Restriction Fragment Length Polymorphisms Associated with Water Use Efficiency in Tomato

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Improvement of crop water use efficiency (WUE) has not been successful because evaluation for this component of drought resistance is unreliable in field-grown plants. The composition of stable isotopes of plant carbon ($\delta^{13}C$) was earlier shown to be an excellent indicator of WUE in tomato and other species. It is now reported that $\delta^{13}C$ can be satisfactorily predicted from three restriction fragment length polymorphisms (RFLPs), which are genetic markers for discrete DNA sequences within the genome. An additive type of gene action was observed for all three RFLP markers, and for one of them there also was a significant nonadditive component. Combining the $\delta^{13}C$ and RFLP technologies may aid in identifying genes associated with WUE and in studying mechanisms contributing to WUE. These technologies will also be useful for crop improvement.

CONSIDERABLE VARIATION FOR PLANT water use efficiency (WUE) exists both among and within species (*1*). WUE, an important component of drought tolerance, greatly affects yield under water-limited conditions. Yet, little crop improvement has been achieved because of the difficulty of assessing this trait in field-grown plants.

Season-long WUE (moles of carbon gained per mole of water used, or grams of dry biomass per gram of water used) can be accurately determined in container-grown plants (*2, 3*). However, because of the tremendous labor requirement, this measure is not useful to the plant breeder. In addition, it has never been demonstrated that container-grown plants accurately reflect the genotypes of field-grown plants.

The instantaneous WUE of a plant can be approximated by the following expression (*2*)

$$\text{WUE} = A/E = p_a(1 - p_i/p_a)/1.6\ \Delta w$$

in which A and E are the rates of photosynthesis and transpiration, respectively; p_a and p_i are the partial pressures of CO_2 in the ambient air and in the intercellular air spaces (translatable into the more commonly encountered terms of concentrations of CO_2 in the ambient air and the intercellular air spaces, C_a and C_i); and Δw is the difference in water vapor pressure between the intercellular air spaces and the ambient air. The number 1.6 comes from the difference in diffusion coefficients between water vapor and CO_2. WUE can be altered either by changing the efficiency of the photosynthetic processes associated with the mesophyll cells inside the leaf, or it can be altered by varying the degree of stomatal opening. Whether WUE changes as a result of mesophyll or stomatal alterations, p_i will vary with WUE. Thus, if p_i goes down, WUE increases, and if p_i goes up, WUE decreases. Season-long WUE is the assimilation-weighted time integral of instantaneous WUE and includes a term for respiratory carbon losses. Thus, a reliable measure of the season-long time integral of p_i should be a good indicator of season-long WUE.

Such a measure of p_i is the composition of the stable isotopes, ^{13}C and ^{12}C, of the carbon in the organic matter of the plant. The stable carbon isotope ratio $^{13}C/^{12}C$ of a sample is often related to the isotopic composition of the PeeDee belemnite (PDB) standard (*4*) to yield a $\delta^{13}C$ value (per mil, parts per thousand) defined by the equation

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

in which R is the $^{13}C/^{12}C$ ratio.

A good approximation of the $\delta^{13}C$ value of a plant sample given by Farquhar *et al.* (*5*) is

$$\delta^{13}C_{plant} = \delta^{13}C_{air} - a - (b - a)p_i/p_a$$

The $\delta^{13}C_{plant}$ and $\delta^{13}C_{air}$ (−7.8 per mil) are the carbon isotope compositions of the plant sample and of CO_2 in the air; a (4.4 per mil) is the isotopic discrimination resulting from different diffusivities of $^{13}CO_2$ and $^{12}CO_2$, and b (about 27 per mil) is the isotopic discrimination inherent with ribu-

