IDENTIFICATION AND CHARACTERIZATION

OF GENES ASSOCIATED WITH

WATER-USE EFFICIENCY

IN TOMATO

BY

YALI ZHU

Bachelor of Science
Shanxi Agricultural University
Taigu, China
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Master of Science
Michigan State University
East Lansing, Michigan
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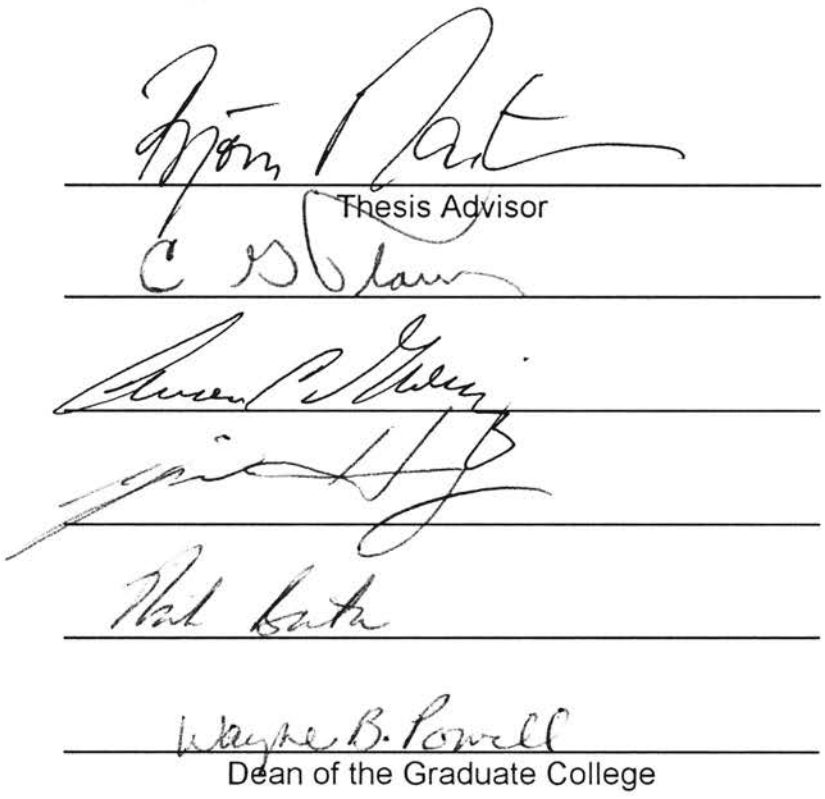

Thesis Advisor

Dean of the Graduate College

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CHAPTER I

ISOLATION AND IDENTIFICATION OF GENES ASSOCIATED WITH WATER-USE EFFICIENCY

I. INTRODUCTION

Rooted in one place, plants have a limited capacity to avoid changes in their surroundings. Being constantly exposed to nature's elements, they have to withstand many environmental stresses which affect their productivity. Unfavorable environments can come in many forms, but lack of water limits US and world agriculture more than any other biotic or abiotic factor. As examples, only 12% of US soils are free of all physicochemical stresses and about 50% of them lack sufficient moisture due to low precipitation or because they are too shallow or sandy to hold much water (Boyer, 1982). Among the various stress factors (such as diseases, water logging, salinity, insect pests, weed, low temperature), drought alone depresses yield of crops by up to 40%.

Different approaches have been followed to minimize the impact of water deficit stress on crops. Artificial irrigation has been invaluable as a partial cure, but in many places irrigation water is not available, or irrigation is not economically feasible. Also, faulty irrigation leads to soil salinization and waterlogging which are very expensive to correct. Meanwhile, legitimate industrial and urban needs are increasingly competing with agriculture for water. Economic and environmental problems are also likely to restrain agricultural water use.

In order to improve crop productivity in environments where growth is limited by the availability of water, the scarce water resource must be used more

efficiently. The challenge is how can plants efficiently use the available water for maximum productivity. Water use efficiency (WUE) was studied by the beginning of this century (Briggs and Shantz, 1913) and it is best defined as the mass of carbon in the plant divided by the mass of water transpired during the period of growth (Farquhar and Richards, 1984). There are now ample data to suggest that WUE is an important component of drought resistance that greatly affects plant yield under water limited conditions (Sinclair *et al.*, 1984). Improvement of plant WUE could be a complement to irrigation. The economic benefit of improved WUE is manifest in either increased productivity at no additional cost, or in maintained yield at reduced production cost.

The traditional approach to improving agricultural productivity has been to select genotypes adapted to existing environments and to manipulate the environment to suit these genotypes. WUE is a very complex trait which depends on several genes (Ehdaie *et al.*, 1993; Masle *et al.*, 1993; Virgona *et al.*, 1990). Progress from classic breeding methods, based on crossing and selection schemes, has been greatly limited. Large agricultural yield improvements have been achieved in many crops and environments by modifying field management practice, plant architecture, and harvest index (Fischer, 1981; Boyer, 1982). These approaches will continue to be useful strategies for future agriculture. However, when agricultural inputs become more costly and scarce, opportunities for large scale modifications of the environments become more limited.

Genetic variation in WUE has been reported for various crop species (Farquhar and Richards, 1984; Hubick *et al.*, 1986, 1988; Hubick and Farquhar, 1989; Martin and Thorstenson, 1988; Condon *et al.*, 1987; Virgona *et al.*, 1990; Ehleringer *et al.*, 1991; Ismail and Hall, 1992). However, many questions remain unanswered. What are the biochemical and molecular mechanisms of this variation? How can the genetic variation be exploited in plant breeding or genetic engineering to produce plants with greater WUE? Answering these questions is an urgent need for the future of agriculture. An improved understanding of the molecular basis for plant WUE would permit one to identify, isolate, and introduce plant genes controlling WUE into crop plants to improve their productivity in adverse environments.

The emphasis of this research was to identify components of gene expression that are involved in WUE. The approach was to identify, clone and characterize genes that are specifically expressed in water-use efficient plants. Tomato was chosen as an ideal species for research on the molecular genetic basis of WUE. The tomato is commercially important throughout the world both for the fresh fruit and the processed food industries. USDA statistics (US Department of Agriculture, 1994) showed that in 1992 there were 400,000 acres of tomato harvested in the US with a cash value of $2 billion of the $11 billion total for vegetables and specialties. The same year, the average American consumed 89 lb. of tomato, of which 15 lb. were fresh market tomatoes and the remainder processed tomatoes. Tomatoes also serve as a significant contributor to human nutrition. For example, tomatoes are rich in vitamins C and

A and an 8 ounce serving of tomato juice provides 60 percent of the recommended daily requirement of vitamin C for an adult (Atherton and Rudich, 1986). Tomato also has a rich source of genetic variation accessible in its wild relatives (Rick, 1960; Rick and Yoder, 1988). *Lycopersicon pennellii* is a wild tomato relative native to the deserts of Peru. It is capable of growing and reproducing with a minimal amount of water and it resists wilting in its environment (Yu, 1972). The cultivated tomato, *L. esculentum*, is drought sensitive, and requires irrigation for satisfactory production in most growing areas. *Lycopersicon pennellii* has greater WUE than *L. esculentum* (Martin and Thorstenson, 1988) and it can be crossed with *L. esculentum* to produce a fertile interspecific hybrid. Together, these two species provide an ideal system for studying plant WUE.

The primary objective of this research was to understand the molecular genetic basis of WUE in tomato by investigating differences in gene expression between the above mentioned two species. Specific objectives were to: 1) isolate and identify WUE associated genes which were differentially expressed in the two *Lycopersicon* species, and 2) characterize expression patterns of WUE associated genes.

II. LITERATURE REVIEW

1. The relation between water use and plant productivity

The relationship between water consumption by plants and overall productivity has been a topic of intense research for almost a century. The pioneers among natural scientists, especially agronomists, took great efforts to study water requirements for growth of a broad range of crop species. Their studies of crops and native plants showed a substantial difference in the relationship between water consumption and biomass production among species as well as among cultivars of a species (Briggs and Shantz, 1913; Shantz and Piemeisal, 1927). Early studies also demonstrated the effects of atmospheric conditions on the balance between carbon gain through photosynthesis and water loss through transpiration (Lemon *et al.*, 1971). Water-use efficiency at the leaf level is defined as the ratio of the rates of net photosynthesis and transpiration. Carbon dioxide and water vapor exchange between leaf and atmosphere are through the same path, stomata, but in different directions. In water-limited environments, a plant with optimum WUE should strive for maximal CO_2 uptake and minimal water loss at the same time.

In C_3 plants, a single carboxylating mechanism results in the net synthesis of carbon compounds through assimilation of CO_2. This mechanism was fully explained in the 1950s by a team led by M. Calvin. In the Calvin cycle, CO_2 is

initially fixed by ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). The reaction yields two three-carbon molecules and serves not only as the starting point for synthesis of carbohydrates, but also for fatty acid and amino acid synthesis. The photosynthetic pathway of C_4 plants was elucidated in the mid-1960s. Since C_4 plants have an auxiliary efficient mechanism of carbon fixation, photosynthesis in C_4 plants can operate at low stomatal conductance, which minimizes water loss, and low intercellular concentrations of CO_2. Therefore, the C_4 pathway brings advantages in WUE to C_4 plants. Later, Crassulacean acid metabolism (CAM) plants were shown to operate at extremely high WUE. The CAM metabolism allows plants to accumulate CO_2 from the air when stomata are open at night and temperature and water loss are at their lowest. Final photosynthetic fixation of CO_2 by rubisco proceeds behind closed stomata during the following day. The process therefore results in minimal water loss and massive CO_2 fixation. The discovery of C_4 and CAM metabolisms gave an explanation of the major differences in WUE among plants having different photosynthesis pathways. Genetic variation in WUE was also observed among species and cultivars within the C_3 pathway, such as wheat (Farquhar and Richards, 1984; Condon *et al.*, 1987), peanut (Hubick *et al.*, 1986, 1988), tomato (Martin and Thorstenson, 1988), barley (Hubick and Farquhar, 1989), sunflower (Virgona *et al.*, 1990), common bean (Ehleringer *et al.*, 1988) and cowpea (Ismail and Hall, 1992).

However, breeding programs have not exploited this variation due to the lack of an effective selection technique. Selection for WUE in the field is often

not feasible since measuring both biomass and water use is difficult, time consuming, and imprecise.

2. Stable carbon Isotope discrimination as an indicator of plant WUE

Substantial differences in the ratio of $^{13}C/^{12}C$ in plant tissue samples have been noticed among plant species. Detailed descriptions of the mechanistic basis of the isotope fractionation processes during photosynthesis were reported in the 1980s (Vogel, 1980; O'Leary and Osmond, 1980; O'Leary, 1981; Farquhar *et al.*, 1982). It was especially the studies of Graham Farquhar, Marion O'Leary, and Joe Berry (1982) that established the biochemical and physiological basis for ^{13}C discrimination in C_3 plants. Their work led to a better understanding of differences in productivity and water relations among cultivars of C_3 crops.

Most elements of biological interest have two or more stable isotopes of which one isotope usually dominates. Carbon dioxide in air contains about 1.1% ^{13}C and 98.9% ^{12}C. Because of the mass difference between $^{12}CO_2$ and $^{13}CO_2$, there is an isotope fractionation in diffusion but the main fractionation in plants is caused by the carboxylation reaction in photosynthesis. The carbon isotope composition, $\delta^{13}C$, is the ratio of $^{13}C/^{12}C$ of a sample (R_{sample}) relative to the corresponding isotope ratio of a standard carbon source ($R_{standard}$) as described by the following equation,

$$\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 1000$$

The most common standard is carbon dioxide obtained from "PDB", a limestone from the Pee Dee formation in South Carolina (Craig, 1957). The carbon isotope discriminatiom, or Δ, is related to $\delta^{13}C$ as follows,

$$\Delta = (\delta^{13}C_{air} - \delta^{13}C_{plant}) / (1 + \delta^{13}C_{plant})$$

Subscripts air and plant define $\delta^{13}C$ of CO_2 in the air and in plant organic matter, respectively. Values in $\delta^{13}C$ notation can be easily converted to values in Δ notation by measuring $\delta^{13}C_{air}$, or by assuming $\delta^{13}C_{air}$ = -7.8 per mil which is the current global average.

The link between Δ (as well as $\delta^{13}C$) and WUE of plants having the C_3 photosynthetic pathway is now well understood. The theory has been developed in detail (Farquhar *et al.*, 1989; Evans and Farquhar, 1991). The Δ and WUE are independently linked to p_i/p_a, i.e. the ratio of the partial CO_2 pressure in the intercellular air spaces of photosynthesizing leaves and in the ambient air, respectively:

$$\Delta = a - d + (b - a)\, p_i/p_a$$

and

$$WUE = [(1 - \Phi_c)\, p_a\, (1 - p_i/p_a)] / [1.6\, (1 + \Phi_w)\, (e_i - e_a)]$$

where a is isotopic fractionation due to diffusion of CO_2 in air (4.4 per mil), d (1-3 per mil) is fractionation occurring during dissolution of CO_2 in water of the cell wall, liquid phase diffusion, and respiration, and b (27 per mil) is the major isotope fractionation caused by Rubisco. The Φ_c is the fraction of photosynthetically fixed CO_2 subsequently lost in respiration, and Φ_w is uncontrolled water loss as a fraction of daytime stomatal transpiration. The e_i and e_a are the partial pressures of water vapor in the intercellular air spaces and in the surrounding air, respectively, and the number 1.6 comes from the difference in diffusion coefficients between water vapor and CO_2. The ultimate

equation for the relationship between Δ and WUE is obtained by combining the previous two equations (Evans *et al.*, 1991):

$$WUE = [(1 - \Phi_c)\, p_a\, (b - d - \Delta)] / [1.6\, (1 + \Phi_w)\, (b - a)\, (e_i - e_a)]$$

Thus, the value of Δ is expected to be a predictor of WUE in plants. A number of studies have related Δ to plant WUE and productivity (Condon *et al.*, 1987; Martin and Thorstenson, 1988; Hubick and Farquhar, 1989; Virgona *et al.*, 1990; Masle *et al.*, 1993). Furthermore, the broad-sense heritabilities reported for Δ in wheat were relatively large, ranging from 74% to 94% (Ehdaie *et al.*, 1991, 1993) and from 62% to 95% (Condon and Richards, 1992), depending on the experimental conditions and on the plant parts sampled. Estimates of broad-sense heritability for Δ in cowpea ranged from 76% to 90% (Hall *et al.*, 1990) and in peanut it varied from 53% to 81% (Hubick *et al.*, 1988). These observations suggested that selection for Δ, under either well-water or water-stressed conditions could indirectly improve WUE. Due to the easier and faster measurement of Δ than total growth and water use, the Δ measurement has opened up new opportunities for the genetic improvement of plant WUE.

