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Interspecific Gene Transfer[1]

Implications for Broadening Temperature Characteristics of Plant Metabolic Processes

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We report here an approach to metabolic engineering to alter the temperature characteristics of an enzyme pool based on the concept of thermal kinetics windows (TKWs), a useful indicator of enzyme performance. A chimeric cucumber NADH-hydroxypyruvate reductase (HPR) gene under the control of a cauliflower mosaic virus 35S promoter was constructed and introduced into the genome of tobacco (*Tobacum tobacum*). The root system of the R_1 generation of the resultant transgenic plants expresses only the cucumber enzyme (the native tobacco HPR gene is light regulated and only found in the aerial portions of the plant). Enzyme isolated from the transgenic root tissues exhibits a TKW centered at 32.5°C, characteristic of cucumber. The pool of HPR in the shoots, containing both tobacco and cucumber enzymes, exhibits a broad TKW consistent with an equal mix of the two forms. These data do not simply demonstrate that an introduced gene can be expressed in a transgenic plant but that the kinetics properties of the resultant enzyme are unaltered and when sufficient enzyme is produced the temperature characteristics of the total pool are altered. This suggests that the temperature characteristics of plant biochemical pathways can be broadened to suit changing thermal environments.

Traditionally, genetic engineering has focused on the alteration of the metabolic capabilities of plants by the introduction of novel genes or the specific inhibition of single genes by antisense technology. Often this results in a phenotype that is of importance to production agriculture (Fraley, 1992). However, there has been little progress in manipulating metabolism to alter the plants' ability to cope with stressful changes in the environment by these means. This is of particular import at this time, when predictions of global environmental changes include major alterations in vegetation and agricultural practices (Daily and Ehrlich, 1990). Murata et al. (1992) altered the chilling sensitivity of tobacco plants by the introduction of a chimeric gene coding for glycerol-3-phosphate acyltransferase from squash or *Arabidopsis* under the control of the CaMV 35S promoter. In this case expression of this enzyme resulted in a change in membrane lipid composition, which in turn led to an increase in chilling tolerance. The native glycerol-3-phosphate acyltransferase enzyme was not readily detectable, and thus, the modification was achieved by the introduction of an activity at high levels that was normally at a minimal level.

We have taken an approach that does not require the effective replacement of an enzyme activity but rather supplements an existing enzyme pool to broaden the temperature characteristics of the enzyme to better match the temperatures experienced in the dynamic thermal environments of the field. We have based our approach on a concept that links plant temperature optima to the kinetics properties of enzymes and biochemical pathways: the concept of TKWs (Burke et al., 1988). We use the apparent K_m of an enzyme reaction as an indicator of the functional capacity of an enzyme and the effect of temperature on this value to establish the temperature optima for the metabolism of the plant species under investigation. This approach was used successfully in many early studies designed to investigate the role of temperature adaptation of enzymes in protein evolution by isolating lactate dehydrogenase from teleost fishes living in vastly different temperature environments (Somero and Low, 1976). By measuring the temperature range within which the apparent K_m remains within 200% of the minimum value, one can estimate the limits of optimal enzyme function. The resultant temperature range is termed the TKW. The use of other kinetics parameters is not as reliable as the use of apparent K_m in indicating the optimal thermal range of an enzyme (Somero, 1978), even though they may more accurately reflect actual enzymatic efficiencies in vivo. Thus, the TKW serves as a reasonable and practical estimate of the temperature limits beyond which, hotter or colder, the plant experiences a stressful thermal environment. This concept has established that stress occurs well before the extremes of temperature normally considered as stressful, i.e. heat

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Abbreviations: CaMV, cauliflower mosaic virus; HPR, hydroxypyruvate reductase; SSU, small subunit of Rubisco; TKW, thermal kinetic window.

shock and chilling injury, and there is an impact on productivity at what could be considered mild temperatures (Burke et al., 1988). Thus, if the temperature characteristics of a particular metabolic process, especially a process that is involved in stress protection or disease resistance or that has a direct impact on crop productivity, could be broadened by even a moderate amount, the agronomic benefits could be substantial.

