IDENTIFYING, MAPPING, AND ANTISENSING CANDIDATE HP1 SEQUENCES IN TOMATO

A Senior Honors Thesis

By

JONATHAN DEAN HOMMEL

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2000

Group: Cell Biology 1

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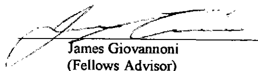

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April 2000

Group: Cell Biology 1

ABSTRACT

Identifying, Mapping and Antisensing the Candidate HP1

Sequences in Tomato. (April 2000)

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The tomato has been an agriculturally important crop for centuries. For that reason, learning about the fundamental biochemistry and physiology of the plant has been an active research area for decades and has lead to increased crop production. Size, color, nutritional content, herbicide resistance, and insect resistance characteristics have all been successfully manipulated due to the knowledge base established by research and the tools of biotechnology. One main area of research in tomato focuses on fruit ripening. Photo-perception plays a major role in the fruit ripening process and is controlled in part by two *high-pigment* (HP) genes, HP1 and HP2. When these genes are mutated, the plant appears to have a decrease in hypocotyl length, enlarged cotelydons, and increased anthocyanin levels when germinated in the dark. The fruit appearance of mutants grown in the light includes elevated sucrose, lycopene, chlorophyll and anti-oxidant levels. These characteristics have substantial commercial value. While both mutations are well understood, only the HP2 DNA sequence has been discovered. It was discovered by comparison to a known DNA sequence from *Arabidopsis* because of the extensive synteny between *Arabidopsis* and tomato. I have recently discovered three candidate DNA sequences in tomato that show a high degree of homology to other known negative regulators in the light signal transduction pathway of *Arabidopsis*. These candidates were discovered by using detailed homology searches based on the Expressed Sequence Tag (EST) database generated by The Institute for Genomics Research (TIGR). After identifying these candidates, I began the basic mapping procedure for identifying general chromosomal location through Restriction Fragment Length Polymorphism (RFLP) mapping. I have also generated antisense mutants of the candidate genes in wild type *Lycopersicon esculentum*. These mutants are currently in the late stages of tissue culture and may ultimately be used as a tool to clone the HP1 mutant or to identify and clone other genes involved in the light signal transduction pathway of tomato.

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I would like to thank the following people:

- Dr. James Giovannoni who was my advisor and provided the means for me to complete this project.
- Ashlee McCaskill who was instrumental in being patient with me and teaching me the details that I needed to know.
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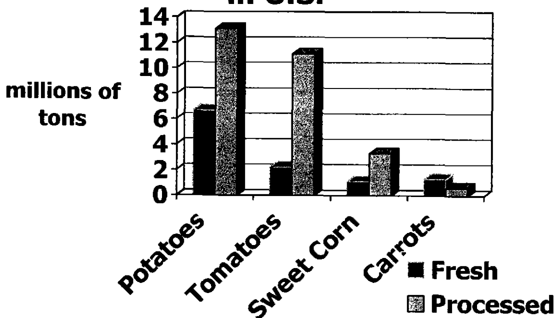
Introduction

Due to the agricultural importance of tomato, learning about the fundamental biochemistry and physiology of the plant has been an active research area for decades and has lead to increased crop production. Size, color, nutritional content, herbicide resistance, and insect resistance characteristics have all been successfully manipulated due to the knowledge base established by research and the tools of biotechnology.

Figure 1



Tomato is the second leading produce in the United States behind the potato. Americans consume an average of 13.3 million tons of tomato products annually thus establishing a large market for improved tomatoes. The excellent nutritional value of tomato contributes to its wide consumption. It contains substantial levels of:

- folate

- potassium
- vitamin A (beta-carotene form)
- vitaminC
- lycopene

Lycopene, the pigment that makes tomatoes red, is an antioxidant that has been proven to fight cancer. Out of 72 studies done, 57 reported an association between Lycopene and reduced cancer rates.

In order to control the nutritional quality of tomatoes, we must study the mechanisms of fruit ripening and plant growth. The main aspect associated with the ripening of tomato is its color change from green to red. This is due to a spike in lycopene levels of the fruit. The color change is accompanied by a fruit softening process caused by increased levels of hydrolytic enzyme activity which causes pectin depolymerization. The metabolic activity of tomato also changes dramatically as it focuses on the ripening process.