3. Molecular markers associated with plant WUE

Molecular markers have been widely used in plant breeding and genetic analysis. Marker assisted selection by means of restriction fragment length polymorphism (RFLP) or other molecular markers offers an important alternative for selection of hard to measure traits (Tanksley *et al.*, 1989). Several studies (Ehdaie *et* al., 1993; Masle *et* al., 1993; Virgona *et* al., 1990) have shown that WUE is a quantitatively inherited trait. The identification of quantitative trait loci

(QTL) would allow the analysis and selection of this complex quantitative trait as a set of linkage groups containing the genes of interest. In 1989, Martin *et al.* first published very encouraging results regarding the genetic control of WUE, based on analysis of DNA polymorphisms between two species of *Lycopersicon*. They showed that 70% of the variation in carbon isotope discrimination (Δ) between *Lycopersicon esculentum* and *L. pennellii* was associated with three DNA fragments. These three DNA fragments were mapped to three different RFLP linkage groups. They had previously shown that the variation in Δ among the two species and their F1 progeny was strongly correlated with WUE (Martin and Thorstenson, 1988). Later, an analysis of DNA polymorphisms among *Arabidopsis thaliana* ecotypes differing in Δ was reported (Masle *et al.*, 1993). The RFLP analysis was conducted on three high and three low Δ ecotypes, and all pairs of low-high Δ ecotypes were polymorphic to a varying degree, with two pairs being highly polymorphic. In these two studies, carbon isotope discrimination was chosen as a criterion rather than WUE directly because a good and stable correlation had been found between the two characteristics across a broad range of species (Farquhar *et al.*, 1989) and because Δ is so much easier to measure. The results from these indirect mapping studies of QTL for WUE indicated the possibility of directly mapping of QTL conditioning WUE, provided the phenotypic measurements of WUE can be accomplished. In 1996, Mian *et al.*, identified six RFLP markers associated with WUE in soybean. They found four of the six markers to represent putatively independent QTL. No epistatic interactions were detected among the four putative independent

markers. These four marker loci were additive to one another and if combined would explain 38% of total variation in WUE. The heritability of this trait was 50% and most of the genetic variation was explained by the four putatively independent QTL. Recently, a set of putative QTLs responsible for WUE have been identified in Martin's laboratory (Lin *et al.*, 1998, personal communication) using RFLP analysis.

The RFLP analysis allows the localization of QTLs for WUE and determination of the relative magnitude of their effects on the trait. Indirect selection for WUE via molecular markers may improve selection efficiency. Also, once the molecular markers have been identified and mapped, the genes involved can be cloned.

There are three main steps in map-based gene cloning: (1) identifying markers that are tightly linked to a target gene, (2) walking to the gene using a yeast artificial chromosome (YAC) library, a bacterial artificial chromosome library (BAC) or various genomic libraries constructed in λ vectors, and (3) complementing the recessive phenotype by transformation. The RFLP analysis presented above is relevant to the first step. Now, YAC, BAC and cosmid libraries constructed with large-insert genomic DNA are available for some species, such as *Arabidopsis*, tomato, maize, sorghum, rice etc. (Grill and Somerville, 1991; Martin *et al.*, 1992; Edwards *et al.*, 1992; Woo *et al.*, 1994; Umehara *et al.*, 1995). Gene cloning by map-based chromosome walking is much more realistic than in the recent past. Already, the technology has resulted in the successful isolation of the first disease-resistance genes in

tomato (Martin *et al.*, 1993) and *Arabidopsis* (Mindrinos *et al.*, 1994) and several of the first genes known to be involved in the plant hormones ABA (Giraudat *et al.*, 1992; Leung *et al.*, 1994; Meyer *et al.*, 1994), auxin (Leyser *et al.*, 1993), and ethylene (Chang *et al.*, 1993) signalling. No doubt, the major genes involved in controlling polygenic traits like WUE can also be identified and isolated by this technology.

4. Transgenic plants with improved plant water status

During the last decade, tools have been developed which allow the application of molecular biology to study and manipulate complex physiological traits. The methodology to generate transgenic plants is available for many species. This technique reduces the dependence of breeding on sexual reproduction and, in principle, genes from any living organism can rapidly be incorporated into a plant. The incorporation of genes in plants may provide improved performance under environmental stress and allow a way in which the plant can better endure its environment. Tobacco plants have been modified by the introduction of the *Escherichia coli* mtlD gene, which encodes mannitol-1-phosphate dehydrogenase, under the control of a constitutive promoter (Tarczynski *et al.*, 1993). The transgenic tobacco plants can accumulate mannitol up to 6 μmol g^{-1} fresh weight and have a growth advantage over control plants in terms of better height gain, less fresh weight loss and more new leaf and root production in the presence of 250 mol m^{-3} NaCl. Similarly, the genes encoding the synthesis of fructan isolated from bacteria have been introduced into tobacco and potato (Ebskamp *et al.*, 1994; van der Meer *et al.*, 1994). In

addition to accumulating a large amount of fructan, fructan-producing tobacco plants performed significantly better under polyethyleneglycol-mediated drought stress than the wild type tobacco. The growth rate of the transgenic plants was significantly higher, as were fresh weight and dry weight yields (Pilon-Smits *et al.*, 1995). Since mannitol and fructan are soluble, they may play a role in osmotic adjustment. The osmotic potential inside the cell is lowered by the accumulation of inorganic solutes such as K^+, Na^+, Cl^-, NO_3^-, and organic solutes such as carbohydrates, amino acids and other organic acids. The decrease in osmotic potential causes a reduction in cellular water potential below the external water potential and allows water to move into the cell. Thus, lowering of the osmotic potential results in turgor maintenance and maintenance of processes like growth and stomatal opening that depends on turgor. Proline, a natural osmolyte (Yancey *et al.*, 1982), has been overproduced in transgenic tobacco plants by overexpressing mothbean Δ^1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme able to catalyze the conversion of glutamate to Δ^1-pyrroline-5-carboxylate, which is then reduced to proline (Kavi Kishor *et al.*, 1995). The transgenic plants produced a high level of the enzyme and synthesized 10- to 18- fold more proline than control plants. Overproduction of proline facilitated lowering of osmotic potential and maintenance of turgor during water stress and significantly enhanced biomass production and flower development under salt-stress conditions. As a consequence, the WUE of these transgenic plants increased under water stress compared to control plants.

Under unstressed control conditions, the presence of mannitol or fructan had no significant effect on growth rate and yield. Also, when grown under greenhouse conditions (unstressed), transgenic plants with overproduction of proline were not statistically different from the control plants. These results may further explain the relationship between WUE and drought tolerance. Only under limited water conditions is WUE an important component of drought tolerance. Genes involved in drought tolerance may be only expressed in water stressed environments, but none of them have been directly associated with WUE. Genes responding to water stress and genes associated with WUE are not necessarily the same genes. If those two sets of genes were incorporated into one plant, this plant may have greater WUE in all conditions: well watered, water stressed, and salt stressed.

5. Prospects of isolation of genes associated with WUE

Due to the complexity of WUE, there has been no report to date of isolation of WUE genes. One promising isolation method is map-based cloning that depends on the availability of molecular markers tightly linked to the target genes and a linkage map with molecular markers on it. Chromosome walking is a map-based cloning technique, but chromosome walking is technically demanding and none of the steps in chromosome walking are trivial. Most of the steps require large amounts of work and careful thought in order to maximize the chances of obtaining the correct genes.

In recent years, polymerase chain reaction (PCR) based cloning has become an invaluable tool for molecular biologists. PCR can amplify a DNA

sequence *in vitro* from picogram quantities of DNA or even a single copy of a gene within a matter of hours. More important, PCR can be used not only to isolate DNA fragments but also to modify them to aid cloning or to obtain mutants. There are many instances where the easiest way is to use PCR to amplify a known coding sequence or promoter for characterization, and to clone or generate constructs. In such cases, the procedure is quite straightforward. In 1992, another technique, differential display polymerase chain reaction (Liang and Arthur, 1992) was developed. This technology makes finding unknown sequences, like genes for WUE, even more feasible. The PCR reactions are performed using an oligo(dT) primer and a random primer. Radiolabel is included in the reaction and the products are visualized on sequencing gels. Polymorphic bands that are identified can be isolated by excision from the gel, elution and PCR reamplification, and the product used either for screening or for cloning.

The differential display technique was first used in animal systems to identify novel genes expressed in mammary tumor cells but not in normal cells (Liang and Arthur, 1992). Because it is more sensitive and speedy compared with other gene cloning techniques, differential display has been expanded into almost all scientific areas. Differential display has been widely used to study differential gene expression in plants subjected to environmental stresses and in plants at different developmental stages. A novel *Arabidopsis* cDNA that is differentially regulated in response to exposure to ozone was isolated using differential display (Sharma and Davis, 1995). This technique also successfully

identified a full-length and a partial length cDNA showing response to pathogen stress of alfalfa (Truesdell and Dickman, 1997). Differential display has also been used to study developmental processes in plants at the RNA level. Fruit ripening is a complex developmental process that involves specific changes in gene expression and cellular metabolism. Using differential display, enhanced expression of mRNAs in nonclimacteric fruit were identified in ripening strawberry fruit (Wilkinson *et al.*, 1995). Developmentally regulated genes of somatic embryogenesis in eggplant have also been identified (Momiyama *et al.*, 1995), as have sucrose-regulated genes in cultured rice cells (Tseng *et al.*, 1995) and genes involved in cotton fiber development (Song, 1995). The many genes that have been cloned make it evident that differential display provides a comparatively quick way to clone genes involved in all sorts of regulatory pathways.

Significant genetic variation in plant WUE has been demonstrated in numerous experiments (Farquhar and Richards, 1984; Hubick *et al.*, 1986, 1988; Condon *et al.*, 1987; Martin and Thorstenson, 1988; Hubick and Farquhar, 1989; Virgona *et al.*, 1990; Ehleringer *et al.*, 1991; Ismail and Hall, 1992). The cultivated tomato has wild relatives that exhibit excellent drought tolerance and have high WUE (Yu, 1972; Martin and Thorstenson, 1988). The high WUE is most probably related to expression of specific genes. By using the differential display strategy, it is possible to screen a wide range of mRNA species that show positive correlation with high WUE of tomato and clone the WUE specific genes. Ultimately, transgenic plants that are more water-use efficient, and

therefore, more agronomically superior than presently available crops, will be developed this way.

III. MATERIALS AND METHODS

The differential display reverse transcription polymerase chain reaction (DDRT-PCR) and cDNA library screening approaches were applied to clone and identify genes putatively associated with WUE. The technique of DDRT-PCR is principally based on the isolation and purification of individual mRNAs from plants subjected to altered conditions. The DDRT-PCR method utilizes reverse transcription and polymerase chain reaction (PCR) using sets of oligonucleotide primer combinations.

1. Plant materials and growth conditions

The wild relative (*Lycopersicon pennellii*) of the cultivated tomato (*L. esculentum*) cv. UC82B requires less water during its growth period and has greater WUE. *L. pennellii* can produce a fertile interspecific hybrid with *L. esculentum* when it is used as the pollen parent. For this experiment, we used *L. pennellii*, *L. esculentum* and the F2 generation of selfed F1's obtained by crossing the two *Lycopersicon* species as sources for differentially expressed mRNAs. Seeds from the three genotypes were planted in flats and allowed to germinate in a growth chamber (Conviron, Controlled Environments Limited, Winnipeg) maintained at 14h/10h day/night length and 30°C/20°C day/night temperature. When 3-5 cm tall, individual seedlings were transplanted into containers holding 7.5 L of a standard soil mix and allowed to grow in a

greenhouse at 30°C/20°C average day/night temperature. Each container was watered every day to a weight resulting in a soil moisture level of 100% of field capacity. After 6 weeks, leaf material was collected from each plant separately, frozen in liquid nitrogen and stored at -80°C for RNA extraction.

WUE was calculated by dividing the total plant dry weight by total water use over the entire growing period, and by correcting for soil evaporation estimated from the weight losses of plantless containers as described by Martin and Thorstenson (1988). Ten F2s with the highest WUE were pooled for the differential display step as were ten F2s with the lowest WUE.

2. RNA extraction

Total RNA from the two pools of F2 materials and from the two parents were extracted following an RNA isolation procedure modified from Logemann *et al.* (1987).

Five grams of tomato tissue was gound thoroughly in liquid nitrogen, and homogenized further by adding 2 volumes guanidine buffer which consisted of 8 M guanidine hydrochloride, 20 mM MES (4-morpholineethansulfonic acid), 20 mM EDTA, and 50 mM mercaptoethanol at pH 7.0. The guanidine hydrochloride extract was set at room temperature for 20-30 min and centrifuged at 12,000 g at 4°C for 10 min. The RNA-containing supernatant was filtered through one layer of miracloth to get rid of floating particles, and extracted twice with 1 volume phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v). The aqueous phase which contains RNA was recovered by centrifugation at 12,000 g at 4°C for 45 min.

The RNA-containing aqueous phase was collected, mixed with 0.7 volume of precooled ethanol and 0.2 volume 1 M acetic acid and incubated at -20°C overnight to precipitate the RNA. The precipitated RNA was pelleted at 12,000 g for 10 min and washed twice with sterile 3 M sodium acetate at pH 5.2 to remove the low-molecular-weight RNAs and contaminating polysaccharides. The salt was removed by a final wash with 70% ethanol and the RNA pellet was subsequently dissolved in sterile water.

The isolated RNA was further purified by LiCl precipitation. One volume 4 M LiCl was added to an RNA sample obtained from the previous step and precipitated at 4°C overnight. The RNA pellet was recovered at 12,000 g at 4°C for 30 min, dissolved in sterile water, and LiCl precipitated one more time. The RNA pellet was recovered, washed with 70% ethanol, dissolved in sterile water, and then quantitated prior to further use.

3. Differential display screening

Total RNA was further cleaned by removal of chromosomal DNA contamination with Rnase-free Dnase from MessageClean™ Kit (GenHunter Corp., Nashville).

3.1 Reverse transcription of mRNA (RT)

First-strand cDNAs were synthesized from 0.2 μg of the total DNA-free RNAs with oligo-dT primers anchored to the beginning of the poly(A) tail using the RNAimage™ system (GenHunter Corp., Nashville). The anchored oligo-dT primers contain 11 T residues followed at the 3' end by one of three additional

nucleotides (e.g.$T_{11}A$, $T_{11}C$, and $T_{11}G$). The RT reactions were catalyzed by Moloney murine leukemia virus (MMLV) transcriptase at successive steps of 65°C for 5 min, 37°C for 60 min, and 75°C for 5 min.

3.2 Polymerase chain reaction (PCR)

Two μl of the cDNA synthesis product was used to prepare a 20 μl PCR reaction. The reaction was carried out using one of several available arbitrary primers (AP) along with the same anchored primer and ^{33}P radiolabeled dATP to amplify a portion of the cDNA population constructed during the RT reaction. The PCR reaction contained 1x PCR buffer (GenHunter Corp., Nashville), 2 μM dNTP, 0.2 μM of each primer, 0.25 μl α-[^{33}P]dATP (2,000 Ci/mmole, Amersham Life Science Inc., Arlington Heights), and 1 unit AmpliTaq DNA polymerase (Perkin-Elmer Inc., Branchburg). PCR was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc. Watertown). The cycling program consisted of 40 cycles at 94°C for 30 sec, 40°C for 2 min, and 72° for 30 sec, followed by a final extension at 72°C for 5 min.

3.3 Size fractionation

A 6% acrylamide/7 M urea denaturing sequencing gel was prepared in TBE buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.0) and polymerized overnight. Before using, the gels were prerun for 45 to 60 min. Three and a half μl of each PCR sample mixed with 2 μl of loading dye was incubated at 80°C for two min, loaded immediately onto the gel, and electrophoresed for about 4 hr at 50°C, at 100 watts constant power until the xylene dye reached the bottom. The gel was blotted on a piece of 3M Whatman filter paper, covered with plastic

wrap, and dried under vacuum on a gel dryer at 80°C for 1.5 hr. The dried gel was exposed to X-ray film for a time ranging from overnight to 72 hr and developed for band selection.

3.4 Reamplification of cDNA fragments

After developing the film, the autoradiogram was oriented with the gel on a light box and the bands of interest were located by punching through the film with a needle at the four corners of each band of interest. The located bands were cut out with a clean razor blade.

The slice of gel and 3M paper was soaked in 100 μl dH_2O overnight, boiled for 15 min, and spun for 2 min at full speed in a microcentrifuge to pellet the gel and paper debris. The supernatant was transferred to a new microfuge tube and precipitated with 1/10 volume of 3 M sodium acetate, 5 μl glycogen (10 mg/ml) and 5 volumes of 100% ethanol for 30 min in a -80°C freezer. The DNA was pelleted for 10 min in a microcentrifuge at full speed at 4°C. The DNA pellet was washed with 200 μl ice-cold 85% ethanol, air dried, and dissolved in 10 μl dH_2O. Four μl of the DNA was used for reamplification using the same sets of primers and PCR conditions as in the initial differential display PCR reactions, except the dNTP concentration was 20 μM and no radiolabel was used.