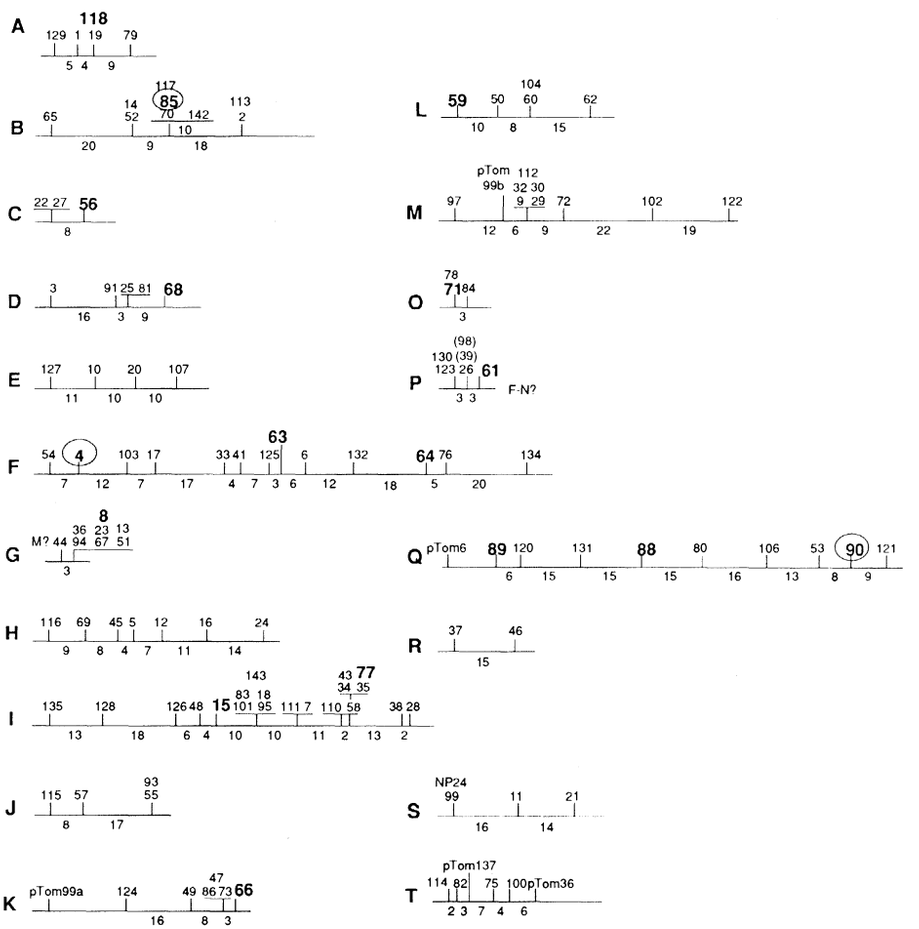

Fig. 1. Genetic linkage map of tomato. Numbers above the lines designate RFLP genetic markers; those below the lines denote the recombinational values in percent between neighboring markers. Markers used in the analyses are indicated in bold. Markers found to be associated with the variance for $\delta^{13}C$ are circled. Underlined clones are in unknown order.

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lose-1,5-bisphosphate carboxylase, the primary carboxylating enzyme of plants having the C_3 photosynthetic pathway. The $\delta^{13}C$ of a plant sample is an indicator of the assimilation-weighted time integral of p_i during the period of tissue formation, and since WUE is also correlated with p_i, the value of $\delta^{13}C$ is expected to be a predictor of WUE.

That $\delta^{13}C$ is tightly correlated with p_i, as theory predicts (*5–8*), has been shown in numerous studies (*5, 9–11*), and experimental data have shown the usefulness of $\delta^{13}C$ as an indicator of WUE in crops like wheat (*2*), peanut (*12, 13*) and barley (*13*) studied in isolated plants, and in peanut (*13–15*) in closed canopies in the field. We found that the correlation is also good in tomato (*3*). We have now defined discrete chromosomal fragments [restriction fragment length polymorphisms (RFLPs)] that function as genetic markers for the expression of WUE. We used the $\delta^{13}C$ technology to rank a number of field-grown tomato genotypes for this trait.