This ripening process is controlled by hormones and light transduction.

1. Hormone ethylene- Ethylene binds to receptor sites on outside of the plasma membrane which causes a phosphorylation cascade inside the cell.
2. Light transduction phytochromes- Phytochromes are light receptor proteins that change conformation upon light irradiation.
3. Light transduction negative regulators- Negative regulators lie downstream of phytochromes and act as an intermediate in the pathway by binding to specific conformers of the phytochromes. The HP1 gene is thought to be a negative regulator.

Background

Characteristics of HP1

The light transduction pathway is controlled in part by two high-pigment (HP) loci, HP1 and HP2. These loci have been shown to be non-allelic (van Tiunen et al. 1997), negative regulators that have very similar effects (Mustilli et al. 1994). The HP1 gene has been studied in depth and found to have many regulatory effects on tomato (Yen et al. 1997). One of these effects includes photomorphogenesis. Photomorphogenesis involves light response with respect to plant germination and initial growth stages. Wild type plants undergo etiolation or very rapid growth with extended hypocotyl length and unexpanded cotyledons in the dark. Then, when wild type plants are exposed to light, the etiolation process ends and the following events occure:

- the hypocotyl growth slows
- cotyledons begin to expand
- anthocyanin levels increase (Peters et al. 1989).

Anthocyanins are purple flavonoid pigments that become visible in the hypocotyl. This period of etiolation followed by slowed growth and expanded cotyledons in the presence of light is obviously a mechanism plants have developed to break through the soil and reach sunlight for photosynthesis. The hp1 mutants, however, never undergo an etiolation stage of development suggesting that the HP gene is a negative regulator for light response.

The fruit of the hp1 mutants also show significant phenotypic differences when compared to the wild type. These differences include:

1. There are high levels of sucrose in mature hp1 fruit but no detectable sucrose in wild type fruit. However, this increase comes at the expense of decreased glucose and fructose levels.
2. The hp1 mutants show increased lycopene accumulation in ripe fruit. This pigment increase causes a vividly colored fruit that is more appealing to the consumer and a fruit that may have an increase cancer fighting ability.
3. There are increased chlorophyll levels throughout immature fruit development. It must also be noted that this increase in chlorophyll is seen in leaves and roots as well.

4. They have elevated levels of the flavonoid compound quercetin. This 13-fold increase has novel implications because quercetin has been proven to inhibit cancer tumor formations in laboratory animals (Verma et al. 1988).

Project Objectives

Recently, a DEETIOLATED-2 (DET2) gene has been cloned in *Arabidopsis*, and this represents an opportunity to identify tomato mutants based on synteny between tomato and *Arabidopsis*. Homologous sequences of DET2, that may be discovered in the tomato gene database, are strong candidates for the HP1 gene because of the similarities between the phenotypes of DET2 and hp1. My objective is to identify and map the candidate HP1 genes, which may represent the possible tomato DET2 (tDET2) gene, and to create transgenic plants by anti-sensing the homologous tomato sequence of the DET2 gene thus providing a tool for cloning HP1. This will allow me to ask the question of whether the resulting phenotype is similar to that of the hp1 mutant.

MATERIALS AND METHODS

Identification

The database search was done on the Internet. The Institute for Genomics Research (TIGR) has established a database of tomato Expressed Sequence Tags (ESTs). These ESTs were searched using the BLAST-P program which converts the impute DNA sequence to the related protein sequence in all reading frames. The impute sequences, consisting of known negative regulators of the light signal transduction pathway from *Arabidopsis*, were then compared to all of the database sequences and a list of similar EST's was generated.