3.5 Cloning of cDNA fragments

Fifteen μl of reamplified PCR products were run on a 1.5% agarose gel to visualize distinct bands of the expected size. After a distinct band was obtained, 1 μl of the PCR product was ligated with pCR2.1 through the 3' terminal deoxyadenosine overhang at the LacZα gene of the linearized plasmid. *E. coli*

competent cells (INVαF') were transformed with the plasmid containing the insert for multiplication. Selection and screening were accomplished using ampicillin selection media and the blue-white (LacZα) complementation test, respectively. The Original TA Cloning Kit (Invitrogen Co., Carlsbad) was used in all procedures following the instruction manual.

4. Differential screening

Differential display was first utilized to detect cDNAs representing differentially expressed mRNAs. It was then used to further verify the differentially expressed mRNAs. Two steps were involved in the differential screening: cDNA labeling and differential screening.

4.1 Labeling of cDNA probes

The cDNA labeling was performed using the ReversePrime™ cDNA labeling kit (GenHunter Corp., Nashville) as instructed. The total RNA used here was the RNA purified from *L. pennellii* and *L. esculentum* from the previous extraction step (section 2). The cDNA probes were prepared from 20 μg of each of the two RNA samples by reverse transcription in a 40 μl reaction which consisted of 1xRT buffer (GenHunter Corp., Nashville), 30 μM dNTP (without dCTP), 1 μM T_{20} primer and 100 μCi α-[^{32}P]dCTP (IGN Pharmaceuticals Inc., Costa Mesa). The parameters for the reverse transcription reaction were identical to those in the differential display protocol. After the reverse transcription, non-incorporated α-[^{32}P]dCTP was removed from the labeled cDNAs by gel filtration using a Sephadex G-50 spin column. One μl of each

labeled cDNA was counted in a scintillation counter to determine the efficiency of cDNA labeling.

4.2 Differential screening (reverse northern)

Differential screening by colony hybridization was conducted following the instruction manual of GenHunter Easytransfer™ Replica plating tool with some modifications.

The transformed INVαF' cells were diluted to 1:1000 and 20-50 μl of the diluted cells were spread on separate LB agar plates containing 50 μg/ml ampicillin. After overnight incubation at 37°C, 50-200 ampicillin resistant colonies were obtained from each cDNA clone. The ampicillin resistant colonies from each plate were transferred onto duplicate nylon membranes. All duplicate membranes were incubated on fresh LB agar plates at 37° (6-7 hr) until colonies of 0.5-1 mm diameter were obtained. The membranes were carefully removed from the plates and placed, colony side up, for 7 min on a 3M absorbent filter paper soaked in denaturing solution (0.5 M NaOH/1.5 M NaCl). The membranes were then transferred to a filter paper soaked in neutralizing solution (1.5 M NaOH/0.5 M Tris-HCl pH 7.2/0.001 M EDTA) for 3 min. The neutralizing step was repeated once with a fresh paper soaked in the same solution. The membranes were then rinsed in 2xSSC solution and air-dried on a piece of 3M Whatman paper. The DNAs were fixed onto the membranes by UV cross-linking. The duplicate membranes were separated into "plus" and "minus" groups in separate containers and prehybridized in a shaking water bath at 65°C for 6-7 hr. Equal counts of cDNA probes from *L. pennellii* and *L. esculentum*

were heat denatured for 10 min, chilled immediately on ice, added into its corresponding container representing "plus" and "minus" groups respectively, and hybridized overnight. Afterward, the membranes were washed once for 15 min in 2xSSC, 0.1% SDS at 65°C, once for 15min in 1xSSC, 0.1% SDS and once for 15 min in 0.1xSSC, 0.1% SDS. The membranes were air-dried and the duplicate membranes were processed for autoradiography in the same cassette at -80°C with an intensifying screen. After a 24 to 72 hr exposure, the X-ray films were developed and evaluated by comparing the hybridization signals on the corresponding duplicate filters. Those bacterial colonies containing the cDNA inserts corresponding to the differentially expressed mRNAs were identified and selected for the next step.

5. WUE cDNA library construction

In order to isolate the full length cDNAs corresponding to those cDNA fragments identified by differential display, a high WUE cDNA library was constructed using the ZAP-cDNA synthesis and Gigapack II Gold packaging system offered by Stratagene (Stratagene Corp., La Jolla).

The total RNA from *L. pennellii* was extracted as previously described (section 2). The RNA was applied to an oligo(dT) cellulose spin column (5 Prime → 3 Prime Inc., Boulder) in 0.5 M NaCl solution. The column was then washed with 0.5 M NaCl buffer three times and 0.1 M NaCl buffers two times by centrifugation at 200 g for 10 sec to remove poly(A)$^-$RNA. Poly(A)$^+$mRNA was spin eluted using a buffer (10mM Tris-HCl and 1mM EDTA, pH 7.5) that was

prewarmed to 65°C. The whole procedure was repeated once to make sure all poly(A)$^-$RNA was removed. The resulting poly(A)$^+$mRNA was then ready to serve as an efficient template for first strand cDNA synthesis.

The Stratagene's ZAP-cDNA synthesis protocol outlined in Fig. 3 was followed. A 50-base oligonucleotide primer-linker was used to prime first strand cDNA synthesis and provide an *Xho* I restriction enzyme recognition site and an 18-base poly(dT) sequence. The poly(dT) region bound to the 3' poly(A) region of the mRNA template, and the first strand cDNA was synthesized by MMLV reverse transcriptase.

During second strand cDNA synthesis, the RNA bound to the first strand cDNA was nicked by Rnase H to produce a multitude of fragments. These RNA fragments served as primers for DNA polymerase I to synthesize second strand cDNA. *Eco*R I adapters were ligated to the blunt ends, and the resulting cDNA-adapters were cleaved with *Xho* I to generate the directional cDNA.
The finished cDNAs were size fractionated on a drip column containing Sepharose CL-2B gel filtration medium. Eight μl of each collected fraction were electrophoresed on a 5% nondenaturing acrylamide gel to determine which fraction would be used for ligation. Afterward, the fractions were precipitated with ethanol to recover the size-selected cDNAs (> 500 bp). The recovered cDNAs were ligated into the Uni-ZAP XR lambda vector and *in vitro* packaged with Gigapack II packaging extract. The Uni-ZAP XR library was amplified to make a large, stable quantity of a high-titer stock of the library.

6. WUE cDNA library screening

The DNA screening protocol from Stratagene was used to screen the cDNAs associated with WUE. The procedure is outlined below.

The library was titered first to determine the concentration. The *E. coli* strain XL1-Blue MRF' served as a host cell for λ phage. About 50,000 pfu, 600 μl of host cell at an OD_{600} of 0.5 and 6.5 ml of NZY top agar were spread evenly on a large 150-mm NZY agar plate (20 plates to screen 1 x 10^6 plaques), and the plates were incubated at 37° for 4-6 hr. The plates were then chilled for 2 hr at 4°C to prevent the NZY top agar from sticking to the MSI nylon membrane (Micron Separation Inc., Westborough). The plaques were transferred to duplicate membranes, with a 2 min exposure with the first membrane and a 4 min exposure with the second one. The membranes were denatured for 5 min in 1.5 M NaCl/0.5 M NaOH, neutralized for 7 min in 1.5 M NaCl/0.5 M Tris-HCl pH 8.0, and rinsed for 2 min in 2xSSC solution. The DNA was fixed to the membranes using a UV crosslinker. Prehybridization and hybridization were carried out at 42°C in a solution containing 5xSSPE, 5x Denhardt's solution, 0.1% (w/v) SDS, and 100 μg/ml salmon sperm DNA for 6 and 24 hr, respectively. The probes were the cDNA fragments confirmed from differential screening and labeled with α-[^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1983). The membranes were washed twice at 52°C for 30 min and rinsed with 2xSSC. The membranes were placed between two sheets of plastic wrap and exposed to X-ray film at -80°C for 24-72 hr. After autogradiography, the plaques were identified and the corresponding clones were selected and titered with host cells

for the second screening. All steps were the same as above except that prehybridization and hybridization were conducted at a higher stringency 62°C in a solution containing 3xSSC, 2x Denhardt's solution, 0.5% SDS, and 50 μg/ml salmon sperm.

The positive plaques isolated from the second screening were excised *in vivo* using ExAssist helper phage with SOLR strain to obtain the insert-containing pBluescript phagemids. The phagemids were purified using standard molecular techniques (Sambrook *et al.*, 1989). The purified plasmids were digested with *Xho* I and *Eco*R I at 37°C for 2 hr. The digest was electrophoresed on 1% agarose gel, transferred to Hybond-N nylon membranes, and probed with the same probes used in the cDNA library screening. After a washing step (section 4.2), the membranes were exposed to X-ray films overnight and developed to identify plasmids which contained the inserts. The identified plasmids were purified using Wizard^{R} Minipreps (Promega Co., Madison) according to the manufacturer's instructions and sequenced automatically by a 373 DNA sequencer (Perkin-Elmer Inc., Branchburg) by the Nucleic Acid and Protein Resource Facility at Oklahoma State University. Sequence analyses were conducted using the BLAST network service (NCBI) and the MacVector program (Oxford Molecular Co., Medison) to identify the cDNAs.

IV. RESULTS

1. Determination of WUE and selection of F2s for DDRT-PCR

Rather than comparing genes expressed in stressed and unstressed plants, all plants were grown under unstressed conditions. Parents and pooled F2s were used to search for genes that were differentially expressed in plants with great WUE. Pooling of F2 individuals minimizes the risk of finding differences between parental species independent of WUE.

At the end of the growth period, plants were cut at the soil level. The roots were gently rinsed with water to remove soil. Shoots and roots were dried at 70°C to constant weight and total plant dry weight was determined. The water use by each plant was calculated by summing daily water additions over the growing period, and correcting for water evaporated from the soil. The WUE (g dry weight / kg water) was calculated by dividing total plant dry weight by water use during the growing period.

The WUE of *L. pennellii* (4.56 g / kg) was 33% greater than the WUE of *L. esculentum* (3.43 g / kg). Based on the value of WUE, ten F2 plants with the highest WUE and ten with the lowest WUE were selected. The average WUE value of the high pool of F2s (3.96 g / kg) was 35% greater than WUE of the low pool of F2s (2.94 g / kg). The analysis of variance test showed the differences in WUE between parents and the two pools were significant (Fig. 4).

Five grams leaf tissue with equal amounts from each F2 individual in a pool was collected for RNA extraction. The pools were named the high pool and the low pool. RT-PCR samples from *L. pennellii*, the high pool, *L. esculentum*, and the low pool were run side by side on a denaturing sequencing gel, so that the mRNA abundance from each sample could be directly compared (Fig. 5).

2. Cloning of cDNA fragments by DDRT- PCR

Differential display is a very sensitive, rapid and economical method for identifying mRNAs from differentially expressed genes isolated from many types of tissues or developmental stages. Although most of its original applications have been designed for the studies of differential gene expression in animal systems, the technique has already gained popularity in plant systems due to its simplicity and relative ease to use. With this technique, a total of 20 cDNA fragments were found differentially expressed among the four tomato populations. Five of them appeared in *L. pennellii* and high WUE F2s, but not in *L. esculentum* and low WUE F2s. Eight were not visualized in *L. pennellii* and high WUE F2s, but showed up in *L. esculentum* and low WUE F2s. Seven were found highly expressed in *L. pennellii* and high WUE F2s, but expressed at greatly reduced levels in low WUE F2s and absent in *L. esculentum* (Fig. 5). The use of pooled F2 individuals was intended to minimize interference by genes unrelated to WUE.

3. Confirmation of cDNA fragments by reverse northern

A screening step was used to verify the cDNA fragments identified by differential display. The standard protocol recommends the use of reamplified cDNA directly as a probe for Northern blot verification. A different approach is to clone the reamplified cDNA and then use it as a probe. Both approaches have their advantages and disadvantages. Reamplified cDNA from differential display sometimes contains a mixture rather than a single cDNA due to contamination by other co-migrating cDNA fragments. As a result, direct use of reamplified cDNA may cause multiple mRNAs to be detected, making the identification of the truly differentially expressed gene difficult. On the other hand, if the cDNA is cloned, the choice of which clone to use as a probe may be critical depending on the extent of cDNA contamination. The laborious screening and sometimes the limited amount of RNA samples are limitations in using the Northern blot technique.

By using reverse northern and colony hybridization one can avoid the problems mentioned above in screening for the cDNA fragments that truly represent differentially expressed mRNAs. First, this approach reduces the step of electrophoresis and transference of RNA which could be one of the sources causing RNA degradation. Second, many membranes representing different cDNA fragments obtained by differential display can be screened simultaneously with the same set of probes. In this study, ten cDNA fragments expressed in high WUE plants were verified using reverse northern. Seven of them were

selected based on the differential expression evidenced by the reverse northern (Fig. 6). The differences suggest they are positively related to WUE.

4. Isolation of cDNA clones from the high WUE cDNA library

Using the ZAP-cDNA Gigapack II system, a good representative primary WUE cDNA library was obtained, which contained ~5 x 10^6 clones. The seven cDNA fragments verified above were used as probes to screen the library. After a first and a second screening, plaques showed strong hybridization signal with four of the confirmed cDNA fragments. The other three cDNA fragments did not produce a hybridization signal. It is possible they represent extremely rare mRNAs. In this case, the standard screening protocol may need modification. The positive plaques were isolated and excised *in vivo* from the lambda vector.

Phagemids resulting from *in vivo* excision were purified, digested with *Eco*R I and *Xho* I, and hybridized with the same probes used above. If the plasmid harbors the appropriate insert, the probes should show hybridization signals. As a result, the A2 ($T_{11}A$ combined with AP2) probe hybridized with a fragment of ~1300 bp, and the A14 ($T_{11}A$ with AP14) probe hybridized with a fragment of ~1000bp. The A6 ($T_{11}A$ with AP6) and the C13 ($T_{11}C$ with AP13) probes also showed hybridization signals (data not shown).

5. Sequence similarity between A2 and Rubisco activase

The A2 cDNA clone was sequenced in full length from both strands. The sequence is presented in Fig. 7. The tomato A2 cDNA clone consists of 1675 base pairs. The putative open reading frame starts at bp 50 and ends at 1429,

and encodes a protein of 459 amino acid residues. A homology search in the GenBank database showed that the full length DNA sequence of the A2 clone has considerable nucleic acid and predicted amino acid sequence similarity with the RCA gene that encodes rubisco activase. A global alignment of A2 and other RCA amino acid sequences (Fig. 8) reveals that the putative tomato A2 protein is most similar to RCA from tobacco. The predicted amino acid sequence of A2 has 85.7% identity with RCA from tobacco, 74.8% identity with the apple RCA protein, and 71.9% identity with the *Arabidopsis* RCA protein. The A2 cDNA likely encodes a tomato rubisco activase.

The A14 cDNA clone was also sequenced in its full length. The homology search showed a good score match with plant ribosomal protein L17. cDNA clones A6 and C13 were partially sequenced. The partial sequence of C13 showed similarity to the small subunit of Rubisco, and A6 showed similarity to *Themophila* cystein protease. The tentative identity of A6 as a protease may explain the difficulty encountered in attempting to amplify this plasmid using a standard strategy.