The first step in determining whether this is indeed a realistic possibility is to ascertain whether the in vitro temperature characteristics of an enzyme are maintained for an enzyme isolated from a foreign host environment and whether sufficient enzyme could be produced in a transgenic plant such that the total enzyme pool would express a broadened temperature range. Overexpression is not the goal with this approach because it could simply alter the TKW of an enzyme pool to a different narrow range that would not enhance the overall ability of the pathway, or perhaps the plant, to survive under changing environmental conditions. To answer these two questions we introduced a chimeric NADH-HPR gene into the genome of tobacco (*Tobacum tobacum* cv SR1) under the control of the CaMV 35S promoter. The rationale for using this particular construct and enzyme is based on four aspects of this system: (a) HPR from cucumber was chosen because the full-length cDNA was available (Greenler et al., 1989) and cucumber enzymes exhibit a TKW that centers around 32.5°C. This is considerably different from the TKW of the tobacco enzyme (which centers around 25°C), from which it had to be distinguished. (b) The native tobacco peroxisomal HPR gene is light regulated and, as such, the enzyme is not localized in the root tissues of the plant. The introduced cucumber chimeric HPR gene is under the control of the 35S promoter, which is not tissue specific (Benfey et al., 1989) and may indeed exhibit somewhat higher expression in root tissues. Thus, in the transgenic plants it should be possible to isolate a mixture of the cucumber and tobacco enzymes from shoot tissues and the cucumber enzyme alone from roots for purposes of comparison. (c) Overall, the 35S promoter is highly active in plants (Sanders et al., 1987) and as such is expected to yield enough gene product to provide the necessary amount of cucumber HPR to broaden the overall temperature characteristics of the total HPR pool in transgenic tobacco. (d) The use of the peroxisomal HPR as the target enzyme was also advantageous because we had examined the temperature characteristics of this enzyme in other studies of plant temperature optima and had determined that the tobacco and cucumber enzymes can be readily distinguished biochemically. In this report we document the success of this approach in broadening the temperature characteristics for the total leaf pool of HPR of tobacco.

MATERIALS AND METHODS

Construction of the Chimeric Cucumber HPR Gene

The full-length cucumber HPR cDNA sequence contained in the plasmid pBSH18 (Greenler et al., 1989) was excised with *Eco*RI and purified by electroelution from a 0.7% agarose gel. This fragment was inserted into the plant expression vector pMON 316 (described by Rogers et al., 1987) at the *Eco*RI site situated between the CaMV 35S promoter and the nopaline synthetase 3′ untranslated region. This created a cucumber HPR gene driven by the 35S promoter of CaMV and completed by the terminator sequence of the nopaline synthetase gene from the Ti plasmid pTiT37.

Conjugation into *Agrobacterium tumefaciens* and Transformation of Tobacco

The pMONHPR construct was conjugated into *Agrobacterium tumefaciens* by the triple mating procedure described by Fraley et al. (1985). In these conjugations the *A. tumefaciens* strain GV3111SE was used as a recipient. This strain contains a disarmed Ti plasmid, pTiB6S3-SE, in which the T-DNA phytohormone biosynthetic genes, the T_L DNA right border, and all of the T_R DNA have been deleted and replaced with a bacterial kanamycin-resistance marker (Fraley et al., 1985). Transformation of sterile leaf segments of *Tobacum tobacum* cv SR1 and regeneration of transformed plants was achieved using the procedure described by Horsch et al. (1985). Putative transformants, plantlets surviving on kanamycin at 100 μg/mL, were further screened for the production of nopaline as described by Otten and Schilperoort (1978). After *A. tumefaciens* was cured, plants were grown in the laboratory at room temperature in 4-inch pots containing a peat/soil/vermiculite mix under a 16-h day/8-h night light regime in which they were irradiated with approximately 50 μE m^{-2} s^{-1}. Eighteen R_0 plants expressing high levels of the cucumber HPR RNA were identified by isolation and analysis of leaf tissue RNA (as described below). Selected plants were selfed and the R_1 seeds collected and germinated on Murashige-Skoog medium containing 200 μg/mL of kanamycin. R_1 plants from each original transformant were grown and retested for high cucumber HPR mRNA accumulation in leaf tissue, and from these R_1 lines one line, R6+3, that accumulated the highest levels of cucumber HPR transcript was chosen for use in the remainder of the study.

