An alternative agriculture system is defined by a distinct expression profile of select gene transcripts and proteins

Vinod Kumar*, Douglas J. Mills[†‡], James D. Anderson[§], and Autar K. Mattoo*[¶]

*Vegetable Laboratory, [†]Sustainable Agricultural Systems Laboratory, and [§]Plant Sciences Institute, Henry A. Wallace Beltsville Agricultural Research Center, United States Department of Agriculture, Agricultural Research Service, Building 010A, Beltsville, MD 20705-2350

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Conventional agriculture has relied heavily on chemical inputs that have negatively impacted the environment and increased production costs. Transition to agricultural sustainability is a major challenge and requires that alternative agricultural practices are scientifically analyzed to provide a sufficiently informative knowledge base in favor of alternative farming practices. We show a molecular basis for delayed leaf senescence and tolerance to diseases in tomato plants cultivated in a legume (hairy vetch) mulch-based alternative agricultural system. In the hairy vetch-cultivated plants, expression of specific and select classes of genes is up-regulated compared to those grown on black polyethylene mulch. These include N-responsive genes such as *NiR*, *GS1*, *rbcL*, *rbcS*, and *G6PD*; chaperone genes such as *hsp70* and *BiP*; defense genes such as chitinase and osmotin; a cytokinin-responsive gene *CKR*; and gibberellic acid 20 oxidase. We present a model of how their protein products likely complement one another in a field scenario to effect efficient utilization and mobilization of C and N, promote defense against disease, and enhance longevity.

Agricultural research in the past century made significant strides toward developing improved germplasm, devising integrated pest management, and defining cultivation practices. This has led to increased crop production and contained losses caused by pests. Thus, conventional agriculture used knowledge-based technologies to produce food crops that were safe, high yielding, and cost effective (1). Conventional agriculture depends on synthetic nitrogen (N), potassium (K), and phosphorous (P) fertilizer, pesticides, and heavy machinery driven by fossil fuels. Heavy reliance on chemical inputs has, unfortunately, resulted in increased production costs and has detrimentally impacted ecosystems by introduction of agrochemicals, raising serious concerns for human and animal health (2). There has been a continuing reliance on the use of pesticides, particularly for fruits and vegetables, as a means of preserving yield and quality.

Concerns about the environment and ecosystem have catalyzed efforts to seek alternative agricultural practices for long-term sustainability of agriculture (3). The alternative systems approach based on enhancing biological interactions to sustain and enhance production agriculture has had some success. This approach has innovatively reduced off-farm chemical inputs, and put emphasis on improved farm management and conservation of soil, water, energy, and biological resources (2).

In very high chemical input systems, such as fresh market tomato production (4), the use of legume cover crops offers advantages as a biological alternative to commercial fertilizer (5) that reduces soil erosion and loss of nutrients (6), enhances water infiltration, reduces runoff, and creates a "natural" pest–predator relationship (7). An important economical outcome of legume cover crop use has been the stemming of disease incidence or severity in diverse crops (ref. 7 and references therein). The legume hairy vetch (HV) (*Vicia villosa* Roth) has been shown to be an effective cover crop and organic mulch for growing tomato plants (4), resulting in delayed senescence and lesser incidence of foliar diseases (7).

Despite publications that spell out advantages of alternative farming practices, a successful transition from conventional to alternative has not generally occurred. Alternative agriculture practices are still perceived to be in their infancy where science is concerned (1). It is, therefore, imperative that we understand the mechanism(s) underlying beneficial aspects of legume cover crop, because it can provide the scientific legitimacy for adopting alternative farming practices. As a step toward that goal, we sought specific up- or down-regulated gene transcripts and proteins coincident with reduced leaf senescence and increased disease tolerance in HV-grown tomatoes. We demonstrate here existence of a specific interface between HV mulch and tomato crop, which results in a fundamentally distinct expression profile of gene transcripts and proteins in the leaf that may be related to differential hormone(s) signaling.

Materials and Methods

Plant Material and Field Experiments. Field studies (years 1997, 1999, and 2000) were conducted at the Henry A. Wallace Beltsville Agricultural Research Center farms in Maryland. Preparation of black polyethylene (BP) and HV (*Vicia villosa* Roth) fields and drip irrigation were carried out as described (7). Seedlings of 5-week-old tomato (*Lycopersicon esculentum* cv. Sunbeam) grown under natural photoperiod in the greenhouse were mechanically transplanted 0.46 m apart into the raised beds with no soil cover, BP mulch, or HV (4). A starter fertilization solution (9N–19.6P–12.5K, 0.0048 g/ml) was injected with each transplant. At 3-week intervals, urea was applied through the irrigation system on four occasions. Total N input, in the form of urea, was 100 kg per hectare (ha) for the HV beds and 200 kg/ha for the black polyethylene beds (4).