V. DISCUSSION

Lycopersicon pennellii, native to the deserts of Peru, is a wild relative of the cultivated tomato. *Lycopersicon pennellii* requires less water during its growth period and has greater WUE. *Lycopersicon esculentum* uses more water to produce more biomass but has low WUE. *Lycopersicon pennellii* can produce a fertile interspecific hybrid with *L. esculentum* when it is used as the pollen parent. Comparing *L. pennellii, L. esculentum*, and the F1, Martin and coworkers have found that *L. pennellii* consistently shows greater WUE under various conditions than *L. esculentum* or the F1 hybrid in terms of weight of dry biomass produced per unit of water used (Martin and Thorstenson, 1988). This study confirmed the difference between the parents in a green house environment. The high WUE of *L. pennellii* is most probably related to expression or overexpression of specific genes. Such genes have not been identified and isolated from any plant though significant genetic variation in plant WUE has been demonstrated in numerous experiments. Using a relatively new PCR-based technique, we have extended the study on WUE in tomato by identifying genes whose expression is related to plant WUE.

PCR differential display is a method that was originally developed to identify differentially expressed genes in a pair of mammalian cell populations (Liang and Arthur, 1992). In plants, DDRT-PCR has been used in many different kinds of stress conditions and developmental stages such as ozone exposure,

pathogen stress, sucrose-starvation and fruit ripening to isolate genes involved in the regulatory pathway (Sharma and Davis, 1995; Tseng *et al.*, 1995; Wilkinson *et al.*, 1995; Truesdell and Dickman, 1997). The method is based upon comparisons of mRNAs expressed in two or more cell populations, by running reverse transcribed PCR products on sequencing gels in adjacent lanes. The differential expression can be readily detected by eye, the bands cut out of the gel, eluted, PCR amplified, and the products used to recover full-length clones from cDNA libraries.

Several studies (Ehdaie *et al.* 1993; Masle *et al.* 1993; Virgona *et al.* 1990) have shown that plant WUE is a quantitatively inherited trait and affected by environmental conditions. A number of genes that respond to drought and salt at the transcriptional level have recently been described (Ingram and Bartels, 1996; Bray, 1997). The mRNAs of these genes decrease when the plants are released from stress conditions, suggesting that these genes respond to drought or salt stress. These genes appear to have no relation with WUE. The functions of some drought-induced gene products have been predicted from sequence homology with known proteins and are thought to have a role in protecting the cell from drought stress (Ingram and Bartels, 1996; Bray, 1997). But some of them are not necessarily responsible for drought resistance. One example is the study of expression characteristics of genes that are induced by drought stress in tomato (Kahn *et al.*, 1993). Four genes that were expressed under drought were isolated from *L. esculentum*. The genes required an elevated level of ABA for expression (Cohen and Bray, 1990). Studies were

undertaken to compare the expression patterns of the genes in *L. esculentum, L. pennellii* and the F1. The patterns of expression of these genes in *L. pennellii* were similar to that of *L. esculentum*. This suggests that although responsive to drought, these genes play a similar, yet undefined, role in both genotypes rather than being genes that are specifically responsible for the greater drought resistance of *L. pennellii*. In order to detect the genes that really contribute to WUE instead of being simply drought induced, unstressed conditions were used in our study. Members of the genus *Lycopersicon* contain homosequential chromosomes (Rick and Yoder, 1988), and the majority of cDNAs isolated from tomato have homologies in *L. pennellii* (Zamir and Tanksley, 1988). Still, a minority of cDNAs are different between *L. pennellii* and *L. esculentum*. By simply using the two genotypes as a comparison, there would be many species differences unrelated to WUE. In order to avoid this problem, pools of F2 individuals were used as controls to assist screening the mRNAs associated with WUE. Ten F2 plants with the highest WUE and ten with the lowest WUE were selected and pooled. The average WUE value of the high pool was 3.96 g / kg (dry weight/water) and of the low pool it was 2.90 g / kg. The difference in WUE between the two pools was statistically significant.

Twenty putative WUE associated mRNAs were identified by differential display. Five of the mRNAs were strongly expressed in *L. pennellii* and high WUE F2s, but not in *L. esculentum* and low WUE F2s. Seven of them were highly expressed in *L. pennellii* and high WUE F2s, but expressed at greatly reduced levels in low WUE F2s and not in *L. esculentum*. These mRNAs may

be expressed to a greater extent in water-use efficient plants. Eight of the mRNAs were found only in *L. esculentum* and low WUE F2s, which may suggest they are expressed at a lower level in water-use efficient plants. Only the twelve up-regulated mRNAs were further tested by reverse northern. Seven of these mRNAs were confirmed to be differentially expressed in high WUE plants. These mRNAs may encode proteins whose function is specifically associated with plant WUE. The cDNAs corresponding to these seven mRNAs were used as probes to recover the full-length cDNAs from a WUE cDNA library. Four cDNA clones were isolated from the high WUE cDNA library, named A2, A6, A14, and C13. The full length of clones A2 and A14 were sequenced and compared with sequences in available databases. Clones A6 and C13 were partially sequenced and analyzed for tentative functions. The most interesting mRNA identified, in terms of homology and potential function, is A2. Sequence comparison clearly indicates that the protein encoded by the A2 cDNA is a Rubisco activase.

Rubisco activase is a nuclear-encoded, soluble chloroplast protein that regulates the activity of Rubisco *in vivo* (Portis, 1992). The protein was first described by Salvucci *et al.* (1985), who discovered that the *rca* mutant of *Arabidopsis* lacked Rubisco activase and demonstrated the participation of the protein in activating Rubisco *in vitro*. Rubisco activase has been detected in all higher plant species investigated to date (Salvucci *et al.*, 1987). Numerous *in vitro* experiments have shown that Rubisco activase has an ATP requirement and appears to promote the activation of Rubisco by removing bound sugar-

phosphate inhibitors from the active site. Inspection of the derived amino acid sequence of A2 revealed two regions that share homology with the nucleotide binding domains identified in a variety of polypeptides from both animals and bacteria (Duncan *et al.*, 1986; Higgins *et al.*, 1986). These regions are located at amino acid residues 164-171 and 219-228 of the polypeptide. The amino acid sequence of the two regions were identical in *L. pennellii*, tobacco, and other species. These observations are consistent with an ATP requirement for Rubisco activase-mediated activation of Rubisco *in vitro* (Streusand and Portis, 1987). Site-directed mutagenesis of the conserved lysine 169 in the consensus nucleotide-binding domain of spinach abolished the capability of the protein to bind ATP as well as the Rubisco activase and ATPase activities. These results confirm that lysine 169 is an essential residue (Shen *et al.*, 1991). In the *L. pennellii* enzyme, the predicted amino acid sequence containing asparagine (N)71-lysine(K)-leucine(L)-aspartic acid(D)74 matches a highly conserved sequence motif (N/T)(K/Q)XD found in many guanine nucleotide binding proteins (Nuoffer and Balch, 1994). The photoaffinity labeling of tobacco Rubisco activase indicated that this region might interact with the adenine base of ATP (Salvucci *et al.*, 1994). The dependence of Rubisco activase activity on ATP serves an important regulatory function in photosynthesis by coupling the rate of CO_2 fixation via Rubisco to the rate of electron transport activity (Salvucci *et al.*, 1985, 1986). Rubisco activase might promote plant WUE by elevating the rate of CO_2 fixation. Additional work will be required to further identify the tomato

Rubisco activase, its gene expression patterns, and its relationship to plant WUE.

Database searches revealed that clone A14 encodes a ribosomal protein L17 which is required for translation. The observation that a ribosomal protein displays distinctive differential expression was not an anticipated result. It is known that protein synthesis occurs throughout plant development, and a great demand for ribosomal protein activity might be associated with plant growth and consequently affect plant WUE.

A partial sequence showed clone C13 tentatively encodes the small subunit of Rubisco. Rubisco is a critical enzyme involved in photosynthesis. The Rubisco holoenzyme is composed of eight large subunits and eight small subunits. The large subunit is encoded by a single chloroplast gene *rbc*L, whereas the small subunit is encoded by a small family of nuclear *rbc*S genes. Rodermel *et al.* (1988) demonstrated that antisense RNA directed against *rbc*S mRNA can reduce the amount of Rubisco present in the leaves of transformed tobacco plants and reduce the rate of photosynthesis.

The partial sequence of clone A6 has considerable nucleotide sequence homology with a *Thermophila* cystein protease. It is unclear how this protease might be involved in controlling plant WUE. One possibility is that the protease degrades proteins to yield small molecules in the cell which by their osmotic activity allow cells to hold water and increase plant stress resistance.

By using the pooled plant material combined with the differential display technique, we were able to identify and isolate genes that may be associated

with plant WUE. The differential display can be a useful supplement to or replacement for the more routinely used subtractive hybridization and differential plaque hybridization techniques. As a relatively new method, however, there are still problems to be resolved. For example, as mRNA must be isolated and cDNA obtained to act as the template for PCR, the quality and quantity of RNA used can significantly influence the results. The PCR technique itself is so sensitive that there are significant risks of creating artificial products if proper care is not taken. Even with proper replication, northern analysis is a necessary step to further verify the differentially expressed mRNAs. In our experiment, seven of twelve mRNAs were confirmed by reverse northern and four of the seven were recovered to full-length from the cDNA library. The mRNAs that were not confirmed and recovered could simply be artifacts of the PCR procedure or they might be present at extremely low abundance. If relevant, it should be possible to identify them with other techniques. However, with increased application of differential display, the critical determinants of success and utility of the procedure should eventually be well established.