Table 1. Composition of stable carbon isotopes, $\delta^{13}C$, of leaf tissue from *Lycopersicon esculentum*, *L. pennellii*, and F_3 and BC_1S_1 families derived from these species, the RFLP genotypes at RFLP loci B85, F4 and Q90, and the $\delta^{13}C$ values predicted by the multiple regression model in Table 3, for the families selected for RFLP analysis. For Q90, both the linear (L) and quadratic (Q) contrasts are shown. The genotypic codes are −1, 0, 1(L), and 1, −2, 1(Q) for *L. esculentum* homozygote, heterozygote, and *L. pennellii* homozygote, respectively.

Family no.	Generation	RFLP genotype				Observed $\delta^{13}C$ (‰)	Predicted $\delta^{13}C$ (‰)
		B85 (L)	F4 (L)	Q90			
				L	Q		
40	BC_1S_1	0	−1	0	−2	−24.76	−25.69
78*	F_3	1	1	1	1	−24.97	−25.69
09	BC_1S_1	0	0	0	−2	−25.00	−25.45
08	BC_1S_1	0	0	0	−2	−25.00	−25.45
21	BC_1S_1					−25.01	
39	BC_1S_1					−25.01	
93*	F_3	−1	1	0	−2	−25.07	−25.51
37	BC_1S_1					−25.08	
41	F_3					−25.08	
46	BC_1S_1					−25.14	
47	F_3					−25.14	
10	BC_1S_1	0	1	0	−2	−25.15	−25.21
29	F_3					−25.16	
92*	F_3	1	1	0	−2	−25.22	−24.92
70	BC_1S_1					−25.22	
02	F_3	−1	0	0	−2	−25.24	−25.75
31	F_3					−25.24	
79*	F_3					−25.26	
04	F_3					−25.26	
05	F_3					−25.29	
85	F_3					−25.30	
90*	F_3					−25.40	
110	*L. pennellii*					−25.40	
06	BC_1S_1					−25.40	
69	BC_1S_1					−25.42	
27	F_3					−25.44	
87	F_3					−25.51	
53	BC_1S_1					−25.51	
73	BC_1S_1					−25.61	
91*	F_3					−25.61	
07	BC_1S_1					−25.67	
24	BC_1S_1					−25.79	
52	BC_1S_1					−25.79	
56	BC_1S_1					−25.79	
86	F_3					−25.84	
20	BC_1S_1					−25.86	
71	BC_1S_1					−25.86	
28	F_3	1	−1	1	1	−25.89	−25.57
23	BC_1S_1					−25.93	
01	F_3	−1	−1	1	1	−25.96	−26.15
72	BC_1S_1					−26.08	
45	BC_1S_1					−26.09	
54	BC_1S_1					−26.11	
30	F_3	0	1	0	−2	−26.13	−25.21
22	BC_1S_1	0	0	−1	1	−26.21	−26.73
35	BC_1S_1	0	−1	0	−2	−26.24	−25.69
55	BC_1S_1	−1	−1	0	−2	−26.28	−25.99
26	BC_1S_1					−26.36	
25	BC_1S_1	−1	0	0	−2	−26.56	−25.75
03	F_3	−1	0	−1	1	−26.99	−27.03
38	BC_1S_1	−1	−1	−1	1	−27.00	−27.27
101	*L. esculentum, UC82B*					−27.06	
103	*L. esculentum, UC204B*					−27.37	
36	BC_1S_1	−1	−1	−1	1	−27.48	−27.27
34	BC_1S_1	−1	−1	−1	1	−27.89	−27.27

*The domestic tomato variety UC204B was used in crosses instead of UC82B, which was used in all of the other crosses.

In this application, RFLP markers serve to identify traits, like WUE, that are difficult to identify phenotypically (*16*). RFLPs associated with the expression of the complex traits of insect resistance (*16*) and soluble solids (*17*) in tomato were recently identified with the RFLP technology, which allows genetic components to be tracked by the coinheritance of randomly cloned DNA probes (*18*). This technology is particularly useful when attempts are made to evaluate plants for traits that are not associated with easily observable characteristics.