Determination of HPR Activity and Protein Levels in Transgenic Leaf Tissue

Enzyme Inhibition Assays

Leaf, stem, or root tissue of R_1 progeny of R6+3 was ground to a fine powder in liquid nitrogen, the powder was transferred to precooled microfuge tubes, and extraction medium (50 mM Tris-HCl [pH 6.8], 1 mM PMSF, 1 mM EDTA, 10 μM leupeptin, 5.6 μM *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine) was added (50 μL/50 mg tissue). The resulting slurry was vigorously mixed for 3 min at room temperature before it was centrifuged at 14,000 rpm in an Eppendorf[2] microfuge for 5 min.

[2] Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

The supernatant was collected, given a second brief centrifugation at 14,000 rpm, and aliquoted. One aliquot was used immediately to determine HPR activity and protein concentration, whereas the other aliquots were stored at −90°C for later use in western blot analyses.

Enzyme Kinetics Analyses

For enzyme kinetics analyses HPR protein was purified from appropriate tissues according to the method described by Titus et al. (1983).

Enzyme Assay

The HPR activity was determined by the method of Titus et al. (1983) with a Gilford Response spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH). Protein concentration was measured by the procedure of Bradford (1976) using bovine γ-globulin as the protein standard.

Protein Level Determination

The level of HPR protein in the extracts was measured by western blotting using the following procedure. Previously frozen protein samples, containing 10 μg of total protein, were separated using SDS-PAGE (Laemmli, 1970) and transferred to a nitrocellulose filter. Antiserum to HPR (provided by Dr. Wayne Becker, University of Wisconsin, Madison, WI) was used as the primary antibody, and anti-rabbit IgG antibodies conjugated to alkaline phosphatase were used as the secondary antibody. Immunochemical staining was achieved by the method of Billingsley et al. (1987).

Isolation and Analysis of RNA from Transgenic Leaf Tissue

RNA from leaf, stem, or root tissue was isolated by a series of phenol extractions as described by Lane and Tumaitis-Kennedy (1981). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography and resolved by electrophoresis through a 1.2% agarose gel containing formaldehyde as described by Ausubel et al. (1989). The RNA was transferred onto Duralon-UV nylon membranes using the Posiblot Pressure Blotter system (Stratagene, La Jolla, CA) as detailed by the manufacturer. RNA was cross-linked to the nylon membranes by exposure to 120,000 μJ cm^{-2} of UV radiation in a model 1800 Stratalinker (Stratagene).

Probes and Hybridization Conditions

Cucumber HPR

The probe for cucumber HPR RNA was the full-length cDNA fragment isolated by *Eco*RI digestion of the plasmid pBSH18 (Greenler et al., 1989). The cDNA was radiolabeled with [^{32}P]CTP by nick-translation via a kit and protocol supplied by GIBCO-BRL (Gaithersburg, MD). Hybridization was achieved in a solution containing 25 mM potassium phosphate at pH 7.4, 5× SSC, 5× Denhardt's solution, 50% formamide, and 50 μg/mL sonicated salmon sperm DNA at 42°C. Under these conditions the native tobacco HPR mRNA does not hybridize with the probe.

Tobacco HPR mRNA

The probe for the native tobacco HPR mRNA was a 3′ nontranslated region probe isolated from a partial tobacco HPR cDNA by *Eco*RI digestion. The partial cDNA was isolated from a tobacco leaf cDNA library purchased from Stratagene with the use of the cucumber HPR cDNA as a probe. The conditions for use of this probe to isolate the tobacco counterpart were at a stringency that allowed for its hybridization to the tobacco HPR mRNA on northern blots (hybridization at room temperature and washing in 0.5× SSC at 42°C). The tobacco HPR cDNA is 750 bp in length and has been sequenced for 336 bp from the 5′ end and 316 bp from its 3′ end. The identified coding regions exhibit a 79% similarity with the cucumber sequence at the nucleic acid level (86.3% at the amino acid level) and the 3′ nontranslated sequence has no similarity to the cucumber cDNA. The probe, isolated by *Eco*RI digestion, is 305 bp in length and contains the complete 3′ end of the cDNA and 76 bp of the tobacco coding sequence (this small portion of coding region is also approximately 80% similar to the cucumber HPR cDNA). This probe, under the conditions of hybridization used (see conditions for the cucumber HPR RNA described above), is specific for the tobacco HPR.