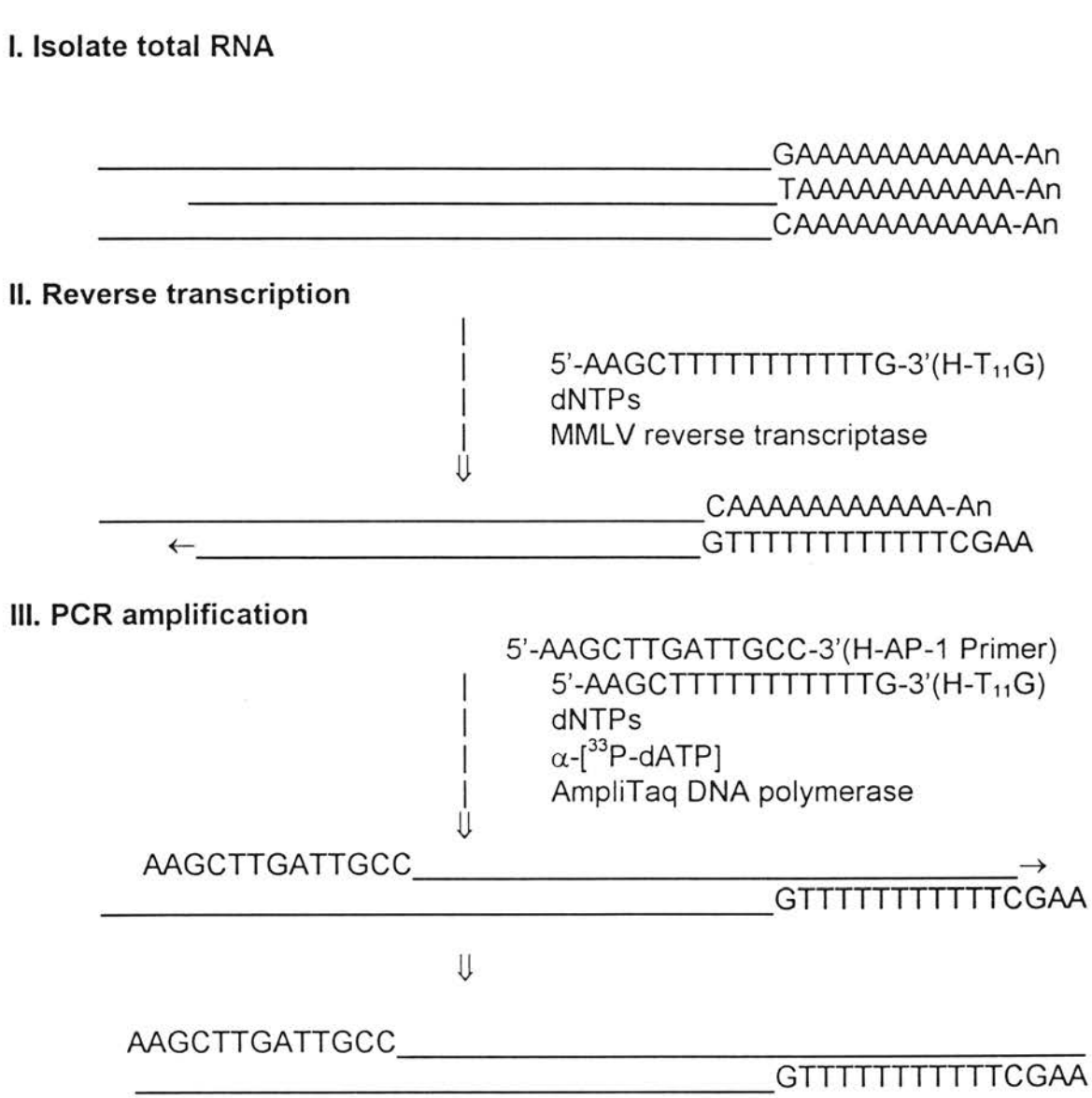

Fig. 1. Differential display schematic diagram

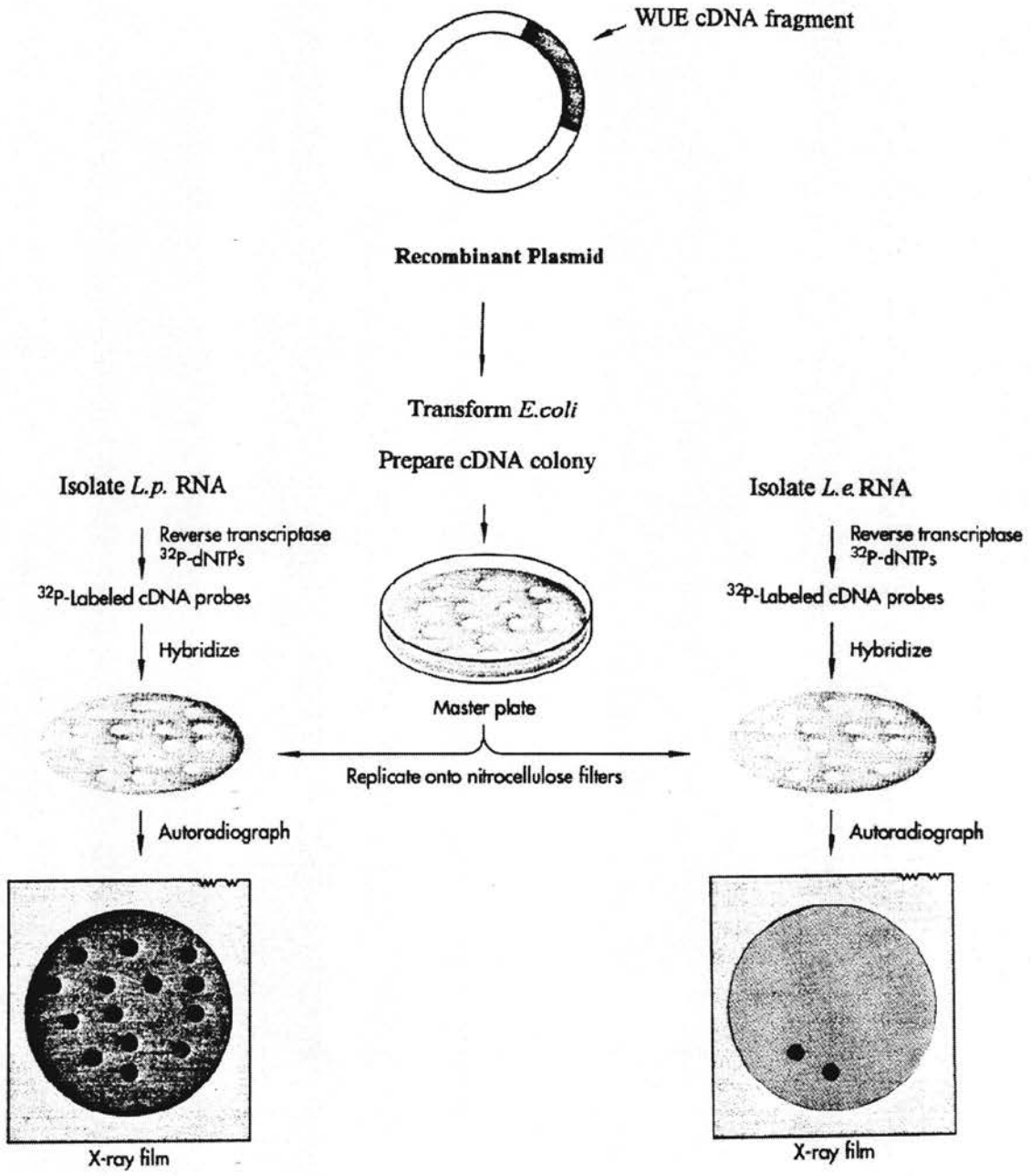

Fig. 2. Reverse northern schematic diagram

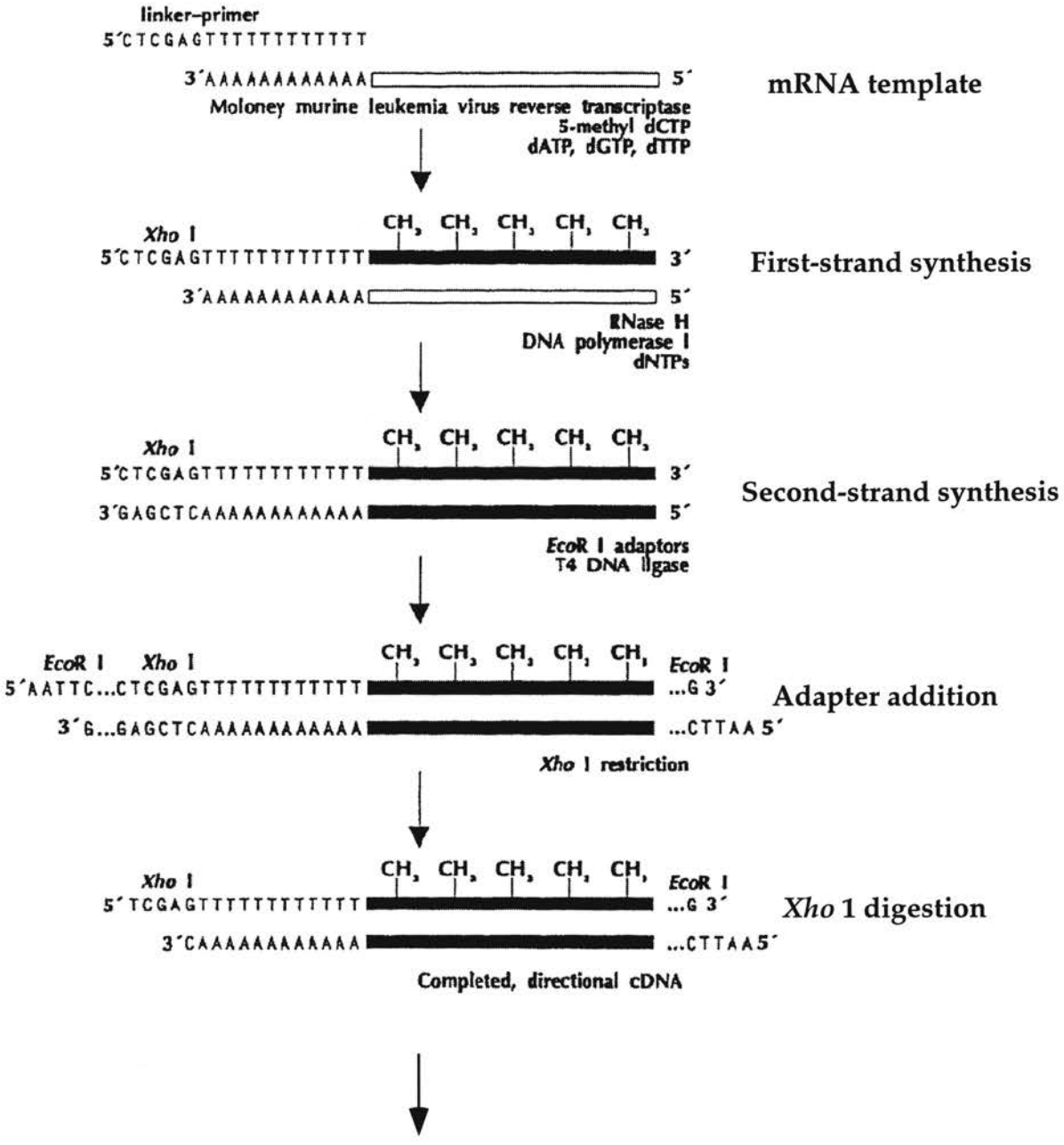

Fig. 3. cDNA library construction schematic diagram

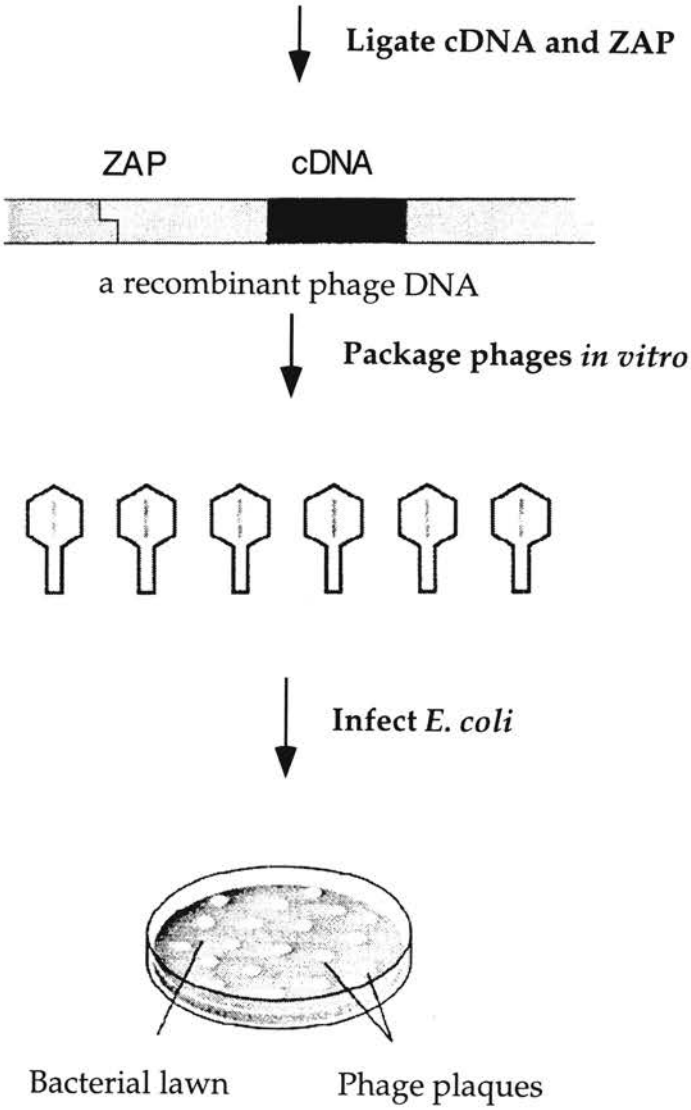

Fig. 3. cDNA library construction schematic diagram (cont.)

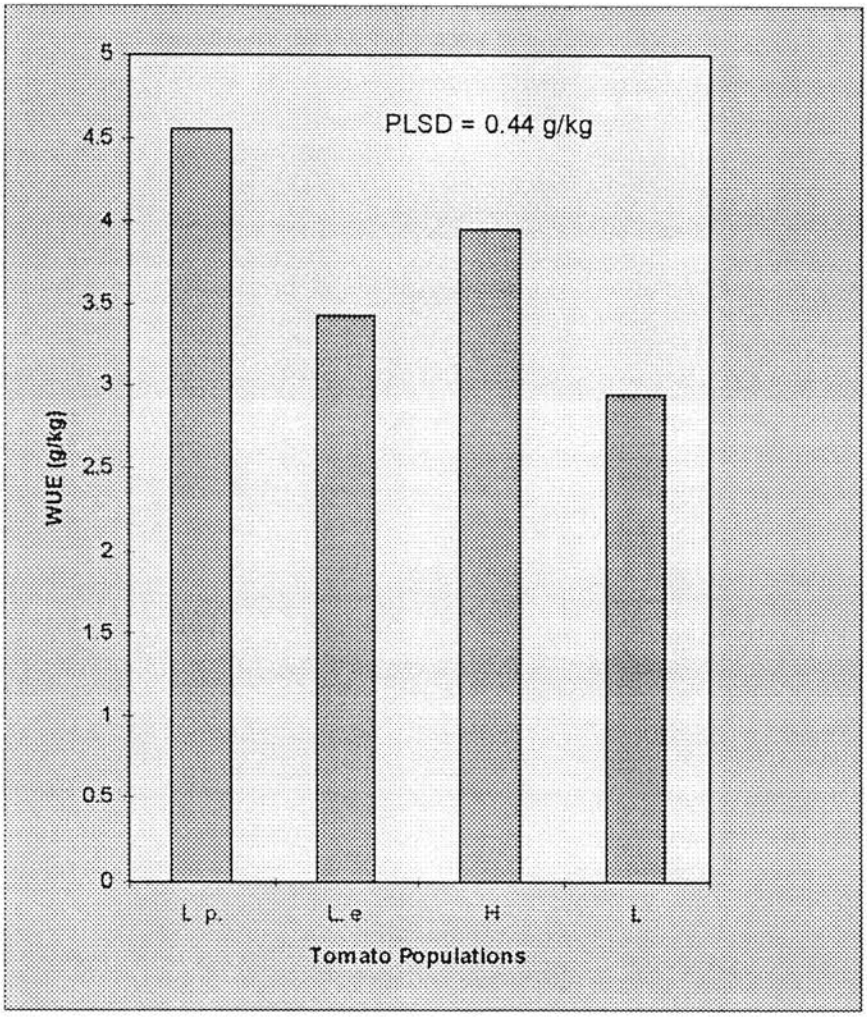

Fig. 4. Water-use efficiency of four tomato populations. *L. pennellii* (L.p.), *L. esculentum* (L.e.), F2 high WUE pool (H), and F2 low WUE pool (L). The differences in WUE between the parents and the two pools are significant. Protected least significant difference (PLSD) at 1% level is 0.44 g/kg.

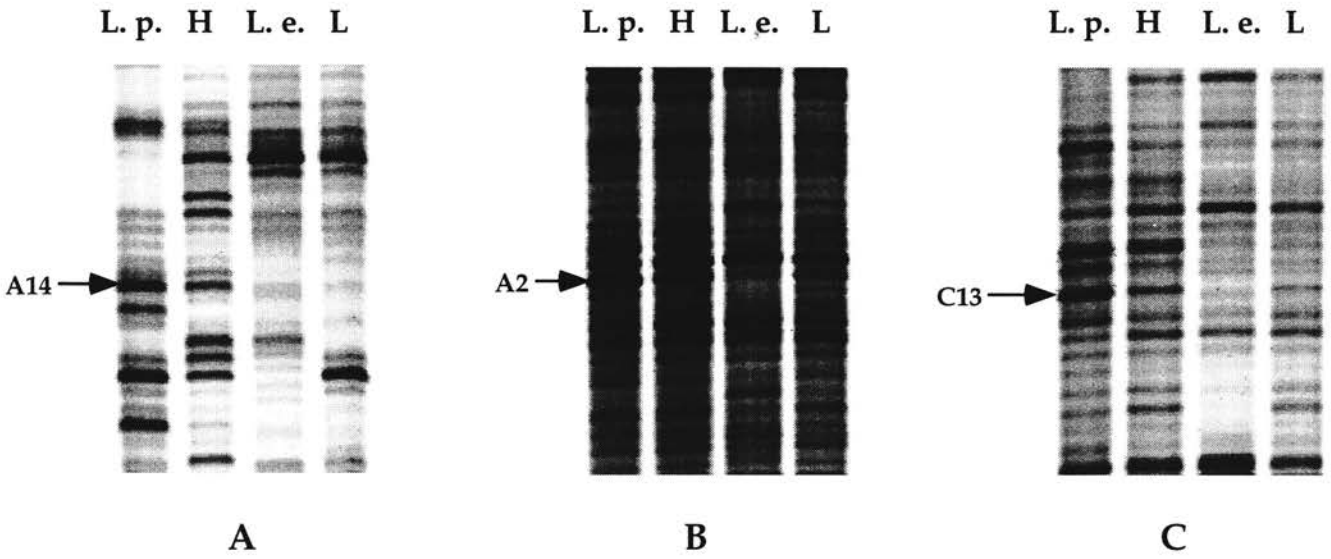

Fig. 5. Differential display of tomato leaf tissue RNA. Four RNA samples from *L. pennellii* (L. p.), F2 high WUE pool (H), *L. esculentum* (L. e.) and F2 low WUE pool (L) were compared by differential display using anchored primer H-T_{11}A in combination with arbitrary primer H-AP14 (A), H-T_{11}A with H-AP2 (B) and H-T_{11}C with H-AP13 (C). The A14, A2 and C13 cDNA fragments are marked by the arrows.