We used a large number of F_3 and BC_1S_1 families obtained by crossing the drought-sensitive, domestic tomato *Lycopersicon esculentum* Mill. (cv. UC82B and cv. UC204B) with the wild, drought-tolerant species *L. pennellii* (Cor.) D'Arcy (*19*). The domestic tomato served as the maternal parent in the initial cross, and the back-crossing was to *L. esculentum*. The statistical treatment was made without differentiating between the two cultivars of *L. esculentum* because they were indistinguishable with respect to the parameters tested.

The genotypes listed in Table 1 were seeded in rows 120 cm apart in a field in Visalia, California, on 13 April 1986. After the plantlets emerged, they were thinned to 20 plants spaced 30 cm apart within rows. Each family was represented twice in a random block design. The soil was kept moist during seedling establishment by application of water every 2 days. On 4 June the interval between irrigations was increased to 6 days, so that the average irrigation rate was reduced to one-third (70 mm per month, or 2.3×10^4 liters per hectare per day) of the normal rate in the area. Reduced irrigation was used to evaluate the performance of the plants under drought—a condition during which WUE becomes increasingly important.

The field study was terminated on 4 August 1986 by collecting one leaf from the outer, exposed position of the canopy from each of ten plants in the middle of each row. The ten leaves were pooled. Dried leaf material was ground to a powder and analyzed as

Table 2. Analysis of variance, mean squares, linear and quadratic contrasts, genotype means, and linear regression coefficients for three RFLP loci associated with expression of $\delta^{13}C$ in tomato.

Source	d.f.	B85	F4	Q90
			Mean squares	
Genotype	2			
Linear*	1	2.91†	3.93†	4.25‡
Quadratic*	1	0.72	0.01	3.19‡
Error	16	0.65	0.72	0.40
			Means ($\delta^{13}C$, ‰)	
Genotype				
L. pen / L. pen		−25.36	−25.31	−25.61
L. pen / L. esc		−25.49	−25.83	−25.51
L. esc / L. esc		−26.49	−26.44	−27.11
Linear regression (coefficient ± SE)		0.66 ± 0.25	0.57 ± 0.23	0.88 ± 0.27

*Values −1, 0, 1 are linear contrasts; 1, −2, 1 are quadratic contrasts. †Significant at $P < 0.05$. ‡Significant at $P < 0.01$.

Table 3. Multiple regression model, with genotypic values at three RFLP loci used to predict $\delta^{13}C$ (‰) in tomato.

Parameter	Contrast	Estimate	SE
Intercept		−25.94	0.16
B85	Linear	0.29	0.22
F4	Linear	0.24	0.19
Q90	Linear	0.56	0.24
Q90	Quadratic	−0.24	0.10

previously described (*20*). Briefly, small subsamples (<5 mg) of powdered tissue were combusted under vacuum. The CO_2 formed by combustion of the organic matter was purified and released into a ratio mass spectrometer to determine the $^{13}C/^{12}C$ ratio. The standard errors of the means of measurements of $\delta^{13}C$ on four plants each of *L. esculentum* and *L. pennellii* in a similar study were 0.16 per mil and 0.05 per mil, respectively. According to the manufacturer's specification the standard error of the mass spectrometric analysis is less than 0.01 per mil.

The $\delta^{13}C$ values of the tomato genotypes ranged more than 3 per mil (Table 1). A similar range of $\delta^{13}C$ values was reported by others for wheat (*2, 21*) and barley (*13*) cultivars, and for varying peanut germplasm (*12, 13*).

The two domestic tomato cultivars (UC82B and UC204B) clustered next to each other at the extreme low end of the range of $\delta^{13}C$ values (Table 1), which indicates poor WUE. The $\delta^{13}C$ of the wild tomato species *L. pennellii* was about 2 per mil less negative, an indication of much greater WUE. The cross with the least negative $\delta^{13}C$ (family no. 40) had a $\delta^{13}C$ value that was 0.6 per mil higher than that of *L. pennellii*, demonstrating that there were progeny with even greater WUE than that of the parent with the highest WUE. This finding is consistent with the observations on tomato (*22*) and peanut (*14*) that some F_2 individuals had a greater WUE than the parent with the best WUE.