Mapping

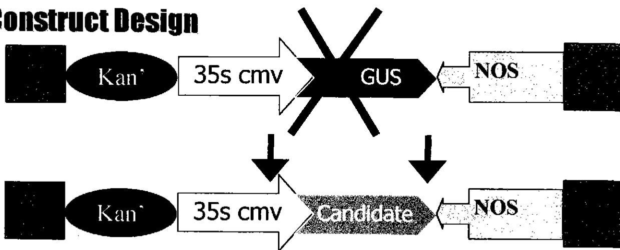
Mapping of the candidate gene will be accomplished by creating a linkage map based on recombination frequencies. Closely flanking Restriction Fragment Length Polymorphism markers will be identified and compared to existing maps. I used Southern Blots made from genomic DNA digested with a series of restriction enzymes. The probes were made by using Polymerase Chair Reaction (PCR) with gene specific primers from the candidate sequences. PCR reactions consisted of 67ul ddH_2O, 10 ul 10X PCR buffer, 10 ul of 10mM dNTP, 0.25 ul Taq polymerase, 1.6 ul forward and reverse primers at 10uM each, 0.5 ng of template DNA. The labeling experiment was done with 150 ng of DNA denatured at 100 C for 5 minutes, on ice for 1 minute. 0.4 ul labeling solution, 1 ul Klenow, and 5 ul P^{32} were added and reaction proceeded overnight. The next day, the reaction was ran through a sephadex column and added to the Southern Blots in hybridization buffer. Hybridization ran for 16 hours and was followed by placement on autoradiography film.

Antisensing

The transformation will be done in *Lycopersicon esculentum*. It will consist of the pBI121 construct, which normally drives expression of the GUS gene. However, the GUS gene will be replaced with the candidate hp1 transforming DNA (tDNA) sequence that is tDET2.

The tDNA strand will actually be the complementary sequence in order to anti-sense the expression. The exact mechanism of anti-sensing with complementary sequences is not well understood. The following components will be included in the construct:

Figure 2



Rt. Bor- (Right Border) The right border sequence is a repeated sequence of DNA that tells the *Agrobacterium* where to <u>begin</u> insertion of construct.

Kan '- (Selectable Marker- kanomycin resistance gene) Only the transformed cells will have this gene and show resistance to kanomycin. Therefore they are allowed to grow and are selected for. Cells that were not transformed are killed in the kanomycin rich media and are selected against.

35s cmv- (Promoter- 35s cauliflower mosaic virus) This promoter is effective because it is constitutively expressed in most plant tissues.

GUS- (Reporter Gene- fluorescent protein) This gene normally provides visual proof of stable DNA integration but it will be removed in this experiment and replaced with the candidate sequences.

NOS Termin.- (Terminator Sequence) This is a terminator sequence that is effective in all transcription termination.

Lt. Bor- (Left Border) The left border sequence is a repeated sequence of DNA that tells the *Agrobacterium* where to end insertion of construct.

Candidate- (Candidate Sequence) This is the HP1 candidate sequence arranged in anti-sense orientation.

This construct, containing the sequences to be transferred between the left and right tDNA borders, will then be transformed directly into the *Agrobacterium*. The sequence between the left and right borders is the only sequence that gets inserted into the plant cell. The GUS sequence was removed from the construct with SacI and SmaI restriction enzymes. The candidate sequence was cut with SacI and XhoI restriction enzymes and was ligated into pBI at 50 ng vector, 1 ul T4 phage ligase, 150 ng of adapter with SmaI and XhoI ends, 1 ul 10X buffer and ddH2O to total volume of 10 ul. The reaction was allowed to proceed for 16 hours at 16 degrees Celsius. The construct was then electroporated into *E. coli* and grown on kanomycin rich Laural Broth (LB) plates. The plasmids were then removed from the *E. coli* with the Qiagen system and electroporated into *Agrobacterium* . The Agrobacterium was grown in 5 ml of Yeast Extract and Peptone rich media (YEP) with 10 ul (50 mg/ml stock) of kanomycin at 27 degrees Celsius for 48 hours.

The process for DNA transfer from bacteria to host has been well documented. These engineered bacteria can then be used to infect cells in wounded wild type cotyledons. The transformed cells are cultured into calluses or clusters of cells. Plant hormones are added to the callus media to stimulate shoots. If a shoot forms, it is added to media with root inducing hormones. If roots develop, the first generation transgenic plant (To) is complete. The resulting plant must then be proven to be transgenic. Using a radioactive 35s promoter probe, which will be unique only to the transgenic plants, on a Southern blot and checking for hybridization is the most widely accepted form of proof. Second generation plants (T1) are self-fertilized progeny of To that will also be proven to have remained transformed with the same previously described method.