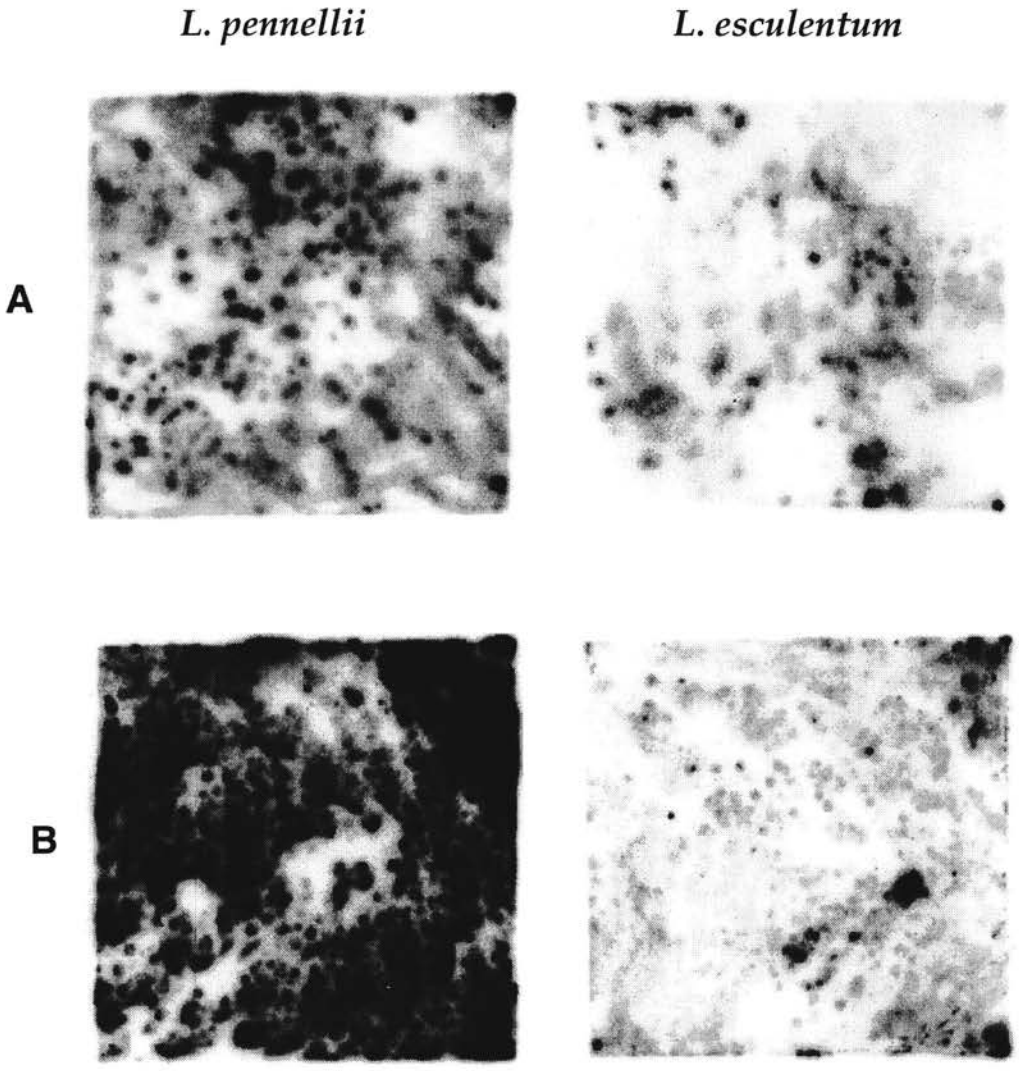

Fig. 6. Reverse northern screening of cDNA fragments obtained from differential display. Ampicillin-resistant colonies from A2 (A) and A14 (B) were transferred onto duplicate filters and processed for differential hybridization with labeled cDNA probes from *L. pennelli* or *L. esculentum.*

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AAGGCAAATCAAGGAAGCTTTTTCATCAATTTCTGTTTAGTCATCAAGAATGGCTGCCTC          60
                                                    M  A  A
AGTGTCAACCATTGGAGCTGCCAGCAAAGCACCATTGAGTTTGAACAACTCAGTTGCTGG          120
S  V  S  T  I  G  A  A  S  K  A  P  L  S  L  N  N  S  V  A
AACTTCCGTTCCAAGCACAGCCTTCTTTGGAAAATCCTTGAAAAAAGTGTATGCAAAAGG          180
G  T  S  V  P  S  T  A  F  F  G  K  S  L  K  K  V  Y  A  K
TGTTTCCAGCCCCAAGGTTTCAAACAGGAACTTGAGGGTTGTAGCTCAAGAAGTAGACGA          240
G  V  S  S  P  K  V  S  N  R  N  L  R  V  V  A  Q  E  V  D
AACGAAAGAGGACAGATGGAAGGGTCTTTATGATAACACATCGGATGACCAACAGGACAT          300
E  T  K  E  D  R  W  K  G  L  Y  D  N  T  S  D  D  Q  Q  D
TGCAAGGGGTAAGGGTCTGGTCGACAGTCTTTTCCAGGCTCCTACCGGTACTGGTACTCA          360
I  A  R  G  K  G  L  V  D  S  L  F  Q  A  P  T  G  T  G  T
CCACGCCATCATGAATTCCTACGAATACGTCAGCCAGGCTCTTAAAACGTACCAATTGGA          420
H  H  A  I  M  N  S  Y  E  Y  V  S  Q  A  L  K  T  Y  Q  L
CAACAAATTGGACGGCTTCTACATTGCCCCTGCTTTCATGGACAAACTTGTTGTTCACAT          480
D  N  K  L  D  G  F  Y  I  A  P  A  F  M  D  K  L  V  V  H
CACCAAGAACTTCTTGACATTGCCCAACATCAAGGTTCCACTCATCTTGGGTGTATGGGG          540
I  T  K  N  F  L  T  L  P  N  I  K  V  P  L  I  L  G  V  W
AGGCAAAGGTCAAGGTAAATCATTCCAATGTGAGCTTGTCTTCAGAAAGATGGGAATCAA          600
G  G  K  G  Q  G  K  S  F  Q  C  E  L  V  F  R  K  M  G  I
CCCCATTATGATGAGTGCTGGAGAATTGGAAAGTGGAAATGCAGGAGAGCCAGCTAAATT          660
N  P  I  M  M  S  A  G  E  L  E  S  G  N  A  G  E  P  A  K
GATTAGGCAAAGGTACAGAGAGGCAGCTGAAATCATCAGGAAAGGAAACATGTGTTGTCT          720
L  I  R  Q  R  Y  R  E  A  A  E  I  I  R  K  G  N  M  C  C
CTTCATCAACGATCTCGATGCAGGAGCTGGTAGAATGGGTGGAACTACCCAATACACCGT          780
L  F  I  N  D  L  D  A  G  A  G  R  M  G  G  T  T  Q  Y  T
CAACAACCAGATGGTGAATGCCACCCTCATGAACATTGCTGACAACCCAACAAATGTCCA          840
V  N  N  Q  M  V  N  A  T  L  M  N  I  A  D  N  P  T  N  V
GCTCCCCGGTATGTACAACAAGCAAGAGAACGCCAGGGTACCCATTATTGTCACTGGTAA          900
Q  L  P  G  M  Y  N  K  Q  E  N  A  R  V  P  I  I  V  T  G
CGACTTCTCCACATTGTATGCTCCTCTTATCCGTGATGGTCGTATGGAGAAGTTCTACTG          960
N  D  F  S  T  L  Y  A  P  L  I  R  D  G  R  M  E  K  F  Y
GGCACCAACTAGGGAGGATAGAATTGGTGTTTGCAAGGGTATTTTCAGAACTGACAACGT         1020
W  A  P  T  R  E  D  R  I  G  V  C  K  G  I  F  R  T  D  N
CCCTGAGGAAGCTGTTGTAAAGATTGTCGATTCCTTCCCTGGACAATCTATTGATTTCTT         1080
V  P  E  E  A  V  V  K  I  V  D  S  F  P  G  Q  S  I  D  F
TGGTGCTTTGAGGGCCCGAGTATATGACGATGAAGTGAGGAAATGGGTTTCAGGAACTGG         1140
F  G  A  L  R  A  R  V  Y  D  D  E  V  R  K  W  V  S  G  T
AATTGAACTCATTGGTGAAAAACTTTTGAACTCTAGAGATGGACCCCCAACTTTTGAGCA         1200
G  I  E  L  I  G  E  K  L  L  N  S  R  D  G  P  P  T  F  E
ACCAAAAATGACCCTTGAGAAGCTCCTTGAGTATGGTAACATGCTTGTTCAAGAGCAAGA         1260
Q  P  K  M  T  L  E  K  L  L  E  Y  G  N  M  L  V  Q  E  Q
GAATGTCAAGAGAGTCCAGTTGGCTGAAACCTATCTTAAAGAGGCAGCTCTCGGAGATGC         1320
E  N  V  K  R  V  Q  L  A  E  T  Y  L  K  E  A  A  L  G  D
TAACGCTGATGCCATCAACACTGGAATTTCTAAGAACTTCACCAATCTCAAAAGTCGTCT         1380
A  N  A  D  A  I  N  T  G  I  S  K  N  F  T  N  L  K  S  R
AAACAATGAAGAGGCGAAAAAAGCGCGACATGTCAACTTCCAGGAGTGATGATGTATTTT         1440
L  N  N  E  E  A  K  K  A  R  H  V  N  F  Q  E

ATTTCTTCAACTTTTTTTGAGGCAATTTGTGAGGGTAGCTATAGAATGTGACTCAACTGT         1500
TTAATATTGTTATGTAAATCAGTTGTGTTCCACTGTTAAGGGAATACAAACTCCAAATAA         1560
TCTTTTGTTTTCAGTTTTTGTCCATTATATTCTTCTGTAATCAACTTGTTGATTTGGAAC         1620
TTCAATATATTTGCTTTGTAAAAAAAAAAAAAAAAACGCGAGACTAGTTCTCTCT              1675
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Fig.7. Nucleotide and predicted amino acid sequences of *L. pennellii* Rubisco activase. The start and stop codons are underlined.

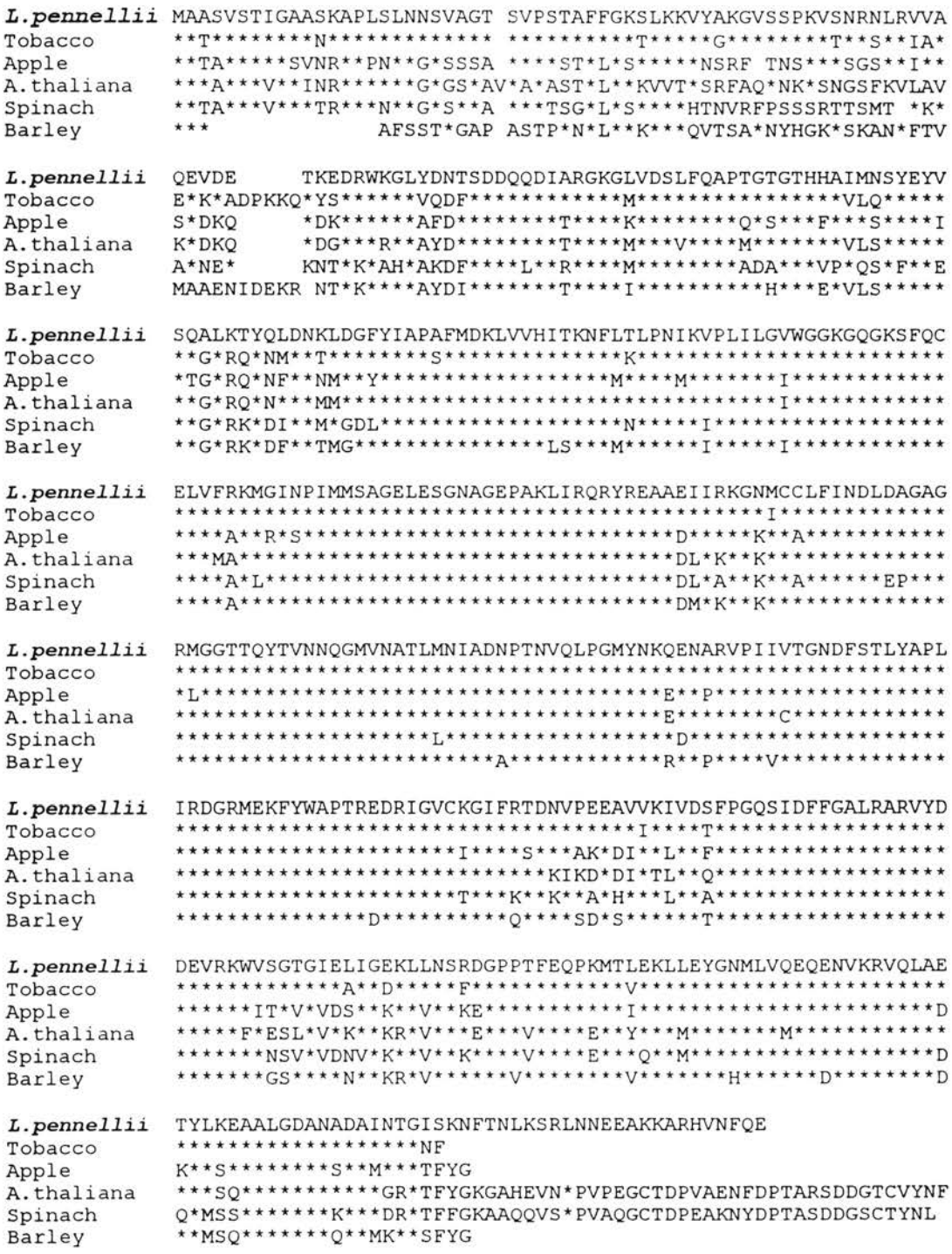

Fig. 8. Predicted amino acids sequences of *L. pennellii* Rubisco activase showing high similarity with those of tobacco, apple, *A. thaliana*, spinach and barley. Asterisks indicate identity and blanks represent gaps introduced to optimize alignment.

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CHAPTER II

CHARACTERIZATION OF GENES ASSOCIATED WITH WATER-USE EFFICIENCY

I. INTRODUCTION

Water-use efficiency at the leaf level is defined as the ratio of the fluxes of CO_2 and water vapor. Either increasing the rate of photosynthesis or decreasing the rate of water loss will result in elevated WUE. It is likely that genes affecting the pathway of ABA biosynthesis which controls the stomatal opening or genes that affect the activity of fundamental enzymes of the Calvin cycle would greatly affect WUE. The previous chapter reported a full length cDNA (A2) that has been isolated and sequenced using the differential display technique. Based on homology to known proteins, this cDNA is thought to encode a *L. pennellii* tomato ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) activase. Rubisco activase is required for activation of Rubisco *in vivo*. Rubisco is one of the most important enzymes in photosynthesis. The significance of Rubisco for photosynthesis and plant growth has been investigated using tobacco transformed with antisense Rubisco small subunit (racS) to decrease the expression of Rubisco (Rodermel *et al.*, 1988; Hudson *et al.*, 1992). The Rubisco content of the transformants was lower by 35, 80 and over 90% than that of the wild-type (Krapp *et al.*, 1994). Decreased WUE was also seen in the antisense racS plants. Compared to the wild type, WUE was marginally lower in plants with 65% Rubisco content, and much lower (up to 4-fold) in plant with 20% Rubisco content. Other studies of antisense racS (Quick *et al.*, 1991; Hudson *et al.*, 1992) also showed that transformants with reduced

Rubisco content exhibited low WUE. The decline was not large in plants with a 30-40% reduction in Rubisco, but was very marked in plants with 5-fold less Rubisco.

Rubisco must be activated to catalyze CO_2 fixation efficiently. Activation of Rubisco in the light is regulated by the stromal enzyme named Rubisco activase (Portis, 1992). This nuclear-encoded protein was first reported by Salvucci *et al* (1985) in studies on the biochemical lesion in a high CO_2-requiring mutant of *Arabidopsis* (Somerville *et al.*, 1982). These and subsequent studies (Portis *et al.*, 1986; Salvucci *et al.*, 1986; Mate *et al.*, 1993; Eckardt *et al.*, 1997) have firmly established an essential role for Rubisco activase in maintaining the activation state of Rubisco in the light at levels that are adequate for photosynthesis. In other words, Rubisco activase causes Rubisco to catalyze CO_2 fixation more efficiently. A high level of WUE is dependent on contributions from high CO_2 uptake, low water consumption, or both. It seems reasonable to propose that Rubisco activase promotes high WUE by elevating the rate of CO_2 fixation. The linkage between Rubisco activase and plant WUE may provide additional insight into the role of the biochemistry of photosynthesis in the control of WUE.

Light, temperature, water, nutrients and carbon dioxide have been proposed to limit growth of plants through effects on the photosynthetic apparatus (Woolhouse, 1983). Since Rubisco activase is involved in the photosynthesis process, these five environmental factors may affect its gene expression. This study was designed to further identify and characterize the

WUE associated genes previously isolated. The specific objectives were to: 1) study expression patterns under different environmental conditions (light, temperature, and water content), 2) study tissue-specific expression patterns of the water-use efficient phenotype using different tissues (leaf, stem, and root), and 3) determine the number of copies of Rubisco activase in the genome of *L. pennellii*.