SSU

The probe for the SSU was a partial fragment of the cDNA clone rbcS-1 from *Mesembranthemum* (de Rocher et al., 1991) received as a kind gift from Dr. Hans Bohnert (University of Arizona, Tucson). The fragment was isolated by *Eco*RI digestion and contains coding sequences as well as the 3′ nontranslated sequence. The probe was used under the hybridization conditions described above for cucumber HPR RNA.

Image Analysis for Quantification of RNA

Autoradiograms derived from the northern analyses were used to quantify individual RNA using a Bio Image Visage 2000 (Bio Image Products, MilliGen/Biosearch Division of Millipore, Ann Arbor, MI) image analysis system. Briefly, the images of the northern blots were scanned, captured, and quantified using a comparative logarithmic software program (whole band analysis), and the results are reported as total integrated intensity in each band.

RESULTS AND DISCUSSION

Transgenic tobacco plants were screened for the expression of the chimeric cucumber HPR gene by northern analysis of leaf RNA extracts and probing with the cucumber HPR cDNA under hybridization conditions specific for the cucumber HPR RNA (see "Materials and Methods"). RNA from SR1 nontransformed plants does not exhibit any signal with this probe under these hybridization conditions (data not shown). Plants that exhibited significant levels of the cucumber HPR RNA as described above were selfed. The R_1 offspring were germinated under high kanamycin selection (a eukaryotic kanamycin-selectable marker gene is co-integrated with the chimeric cucumber HPR gene)

and retested for expression of the *trans*-gene. From these R_1 lines a line that exhibited a high level of expression was chosen for the protein and enzyme analyses. Figure 1A illustrates the level of cucumber HPR RNA in the leaves of this line (R6+3) compared to the levels of the native tobacco HPR RNA and the level of the SSU RNA. Quantitatively, the level of the cucumber HPR RNA (166% of the SSU level) was 10-fold that of the tobacco HPR (14% of the SSU level). The cucumber HPR RNA was found in high levels in the leaves, stems, and roots of the transgenic plants (Fig. 1B), whereas the tobacco HPR RNA was detectable only in the stems and leaves of the same plants shown in Figure 1A for leaf tissue (root tissue RNA did not show a hybridization signal with the tobacco HPR probe; data not shown). HPR RNA was not detected in nontransgenic or transgenic plants transformed with plasmid only when the probe specific for the cucumber form of HPR was used (data not presented).

The expression of the chimeric HPR gene at the level of HPR protein accumulation in different tissues of the transgenic line was investigated by western analysis (Fig. 2). The HPR polyclonal antibody, derived from purified cucumber HPR, did not distinguish between the cucumber and tobacco forms of the enzyme and thus a significant response to the antibody was seen in the protein extract from tobacco leaves. As predicted there was no detectable HPR protein in the untransformed tobacco root extracts (Fig. 2). However, there was a significant level of HPR protein, greater than that seen in the leaf of the SR1 control, in the root extract of the R6+3 *trans*-genote. This demonstrates that the accumulation of the cucumber HPR RNA seen in the tissues of the *trans*-genote is being translated into its protein product in significant quantities. Similar, if not a little higher, levels of HPR protein were seen in the leaf extracts of R6+3 plants, which in all likelihood represented a mixture of both the tobacco and the cucumber forms of the enzyme. That these elevated levels and root accumulation of the protein were not due to the transformation and regeneration procedures was demonstrated by the lack of HPR protein in the roots of *trans*-genotes that express an antisense cucumber HPR chimeric gene (R6–14 ROOT, Fig. 2). It is not clear at this point where in the transgenic tobacco root cells the cucumber form of the enzyme was localized since these cells did not contain the specialized class of peroxisomes found in leaf tissues, which are the normal site of localization HPR (Tolbert, 1982). It is possible that the enzyme was translocated into nonspecialized peroxisomes common in root cells (Newcomb, 1982).