Eight families with high $\delta^{13}C$ and 11 families with low $\delta^{13}C$ (Table 1) were selected for RFLP analysis. Ten plants of each genotype were grown from seeds in the greenhouse in 3-liter pots. The plants were adequately watered and fertilized. The temperature was maintained at 32°C during the day and at 18°C during the night.

When the plants were about 2 months old, healthy leaves were collected and pooled for each genotype. The leaves were lyophilized and ground to a fine powder from which DNA was extracted. (*23*). The DNA was digested with the restriction enzymes Bgl II or Hind III according to the manufacturer's instructions. The DNA was subjected to electrophoresis through a neutral agarose gel, and the fragmented DNA in the gel was blotted (*24*) onto nylon membranes. Seventeen probes from the tomato genetic linkage map of Native Plants, Inc. (*18*), representing 12 linkage groups were hybridized to the blotted DNAs.

The 17 probes we used are spread over 12 of the 19 known linkage groups (*25*) (Fig. 1). The genotype for each marker—homozygous pennellii (*L. pennellii/L. pennellii*), heterozygous (*L. pennellii/L. esculentum*), or homozygous esculentum (*L. esculentum/L. esculentum*)—was determined and is presented in Table 1. Linear and quadratic contrasts among the three genotypic classes were estimated to identify the RFLP loci associated with the expression of $\delta^{13}C$, as well as the gene action—additive or nonadditive—at each RFLP locus (*16*). The three probes circled in Fig. 1 (B85, F4, and Q90) were found to be predictors of $\delta^{13}C$.

When the analysis of variance was partitioned into two orthogonal contrasts, the linear contrast among genotypic classes was significant for all three RFLP markers (Table 2). The quadratic contrast was also significant for Q90. This suggests that the predominant type of gene action associated with the expression of $\delta^{13}C$ is additive and that for Q90 nonadditive gene action was also indicated. The average effect of an allelic substitution was estimated as the linear regression coefficient of $\delta^{13}C$ regressed on gene frequency. It ranged from 0.57 to 0.88 (Table 2).

A multiple regression model (Table 3), which included the linear contrasts for all three RFLP markers plus the quadratic contrast for the marker Q90, led to the predicted values of $\delta^{13}C$ shown in Table 1.

WUE is considered a very complex trait. Therefore, it was surprising that it could be predicted with three RFLP markers. However, each of these genetic markers could be associated with more than one quantitative trait locus. In fact, the RFLP marker Q90 is presumably associated with at least two genes, since it is characterized by both additive and nonadditive gene actions. B85 and F4 could possibly be associated with several genes, all of which have only additive gene action. These technologies may open new ways of studying the genetics and the inheritance of WUE and of evaluating and improving crop WUE, regardless of how many genes are involved. However, the number of genes will be important in probing the anatomical, physiological, and biochemical mechanisms contributing to WUE.

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The Pathway of Infection of *Autographa californica* Nuclear Polyhedrosis Virus in an Insect Host

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An immunohistochemical study was conducted to detect the temporal infection sequence of *Autographa californica* M nuclear polyhedrosis virus in *Trichoplusia ni* larvae. Staining patterns indicated that the initial infection occurred in the midgut, simultaneously in columnar epithelial and regenerative cells, but that subsequently this tissue recovered. A major envelope glycoprotein stained in a polar fashion when it was expressed in columnar epithelial cells, but not when expressed in other cells types. Systemic infection was mediated by free virus for some tissues whereas infected hemocytes appeared to spread virus to other tissues by an unknown mechanism. A cell to cell spread within several tissues was detected. These results have important implications for baculoviruses engineered for improving their pesticide potential.