II. LITERATURE REVIEW

Rubisco activase was first linked to the biochemical lesion responsible for the high CO_2-requiring phenotype of the *rca* mutant of *Arabidopsis* (Somerville *et al.*, 1982), a mutant that lacks the Rubisco activase protein (Salvucci *et al.*, 1985). Studies of Rubisco in the mutant (Somerville *et al.*, 1982; Salvucci *et al.*, 1986) and in transgenic tobacco plants with reduced Rubisco activase levels (Mate *et al.*, 1993) showed that Rubisco can not achieve and maintain an adequate level of activation for growth at ambient CO_2 without the aid of Rubisco activase. Rubisco activase is synthesized on cytosolic ribosomes and imported into the chloroplast. The polypeptide is initially synthesized as a precursor and processed into a mature polypeptide of about 41 to 45 kDa by removal of the transit peptide (Werneke *et al.*, 1988b). In many plant species, including spinach and *Arabidopsis*, there are two forms of Rubisco activase polypeptides that arise from alternative pre-mRNA splicing at the intron nearest the 3' end of the RNA (Werneke and Ogren, 1989). Thus, the longer polypeptide differs from the shorter by having an additional 35 or so amino acid residues at the carboxy terminus. Crafts-Brandner *et al.* (1997) showed that the two forms of spinach activase differ markedly in their thermal stability and the 45 kDa form is more thermostable than the 41 kDa form. Both Rubisco activase polypeptides were absent in the *rca* mutant, which required elevated CO_2 concentration for growth.

Western immunoblots from a few species, such as tobacco and *Chlamydomonas*, indicate the presence of only a single 41- or 42 kDa polypeptide (Salvucci *et al.*, 1987; Roesler and Ogren, 1990). These observations suggest that the larger polypeptide is not essential in these species.

The primary structure of Rubisco activase is highly conserved (>80%) at the amino acid level with the exception of the C-terminal end of the longer form. There is at least one consensus nucleotide binding domain (Werneke *et al.*, 1988b; Shen *et al.*, 1991), which is involved in nucleotide-phosphate binding (Salvucci, 1993). Other regions involved in ATP binding and catalysis have also been identified (Salvucci *et al.*, 1994; Salvucci and Klein, 1994). The expression of Rubisco activase is regulated by light and is tissue-specific. Rubisco activase transcripts are expressed predominantly in leaves and are undetectable in roots (Zielinski *et al.*, 1989), as observed with transcripts for other photosynthetic proteins. The Rubisco and Rubisco activase genes are coordinately expressed with parallel accumulation of Rubisco activase, Rubisco large subunit (*rbcL*), and Rubisco small subunit (*rbcS*) mRNAs in barley leaves (Zielinski *et al.*, 1989).

Some sugar-phosphates bind tighter to Rubisco when the active site of this enzyme is not carbamylated (Portis, 1992). Physiologically the most significant of these sugar-phosphates is the substrate, ribulose 1,5-bisphosphate (RuBP). The tight binding of RuBP to decarbamylated Rubisco prevents spontaneous carbamylation and tends to stabilize Rubisco in an inactive form (Portis, 1992). Other sugar-phosphates bind extremely tightly to Rubisco once

the active site is carbamylated. These compounds are tight-binding competitive inhibitors that inhibit catalysis by preventing the substrate RuBP from binding to the carbamylated enzyme. These sugar-phosphates include carboxyarabinitol 1-phosphate (CA1-P) and 3-ketoarabinitol bisphosphate (KABP) (Gutteridge *et al.*, 1986; Berry *et al.*, 1987; Edmondson *et al.*, 1990). Xylulose bisphosphate is also a potent inhibitor of Rubisco by tightly binding to decarbamylated Rubisco at pH < 7.5 or carbamylated Rubisco at pH > 8.0 (Yokota, 1991; Zhu and Jensen, 1991). When Rubisco activase and ATP are present, Rubisco activity is no longer inhibited by RuBP (Salvucci and Ogren, 1996). Werneke *et al.* (1988a) showed that there is an increase in carbamylation of Rubisco concomitant with the increase in Rubisco activity. Transgenic plants with an antisense RNA against Rubisco activase had reduced Rubisco carbamylation and photosynthesis (Mate *et al.*, 1993). Thus, Rubisco activase overcomes inhibition of enzyme activity by facilitating carbamylation of Rubisco. Similarly, Rubisco activase also relieves inhibition caused by the tight binding of sugar-phosphates to carbamylated Rubisco. Evidence by Portis (1992) and Mate *et al.* (1993) suggests that the major role of Rubisco activase is in accelerating the release of RuBP and other tight-binding sugar-phosphates from Rubisco, thus freeing the active site for activation by CO_2 and elevating catalysis.

The possible mechanism for Rubisco activase involves a physical association between the proteins that alters the Rubisco binding site, but the details of such an interaction remain speculative (Portis, 1992). ATP hydrolysis by Rubisco activase causes global conformational changes that affect the active

site of Rubisco, converting Rubisco into a form that binds sugar-phosphates less tightly. In the new conformation, tight binding sugar-phosphates dissociate more readily from Rubisco, freeing the active site for spontaneous carbamylation by CO_2 and catalysis upon binding RuBP. Evidence from de Jimenez *et al.* (1995) suggests that Rubisco activase belongs to the molecular chaperone family. First, the active Rubisco conformation requires Rubisco activase action in an ATP hydrolysis-dependent process, similar to that reported for molecular chaperones (Goloubinoff *et al.*, 1989; Skowyra *et al.*, 1990). Second, Rubisco activase, as well as other molecular chaperones, is widely distributed in large amounts in living organisms (Salvucci *et al.*, 1987; Ellis and van der Vies, 1991). Finally, the strongest evidence suggesting Rubisco activase is a molecular chaperone is that a large recovery of Rubisco activity is achieved from heat-inactivated Rubisco by addition of Rubisco activase and an energy source, similar to that reported for other heat-inactivated enzymes (Goloubinoff *et al.*, 1989; Skowyra *et al.*, 1990). Eckardt and Portis (1997) found that Rubisco is an extremely heat stable enzyme, whereas Rubisco activase is extremely heat sensitive. Rubisco activase alone does not have the ability to restore the activity of heat-inactivated Rubisco and is unlikely to protect or restore Rubisco activity from heat denaturation in *vivo* because it is more heat-labile than Rubisco. Evidence from Feller *et al.* (1998) indicates that high temperature physically perturbs activase, leading to an inhibition of enzyme activity and the consequent effect on activation of Rubisco. These results support the earlier reports of high-temperature effects on photosynthetic CO_2 fixation (Weis, 1981; Kobza and

Edwards, 1987), indicating that the inhibition of Rubisco activase may be a key regulatory process affected by high-temperature stress.

By using antisense RNA technology, Mate *et al.* (1993) showed that transgenic plants with an antisense gene against mRNA for Rubisco activase had less Rubisco activase and a lower photosynthesis rate. Evidence by Jiang *et al.* (1994) also showed that the rate of photosynthesis and plant growth were reduced in transgenic tobacco with antisence inhibition of Rubisco activase expression at the levels of both mRNA and protein accumulation. The decrease in photosynthesis rate in the antisense plants was accompanied by a corresponding decrease in Rubisco activation. These results indicate that activase has a direct effect on photosynthesis rates in the antisense plants by influencing the activation state of Rubisco. Result from *rbcS* antisense mutants suggests that in high light conditions, control of photosynthesis is largely shared between Rubisco and Rubisco activase (Jiang *et al.*, 1994).

It is likely that Rubisco activase affects plant WUE by affecting carbon uptake. However, no attention has been paid to understanding the control that activase exerts on WUE. As mentioned earlier, high WUE depends on a high rate of carbon acquisition, a low rate of water consumption, or both. The CO_2 assimilation rate is under control of Rubisco activase through its effect on the activation level of Rubisco. It is likely Rubisco activase promotes high WUE by elevating carbon gain.

III. MATERIALS AND METHODS

In order to characterize the cloned cDNAs, gene copy number was investigated by DNA blotting. Gene expression analysis was conducted using RNA blotting.

1. Plant materials and environmental factors

Plants of *L. pennellii* were grown in a growth chamber (Conviron, Controlled Environments Limited, Winnipeg) at 14h/10h day/night length and 30°C/20°C day/night temperature. The plants were watered daily to maintain a soil moisture level of 100% of field capacity. After five weeks, nine plants were transferred to a growth chamber at the same condition as above except the day temperature was 25°C, and nine plants to a chamber at a 37°C day temperature. Light treatment was conducted in one growth chamber at 14h/10h day/night length and 30°C/20°C day/night temperature. In this growth chamber, nine plants were covered with cheesecloth to obtain 385 μmol photons m^{-2} s^{-1} PAR and nine plants to obtain 270 μmol photons m^{-2} s^{-1} PAR compared to the uncovered plants which were exposed to full light of 500 μmol photons m^{-2} s^{-1} PAR. Plants grown at 30°C and full light were used as control plants. Leaf samples were collected separately after 1 hr, 2 hrs, 4 hrs, 7 hrs and 12 hrs exposure to the treatments. Water stress exposure was conducted in a chamber at 30°C/20°C day/night temperature and full light for 2 days, 3 days, 5 days and 7

days without watering. Samples of leaf tissue were collected for analyses of different treatments, and for tissue-specific gene expression, samples from leaf, stem and root were collected. Leaf samples from *L. pennellii* and *L. esculentum* grown at 14h/10h day/night length and 30°C/20°C day/night temperature were harvested for evaluation of gene expression.

2 DNA blotting

Genomic DNA was isolated from leaf samples of *L. pennellii* using a CTAB mini-prep procedure (Haymes, 1996) with some modifications. Two grams of leaf tissue was ground in liquid nitrogen with chilled mortar and pestle. The fine powder was transferred into a cold polypropylene tube and mixed with 10 ml extraction buffer and incubated at 65°C for 10 min. The extraction buffer contained 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 2% hexadecyl-trimethyl-ammonium bromide (CTAB), and 0.4% β-mercaptoethanol. The mixture was partitioned twice against one volume of chloroform/isoamyl alcohol (24:1, v:v). The upper aqueous layer was collected by centrifugation of the mixture at 3000 g for 15 min at 4°C and transferred to a fresh tube. The total DNA was precipitated from the aqueous phase by addition of one volume of isopropanol. The DNA was hooked out, washed with 75% ethanol, air dried and resuspended in 500 μl water.

About 20 μg DNA was separately digested with 30 units of *Eco*R V, *Hind* III, *Hae* III, *Dra* I, *Eco*R I, and *Bst*N I and size fractionated through a 0.8% agarose gel in a 1xTAE buffer. The gel was treated with 250 mM HCl for 10 min

for partial depurination of DNA before blotting. The DNA was then capillary-blotted to a Hybond-N nylon membrane (Amersham Life Science Inc., Arlington Heights) in 0.4 M NaOH. Prehybridization and hybridization were carried out at 65°C for 24 hr. The A2 and A14 cDNA clones obtained from cDNA library screening were excised using *Eco*R I and *Xho* I digestion and purified from the agarose gel by the GENECLEAN protocol (BIO 101 Inc., La Jolla). The purified cDNAs were used as probes for DNA blotting. Afterward, the membranes were washed as described in the Materials and Methods of Chapter I, exposed to X-ray film for 24 hr, and developed for further analysis. The purified cDNAs were also used as probes for tomato RFLP mapping.

3. RNA blotting

Total RNA was extracted as described in the Materials and Methods of Chapter I. About 20 μg total RNA of each treatment were size fractionated through a 1% agarose gel in a 1x Mops buffer, containing 18% formaldehyde. Capillary transfer of RNA onto a MS nylon membranes was carried out in 20xSSC. After transfer, RNAs were fixed onto membranes by UV cross linking. The membranes were then prehybridized at 65°C for 6-7 hr in a solution containing 5xSSPE, 5x Denhardt's, 0.2% SDS, and 100 μg/ml ssDNA. Hybridization was carried out at 42°C overnight in the same buffer containing 50% formamide. The A2 and A14 cDNAs purified from the step above were used as probes. The membranes were washed at 65° in 2xSSC /0.5% SDS solution and exposed to X-ray film for 48 hr.

IV. RESULTS

1. Copy number of A2 in the *L. pennellii* genome

To assess the genomic complexity of A2, genomic DNA hybridization was conducted using A2 cDNA as a probe. The A2 probe hybridized with a single fragment of genomic DNA in each of six different restriction enzyme digests (*Eco*R V, *Hind* III, *Hae* III, *Dra* I, *Eco*R I, and *Bst*N I)(Fig. 9). This indicates that the A2 gene is present in the genome as a low copy gene. Genomic DNA gel blot analysis indicated that Rubisco activase was encoded by a single copy gene in both spinach and *Arabidopsis* (Werneke *et al.*, 1988b). The A2 may also be a single copy gene in the *L. pennellii* genome. It is necessary that further confirmation of the gene copy number be carried out.

2. Mapping of A2 and A14 to the tomato RFLP linkage map

The RFLP mapping technology is extensively used in a wide variety of biological fields. For crop improvement, RFLPs can be employed as tools in breeding programs, and they are also an aid to isolate important genes for subsequent transformation into a suitable background. The A2 and A14 cDNAs were used as probes in an RFLP QTL mapping study to determine where the cDNAs are located and whether they are related to WUE. The RFLP analysis showed the A2 allele is linked to a WUE QTL (LOD = 5.05), and it is located on

chromosome 11. The A14 allele is located on chromosome 12, but it did not show linkage to WUE (Lin, personal communication).

3. Pattern of expression of A2

Although molecular homology is a good criterion for assessing the identity of the cloned cDNA, characterization of its expression pattern in *L. pennellii* can also help to establish its functional role.

Differential expression of the A2 gene was assessed by northern blotting using the A2 cDNA as a probe. Results in Fig.10 show that the probe failed to detect A2 message in total RNA taken from root tissue. A2 RNA accumulation was detected in leaf tissue and to a lesser extent in stem tissue. The size of the RNA molecule was estimated at about 1.7 kb. A decreased amount of A2 mRNA was seen in dark-treated leaf tissue compared to tissue exposed to light, and in *L. esculentum* leaf tissue compared to *L. pennellii* leaf tissue (Fig. 10). A2 RNA accumulation was affected by temperature and light intensity. Expression showed a significant decrease with time of exposure to 37°, but only a very slight change was observed at 25°C with time of exposure (Fig.11). Comparing light intensity treatments, expression was lower at 270 μmol photons m^{-2} s^{-1} PAR exposure than at 385 μmol photons m^{-2} s^{-1} PAR exposure (Fig. 12) These expression patterns are similar to that of Rubisco activase, for which expression is tissue-specific, light inducible and temperature sensitive (Portis, 1992; Eckardt and Portis, 1997). A2 RNA accumulation was also affected by water stress (Fig. 13).

V. DISCUSSION

Rubisco activase is a chloroplast stromal protein that catalyzes the activation of Rubisco *in vivo*. The expression of Rubisco activase is highly regulated. Data gathered from several species indicate that the expression of Rubisco activase is organ-specific, developmentally regulated according to leaf age, and light-inducible (Zielinski *et al.*, 1989; Rundle and Zielinski, 1991; Orozco and Ogren, 1993; Watillon *et al.*, 1993). The expression of Rubisco activase in several species, including *Arabidopsis* (Pilgrim and McClung, 1993), tomato (Martino-Catt and Ort, 1992), and apple (Watillon *et al.*, 1993), is regulated by the circadian clock. Rubisco activase is found to be very sensitive to high temperature, which inhibits its ability to activate Rubisco (Eckardt and Portis, 1997).

The putative *L. pennellii* Rubisco activase was isolated by DDRT-PCR and was found to share 90% nucleic acid and 86% predicted amino acid sequences with Rubisco activase from tobacco. It also contains the consensus nucleotide binding domain, which is involved in ATP binding, and a second region which is also involved in ATP binding.