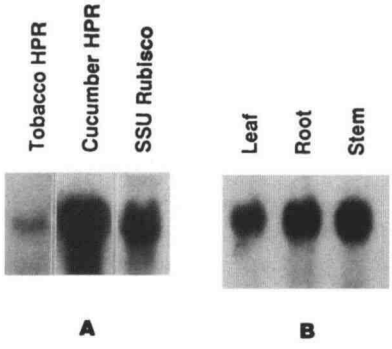

Figure 1. Northern analysis of total RNA extracted from R_1 transgenic tobacco expressing a chimeric cucumber HPR gene. A, Total leaf RNA from R6+3 tobacco plants probed separately with probes specific for tobacco HPR, cucumber HPR, SSU transcripts. B, Total RNA from various tissues from R6+3 plants probed with the cDNA probe specific for cucumber HPR. All lanes contain equal amounts of poly(A)$^+$ RNA (5 μg).

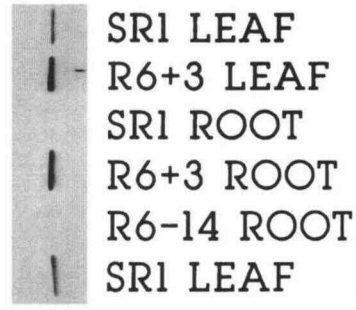

Figure 2. Western blot analysis of HPR protein levels in tissues from transgenic and control tobacco plants. SR1 denotes nontransformed *T. tobacum* cv SR1, R6+3 denotes a transgenic line of SR1 expressing the chimeric HPR gene construct, and R6–14 denotes a transgenic line of SR1 expressing an antisense chimeric HPR gene construct. All lanes contain 10 μg of total soluble protein.

Attempts were made to determine whether the cucumber and tobacco forms of the enzyme, and perhaps heterodimeric forms of the enzyme, could be distinguished using native polyacrylamide gels and activity staining. Unfortunately, although the enzyme could be detected in this way, the similarity of the cucumber and tobacco forms of HPR and the available means of detection made it difficult to distinguish between the two forms. Thus it was impossible to make conclusive determinations of the nature of the association of the two forms in transgenic plants. For this reason we chose a kinetically based determination of the interactions of the two forms of the enzyme as discussed below.

HPR enzymes from cucumber and tobacco exhibited different levels of substrate inhibition, and these differences were sufficient to allow for a diagnostic test of enzyme identity from the tissues of the transgenic plants (Fig. 3). The tobacco leaf HPR was relatively sensitive to substrate inhibition, losing 20% of its maximal activity at only 2 mM hydroxypyruvate, whereas the cucumber leaf enzyme was unaffected at this concentration. It reached a 20% level of inhibition at 5 mM hydroxypyruvate. HPR isolated from the roots of the transgenic tobacco exhibited substrate inhibition kinetics essentially identical with the cucumber leaf HPR, demonstrating that the protein seen in this tissue (Fig. 2, lane 4) was the cucumber form of the enzyme, derived from expression of the chimeric gene. The HPR isolated from the leaves of the transgenic tobacco exhibited inhibition kinetics that were part way between the two extremes, indicating that this enzyme pool was indeed a mixture of the tobacco and cucumber proteins. The kinetics data suggest that the HPR in the transgenic tobacco leaves consists of almost equal amounts of the two proteins (inhibition levels were close to that predicted for equal

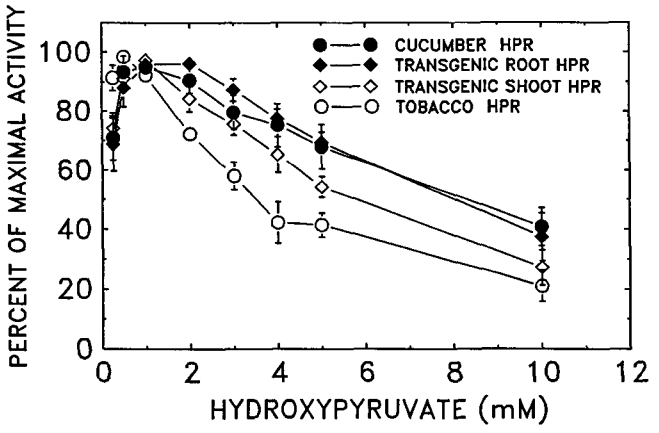

Figure 3. Comparison of substrate inhibition kinetics of HPR from roots and shoots of transgenic tobacco plants expressing a chimeric cucumber HPR gene. Each point is the average value from assays conducted at 20, 25, 30, 35, 40, and 45°C, each of which was conducted in triplicate; bars represent SES.

amounts of the two forms), even though the level of the cucumber HPR RNA in these plants appeared to be higher than the tobacco HPR RNA (even taking into account differences in probe size and strength). This may indicate that either the tobacco HPR RNA is more efficiently translated on tobacco polysomes than is the cucumber HPR RNA or the tobacco HPR protein is more stable (or more efficiently packaged into peroxisomes) than the cucumber protein, at least within tobacco cells, but both possibilities remain to be investigated.