RESULTS AND CONCLUSION

Results- Identification

Three candidate sequences were identified in the TIGR tomato base based on homology with DET2. However, I also ran homology searches with several other known negative regulators of light transduction already cloned in Arabidopsis. Definite trends are observed in the homology numbers (zero represents perfect homology) as seen in figure 3.

Figure 3

Sequence Homology
(based on database search)

	Candidate 1	Candidate 2	Candidate 3
DET 2	10^{-14}	10^{-7}	10^{-8}
COP 1	10^{-15}	10^{-8}	10^{-5}
COP 9	10^{-13}	10^{-8}	10^{-7}
FUS 5	10^{-15}	10^{-12}	10^{-7}
FUS 6	10^{-13}	10^{-12}	10^{-10}
CPD	10^{-18}	10^{-11}	10^{-7}

Results- Mapping

The mapping portion of the project encountered some problems. The generation of the probes was slowed dramatically due to difficulties with PCR amplification. Non specific amplification was observed for unknown reasons but the band of correct length was gel purified.

The survey labeling experiments did work with candidate 1. Polymorphisms observed from the survey show that digestion with Eco RV should give the best resolution on the mapping filter. More difficulties were encountered in getting the probes to hybridize to the Southern Blots and conclusive data for the surveys or maps of candidates 2 and 3 have not yet been obtained.

Results- Antisense Plants

All of the constructs have been electroporated into the Agrobacterium and co-incubated with the tomato tissue. The tissue is still being cultured and calluses are forming with all three candidates. This is promising evidence that the construct is stable in becoming integrated into the genome of the plant because the calluses are being grown in the presence of kanomycin. This means that the kanomycin resistance gene must have been inserted into the genome and is conferring resistance to only the transformed cells and allowing them to grow. The final piece of information will come when the plants finish growing. This will allow PCR and hybridization data on the integrated sequences to be obtained. The plants are currently in their final stages of growth.

Conclusion

The primary objective for the project, to create transgenic plants to be used as tools to clone the HP1 gene, is nearly complete. The transgenic tissue is already been created and this must simply finish growing into the completed plant. There is no preliminary evidence available at this point to get characterization based on basic phenotypic data. As soon as the transgenic plants are finished growing, the tools have been created and can then be used by other scientists to aid in their endeavors to clone light signal transduction genes in tomato. The identification process also has conclusive data. The high homology ratings provide evidence that the identified sequences are in fact strong candidates. They show these high ratings in when compared to many known light transduction regulators from *Arabidopsis*. The survey map for candidate 1 is complete and shows which restriction enzyme will give the best resolution in a mapping filter. The probes have been made and proven on a gel to be clean of foreign length DNA fragments. The mapping data is not complete at this point but information is continuing to be generated about where the candidate sequences are located.

REFERENCES

Mustilli, A.C., Fenzi, F., Ciliento, R., Alfano, F., Bowler, C. (1999). Phenotype of the Tomato high pigment-2 Mutant is caused by a mutation in the tomato homolog of DEETIOLATED1. The Plant Cell. 11:145-157

Peters, J.L., Schreuder, M.E.L., Verduin, S.J.W., and Kendrick, R.E. (1992). Physiological characterization of a high-pigment mutant of tomato. Photochem. Photobiol. 56, 75-82.

Van Tuinen, A., Cordonnier-Pratt, M.-M, Pratt, L.H., Berkerk, R., Zabel, P., and Koornneef, M. (1997). The mapping of phytochrome genes and photomorphogenic mutants of tomato. Theor. Appl. Genet. 94, 115-122.

Verma, A.K., Johnson J.A., Gould M.N., Tanner M.A. (1998). Inhibition of 7,12-dimethylbenz anthracene and N-nitro-methylurea-induced rat mammary cancer by the dietary flavanol quercetin. Caner Res 48: 5754-5760.

Yen, H.C., Shelton, B.A., Howard, L.R., Lee, S., Vrebalov, J., Giovannoni, J.J. (1997). The tomato *high-pigment* locus maps to chromosome 2 and influences plastome copy number and fruit quality. Theor. Appl. Genet. 95:1069-1079

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