of tomato during the winter season. Ultimately, tomato sales influence about 92% of the social economy companies in Almería and 30% of their employment rate on average (Valenciano and Perez Mesa, 2008).

Molecular Analysis of Tomato Seed Germination and Tomato Production in Almería, Spain

Chapter 4

General Conclusions

Theresa T. Nguyen

Tomato is an important vegetable crop that is widely produced, consumed, and studied throughout the world. Successful germination of tomato seeds, the source of tomato, is essential for the propagation and maintenance of tomato plants. Therefore, understanding the mechanisms behind successful tomato seed germination is significant as well. Seed germination is completed with the emergence of the radicle of the embryo, which can only occur if the growth potential of the embryo exceeds the mechanical resistance of the endosperm cap (and testa). The weakening of the endosperm cap cell wall is an important step in reducing the mechanical resistance of the endosperm cap. An important enzyme, endo-β-mannanase, catalyzes the degradation of the cell wall. *SlMAN2*, an EC-specific gene, encodes for endo-β-mannanase.

GeneChip® analysis was performed on four different tomato seed tissues to identify additional EC-specific genes. Genes characterized as cell wall-associated, pathogenesis related-, and hormone signaling-genes were detected as >2-fold EC-enriched. After further streamlining the list of EC-enriched genes to >5-fold enrichment, *NP24* (the most expressed cell wall gene) and *TERF1* (the most enriched hormone signaling gene) were selected to be characterized. A construct containing *NP24* promoter fused with the GFP and GUS coding sequences was used to visualize the EC-specific expression through the tissue printing method. *TERF1* was characterized using RT-PCR to show increased expression during germination of tomato seeds, suggesting its involvement in germination events. The identification of genes enriched in EC helps us hypothesize regulation events occurring during tomato seed germination. It is possible that TERF1 is a key regulator of multiple EC genes. TERF1 may be involved in the

induction of the weakening of the endosperm cap cell wall directly or indirectly through the function of GA.

Tomato is a socially and economically important vegetable in the country of Spain, particularly the province of Almería. With the expansion of the plastic greenhouse industry in Almería, production and exportation of tomatoes in this province is increasing rapidly. Growers in Almería use plastic *parral* greenhouses to mass-produce tomatoes year round, providing the European market with fresh tomatoes during the low-light winter months. Distribution and exportation of tomatoes occur mostly in *'cooperativas'* through an auctioning system, with CASI being one of the largest and most important marketing outlets in the world. The economy in Almería is highly dependent on tomato production and sales, and we, as tomato consumers, are dependent on their productivity as well.

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