To ascertain whether the temperature characteristics of the transferred enzyme were retained in the transgenic plants and whether the mixture of the cucumber and tobacco enzymes in the total leaf pool exhibited mixed temperature characteristics, the apparent K_m's of isolated enzyme pools were determined at varying temperatures. The results of this analysis are presented in Figure 4. The cucumber leaf enzyme exhibited a temperature-dependence curve for apparent K_m that reached a minimum K_m at 32.5°C. This curve was duplicated by the enzyme isolated from the roots of the transgenic tobacco expressing the cucumber HPR chimeric gene, again confirming its identity and the faithful retention of its characteristic kinetics properties. The enzyme from untransformed SR1 tobacco exhibited a curve distinctly different from these two enzyme preparations. It had a minimum K_m at 22.5°C. The mixture of the two forms of the enzyme (cucumber and tobacco) isolated from the leaves of the transgenic tobacco had a temperature-dependence curve for apparent K_m that exhibited a minimum K_m value over a large range of temperatures, 20 to 35°C. This demonstrates that the shoot HPR pool in the transgenic tobacco consisted of a mixture of the two forms of the enzyme and that the level of the cucumber protein was sufficient to alter the temperature characteristics of this pool.

We have demonstrated that the temperature characteristics of the pool of a single enzyme can be broadened by the interspecific transfer of genes. To our knowledge, this is the first demonstration that a complete enzyme pool of a selected biochemical reaction can be designed to better fit the thermal environment of a plant. This now allows us to utilize this approach to determine whether an alteration in the temperature characteristics of the pool of a key enzyme in a pathway can alter the temperature characteristics of an entire metabolic process (or of a key stress-protection enzyme) and thus affect plant productivity in adverse environments. Recent studies (Burke and Oliver, 1993) have shown that the temperature phenotype of an enzyme can be broadened by an increase in the availability of substrate both in vitro and in vivo. This opens up the possibility that increasing the flux through a metabolic pathway at an adverse temperature by altering the temperature characteristics of a control enzyme may have a cascade effect on substrate levels for subsequent enzymes, thus broadening the temperature characteristics of the entire pathway. We are at present testing this possibility.

For a more immediate application, the data we present suggest that the process of introducing novel genes into plants should take into account the species-specific temperature characteristics of the target enzyme or protein, especially where maximum efficiency of the activity of the protein is required.

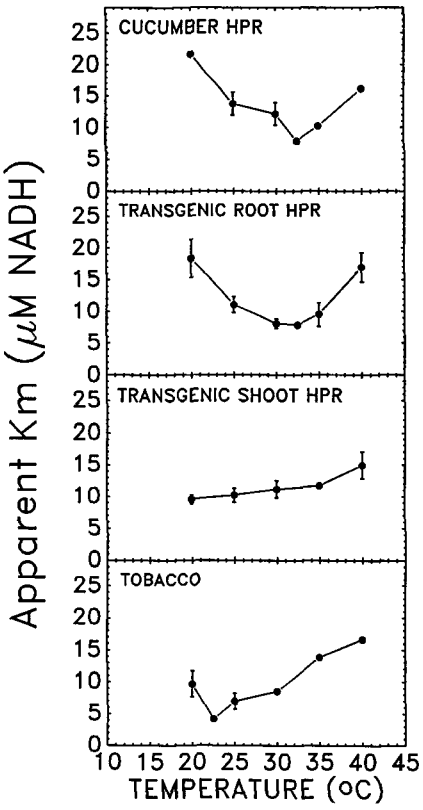

Figure 4. Comparison of the temperature characteristics of purified HPR from roots and shoots of transgenic tobacco plants expressing a chimeric cucumber HPR gene. The temperature-response curves for cucumber HPR, root HPR from transgenic tobacco plants expressing the chimeric cucumber gene, leaf HPR from transgenic tobacco plants expressing the chimeric cucumber gene, and HPR from nontransformed tobacco are shown.

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