Northern analysis using A2 as a probe showed that Rubisco activase mRNA was present in *L. pennellii* leaves and stems, but not in roots. This tissue-specific response is consistent with evidence from barley (Zielinski *et al.*, 1989) and *Arabidopsis* (Liu *et al.*, 1996). The expression of the Rubisco activase gene was found to be greatly reduced after the plants were exposed to

dark for 1 hour, and the mRNA level continued to decline when the plants remained in the dark for nine hours. This result is expected since the transcription of Rubisco activase has been found to be light inducible in other species. One may argue that if the expression of Rubisco activase is highly regulated and light-inducible, the mRNA accumulation should be barely detectable in the dark. In this experiment, the plants used were 5-weeks old and grown in the light instead of etiolated seedlings which have almost no background activity of Rubisco activase. The mRNA observed in the dark exposed plants might be remnants from the earlier light exposure. The promoter of *Arabidopsis* Rubisco activase, which is regulated by light and circadian rhythm, has been identified by Liu *et al* (1996). The sequence between -317 and +13 contains elements sufficient to confer organ-specificity, light induction in etiolated seedlings, and circadian regulation. When plants were exposed to 270 μmol photons m^{-2} s^{-1} PAR and 385 μmol photons m^{-2} s^{-1} PAR, mRNA accumulation based on the A2 probe was found to be reduced compared to the control at 500 μmol photons m^{-2} s^{-1} PAR, which suggests that light intensity affects the transcription of Rubisco activase. In the temperature treatment, the accumulation of mRNA (using the A2 probe) showed a slight decrease at 25°C with time exposure but was significantly diminished at 37°C after 4 hours of exposure. Water status also affects the mRNA accumulation of Rubisco activase (Fig. 13). Northern analysis using the A2 probe showed that plants stressed for two and three days accumulated more mRNA than non-stressed plants. After water-stress for five days, the transcript level was greatly reduced

and it was barely detectable after seven days when the plants were wilted. Water stress may induce Rubisco activase transcription under certain conditions. However, if water stress becomes severe, it affects many metabolic pathways of plants, not just photosynthesis. This study indicates that the expression of *L. pennellii* Rubisco activase is tissue dependent and responds to light. Its transcripts are sensitive to light intensity, high temperature and water stress. The expression patterns of the putative tomato Rubisco activase are similar to Rubisco activase detected from other species. The similarity in both sequence structure and expression patterns is sufficiently high to suggest that A2 encodes a tomato Rubisco activase.

Northern analysis showed that the mRNA of Rubisco activase is more abundant in *L. pennellii* than in *L. esculentum* (Fig. 10). This suggests that Rubisco activase may express differently in these two species and Rubisco activase could play a role in the high WUE of *L. pennellii*. The Rubisco activase gene has been mapped to a tomato RFLP linkage map and linkage to WUE has been detected (Lin, personal communication), which further suggests that Rubisco activase is associated with WUE. Southern analysis suggests that the Rubisco activase gene in the *L. pennellii* genome is a low copy gene. It may be a single copy gene as in spinach and *Arabidopsis* (Werneke *et al.*, 1988b). Further study is needed to confirm this possibility.

In order to extend the characterization of tomato Rubisco activase, protein functional analysis should be conducted. This can be done by overexpressing A2 cDNA in a bacterial system. The overexpressed protein can then be purified

and studied. The cloned Rubisco activase cDNA can provide a foundation to isolate the promoter using the A2 clone as a probe to screen a genomic library. Alternatively, the promoter can be isolated using a PCR-based strategy, such as inverse PCR or TAIL-PCR (Liu and Whittier, 1995). These studies should provide fundamental new information on the genetic control of WUE, WUE gene expression and WUE protein synthesis in tomato and perhaps other plants. Eventually, all these studies can lead to opportunities to genetically alter the expression of WUE in agriculturally important crops.

Fig. 9. Genomic DNA blot analysis of the Rubisco activase gene in *L. pennellii*. Twenty ug genomic DNA from *L. pennellii* was digested with EcoR V, Hind III, Hae III, Dra I, EcoR I and Bst NI, subjected to gel electrophoresis, transferred to nylon membrane filters, and hybridized to radiolabeled A2 cDNA as described in "Materials and Methods." The filters were washed as described, and the results were viewed by autoradiography.

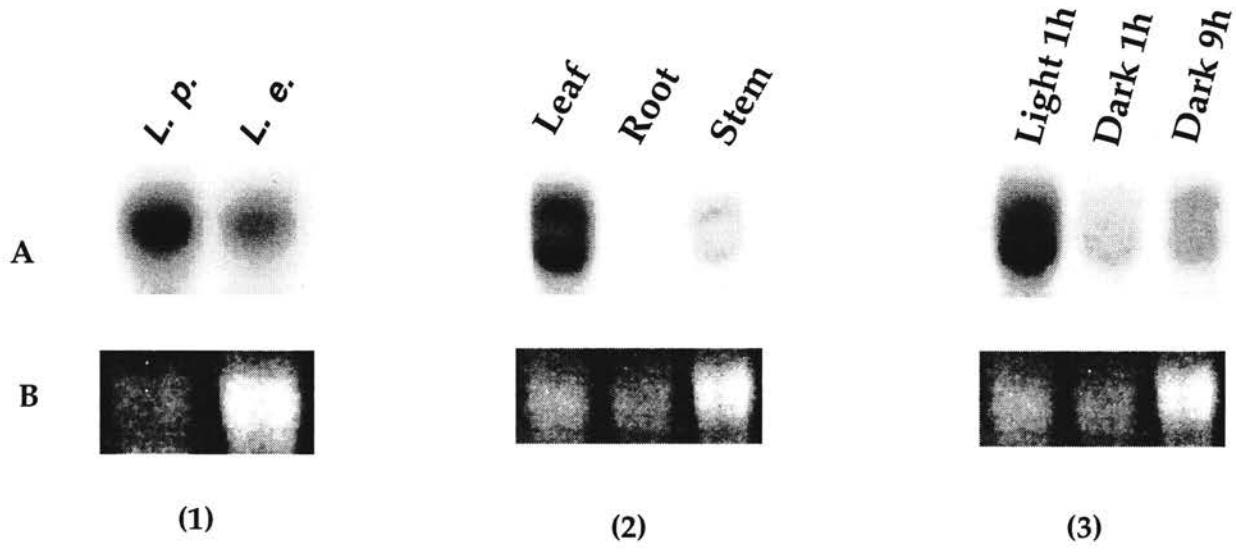

Fig. 10. Tissue-specific and light inducible expression of Rubisco activase in *L. pennellii*. Row A shows the results of northern analysis and row B shows equivalent amount of RNA loaded in each lane. (1). Total RNA from *L. pennellii* and *L. esculentum* leaf, (2). Total RNA from *L. pennellii* leaf, root and stem, (3). Total leaf RNA from *L. pennellii* in light for 1 hour and in dark for 1 and 9 hours. Twenty ug total RNA was subjected to gel electrophoresis, transferred to nylon membrane filters, and hybridized to radiolabeled A2 cDNA. The filters were washed, and the results were viewed by autoradiography.

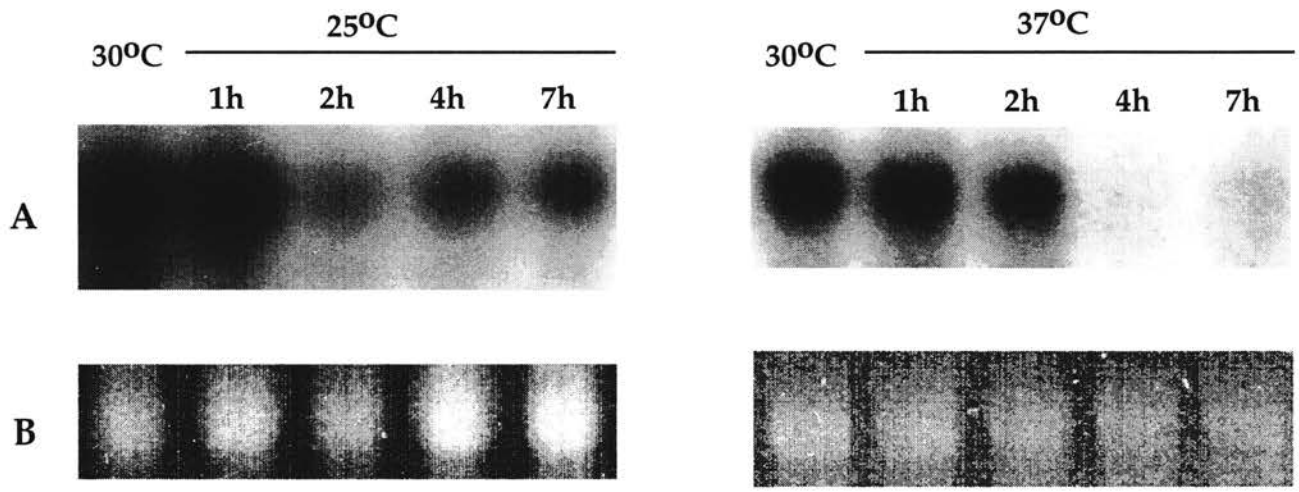

Fig. 11. Temperature effects on Rubisco activase expression in *L. pennellii*. A. Total leaf RNA was isolated from plants exposed to 30°C and from plants exposed to 25°C and 37°C for 1 hour, 2 hours, 4 hours and 7 hours, respectively. Twenty ug total RNA was subjected to gel electrophoresis, transferred to nylon membrane filters, and hybridized to radiolabeled A2 cDNA probe. The filters were washed and the results were viewed by autoradiography. B shows the amount of RNA loaded in each lane, which is approximately equivalent .

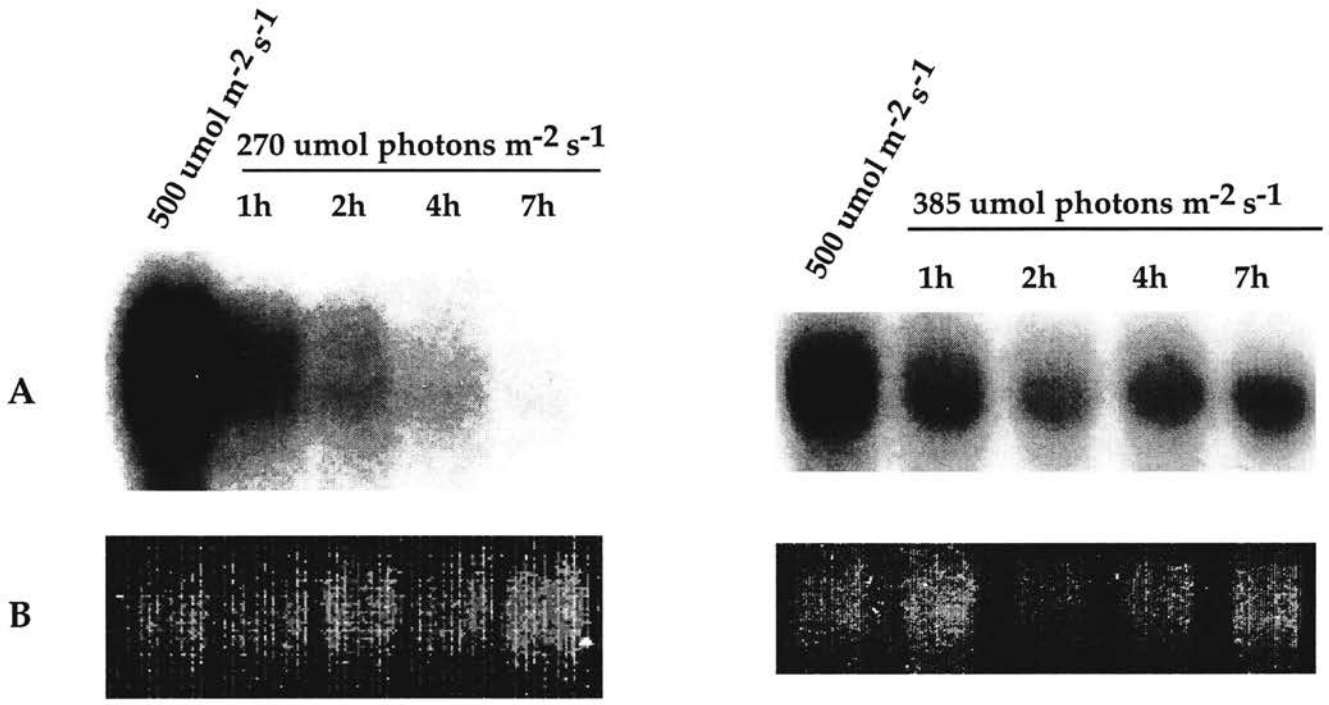

Fig. 12. Light intensity effects on Rubisco activase expression in *L. pennellii.* A. Total RNA was isolated from plants exposed to 500 umol photons m^{-2} s^{-1} and plants exposed to 270 umol photons m^{-2} s^{-1} and 385 umol photons m^{-2} s^{-1} for 1 hour, 2 hours, 4 hours, and 7 hours respectively. Twenty ug total RNA was subjected to gel electrophoresis, transferred to nylon membrane filters, and hybridized to radiolabeled A2 cDNA probe. The filters were washed and the results were viewed by autoradiography. B shows the amount of RNA loaded in each lane, which is approximately equivalent .

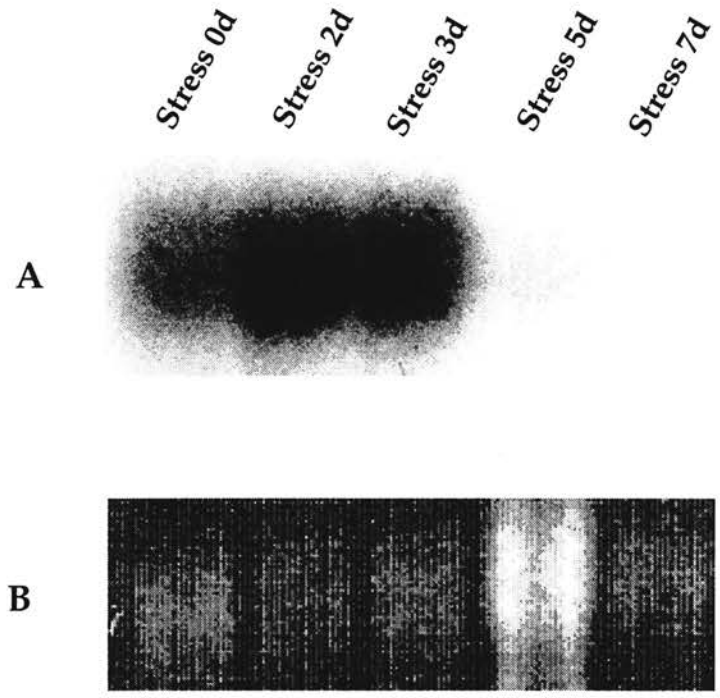

Fig. 13. Water stress effects on Rubisco activase expression in *L. pennellii*. A. Total leaf RNA was extracted from *L. pennellii* water-stressed for 0 day, 2 days, 3 days, 5 days and 7 days, respectively. Twenty ug total RNA was subjected to gel electrophoresis, transferred to nylon membrane filters, and hybridized to radiolabeled A2 cDNA probe. The filters were washed, and the results were viewed by autoradiography. B shows the amount of RNA loaded in each lane, which is approximately equivalent.

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VITA

Yali Zhu

Candidate for the Degree of

Doctor of Philosophy

Thesis: IDENTIFICATION AND CHARACTERIZATION OF GENES ASSOCIATED WITH WATER-USE EFFICIENCCY IN TOMATO

Major Field: Crop Science

Biographical:

Personal Data: Born in Linfen, Shanxi, China, January 4, 1956, the daughter of Yütang Zhu and Lingzhen Peng.

Education: Received Bachelor of Agronomy degree in Agronomy from Shanxi Agricultural University, Taigu, China in January, 1982; received Master of Science in Horticulture from Michigan State University, Michigan, in December, 1994; completed the requirements for the Doctor of Philosophy degree with a major in Crop Science at Oklahoma State University in Spring, 1999.

Experience: Employed as an assistant researcher by Shanxi Academy Agricultural Sciences, 1982 to 1991; Employed as a graduate research associate by Michigan State University, Department of Horticulture, 1992-1994; Employed as a graduate research associate by Oklahoma State University, Department of Plant and Soil Sciences, 1995-1998.

Professional Membership: American Society of Plant Physiologist.