NUCLEAR POLYHEDROSIS VIRUSES (Family: Baculoviridae) are double-stranded DNA viruses that cause disease primarily in lepidopteran insects. These viruses are unusual in that at least two structurally different but genetically identical virions (phenotypes) are produced during a successful infection in an insect host. One virion phenotype consists of one or more bacilliform nucleocapsids enclosed in an envelope with numerous virions occluded within a proteinaceous matrix. These occlusion bodies, called polyhedra because of their shape, are formed within the nuclei of infected cells and are visible with a light microscope. They are responsible for the insect to insect transmission of infection. The second virion phenotype consists of a single nucleocapsid in an envelope derived from a viral-modified cellular plasma membrane acquired as virus buds out of a cell (*1*). This phenotype, referred to as extracellular virus (EV), is involved in the systemic infection of a host.

Autographa californica nuclear polyhedrosis virus (AcMNPV) has received much attention because of the use of a promoter of one of its highly expressed late genes in a very efficient expression system (*2*). AcMNPV and other baculoviruses have been less successfully used as biocontrol agents. Their use as pesticides has not fulfilled their promise, in part because of the time required for an infected insect to cease feeding (*3*). The recently acquired ability to manipulate these viruses at the molecular level provides an opportunity to enhance their virulence and their pesticide potential, but the goal of increasing the effectiveness of these viruses as pesticides while maintaining those properties that make them safe, attractive control agents requires a thorough knowledge of all aspects of the virus-host interaction.

We examined the course of infection of AcMNPV in an insect host, *Trichoplusia ni* (cabbage looper), by immunohistochemical staining. Histopathological studies conducted by light and electron microscopy have revealed many aspects of baculovirus infection, replication, and systemic spread in insect hosts (*1, 4, 5*). However, none has used a probe capable of detecting viral antigen expression before polyhedra production in individual cells. This sensitive approach has enabled us to follow the course of infection initiated at biologically relevant doses and examine representative tissue sections from 43 insects by light microscopy.

Insects were infected (*6*) by inoculating them per os with 1 ng of polyhedra derived virus (designated PDV), which caused 95% mortality, and examined from within an hour of exposure to virus until visible symptoms indicated a widely disseminated disease. Developmentally matched uninfected control insects were also examined. Antibodies used for immunostaining were rabbit polyclonal antibody made against PDV (*7*) and several monoclonal antibodies (MAb) reactive with viral structural proteins as determined by immunoblots and immunoprecipitation (*8, 9*). We used the indirect peroxidase-antiperoxidase (PAP) method with diaminobenzidine as the electron donor (*10*). One monoclonal, MAb B12D5, was specific for the 64-kD surface antigen (designated gp64) exclusive to EV; another, MAb 39P10, was specific for a 39-kD major capsid protein present in both phenotypes (*11*). Whole larvae and entire midguts dissected from bled insects were fixed in Bouin's and embedded in Paraplast Plus (Sherwood Medical Industries, St. Louis, Missouri) (*12*). Serial saggital sections were examined for the distribution of viral proteins. In addition, hemolymph (blood) was titered for virus by plaque assay (*13*) from both infected and uninfected insects. Growth and development of the insects were also monitored for all treatments.

In contrast to previous reports (*1, 4*), the results of this study indicated that the initial infection occurred in the midgut in both columnar epithelial and regenerative cells (Fig. 1A). The simultaneous detection of viral proteins in both these cell types implied that they became infected by the parental inoculum. Since the regenerative cells are not exposed to the gut lumen, this observation suggested that this cell type was infected by nucleocapsids or virions that either passed directly through or between cells but were still retained within the epithelium. Several pathways have been proposed by which different baculoviruses may infect their hosts through the midgut (*1*), including rapid passage through or between cells of the epithelial layer directly into the hemocoel (open circulatory system), or replication within columnar epithelial cells before entry into the hemocoel. We saw no evidence of direct passage into the hemocoel on the basis of staining and plaque assay data (Table 1).

Staining patterns in some of the midgut columnar epithelial cells with MAb B12D5 suggested a polar movement of this viral component toward the basolateral area of these cells (Fig. 1B). The distribution of this protein in this region may help to direct the virus to bud from these membranes as has been previously described for other envelope proteins and viruses in cultured epithelial cells (*14*). Previous observations in elec-

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