Disease and Defoliation Assessment. Foliar disease, developed from field-borne inoculum, included bacterial leaf spot, early blight, and *Septoria* leaf spot. Quantitation of leaf senescence or disease progress was carried out as described (7).

cDNA Subtraction and Cloning of PCR Products. Leaf cDNA synthesis and subtraction were done by using PCR-select cDNA subtraction kit (Clontech) following the manufacturer's protocol. The tester and driver double-stranded cDNAs were prepared from 1 μg of total leaf RNA. Tester cDNAs were ligated to adaptors, whereas driver cDNAs were not. Subtracted PCR products were cloned into *Sma*I-digested pBluescript II KS (Stratagene) and

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Abbreviations: BP, black polyethylene; HV, hairy vetch; CK, cytokinin; CKR, CK receptor kinase; GA_{20}, gibberellic acid 20; rubisco, ribulose-1,5-biphosphate carboxylase/oxygenase.

[‡]Present address: Department of Biology, Georgia State University, Atlanta, GA 30302-4010.

[¶]To whom correspondence should be addressed. E-mail: mattooa@ba.ars.usda.gov.

transformed into competent *Escherichia coli* DH5α cells to make a subtractive library. Alkaline lysis mini plasmid preparations (8) were made from randomly picked colonies, and recombinants were identified by restriction analysis and DNA sequencing.

DNA Sequencing and Analysis. The sequencing reactions (dye-terminator) were run on an ABI 373A automated sequencing machine. Sequences were analyzed by using the 10.2 version of the GCG program package (WISCONSIN PACKAGE version 10.3, Accelrys, San Diego). The GenBank and Swiss-Prot databases were searched for DNA and protein similarities by using BLAST.

RNA Gel Blot and RT-PCR Expression Analysis. Total RNA was isolated (9) from frozen leaf tissue, electrophoresed in a 1.2% formaldehyde-agarose gel, and alkaline blotted onto Hybond-N+ membranes (Amersham Pharmacia). Heterologous DNA probes used for Northern blots were mainly derived through PCR amplification with T3/T7 primers on EST templates cloned into pBluescriptSKmCUadapt vectors (Clemson University Genomic Institute, Clemson University, Clemson, SC). Table 2, which is published as supporting information on the PNAS web site, lists the forward and reverse primers used together with other relevant information for PCR-derived probes described in the legend to respective figure.

Protein Isolation and Immunodetection. Methods for total soluble and membrane protein isolation from tomato leaf, quantification of protein and chlorophyll contents, and SDS/PAGE separation were as described (10). Immunoblots were prepared and subsequently processed following standard procedures (11) using the alkaline phosphatase detection system.

Quantification of Immunoblots and Gene Transcripts. Immunoblots and autoradiographs were scanned and then quantified against internal controls. Analysis was performed by using the public domain NIH IMAGE program (http://rsb.info.nih.gov/nih-image).

Results

Leaf Senescence and Disease. Tomato plants were grown side by side in either BP or HV mulch. Visually, clear differences in plant health began to appear by 65 days after transplantation of the seedlings (Fig. 6, which is published as supporting information on the PNAS web site). Leaf senescence and disease onset data observed in 1997 and 2000 are presented in Fig. 1. Severe drought in 1999 compromised the efficacy of the HV mulch. Senescence was significantly greater in BP as compared to HV-grown tomatoes (Fig. 1*A*). BP mulch plants showed progressive senescence from day 65 onwards, whereas tomato plants grown on HV showed minimal senescence until day 84. These results were also reflected in the timing and duration of natural disease onset (Fig. 1*B*). Disease symptoms were attributed to bacterial leaf spot, early blight, and/or *Septoria* leaf spot. In BP mulch plants, disease onset was readily apparent by day 65 and progressed rapidly from day 84 in the 1997 season; however, disease spread occurred linearly from day 65 onwards in the 2000 season (Fig. 1*B*). In contrast, in both seasons, barely any disease symptoms were seen on HV-grown plants until day 84 after transplanting; thereafter these plants showed disease severity that was significantly lower compared to plants grown in BP, confirming previous observations on reduced disease severity in HV-grown tomatoes (7).

Longevity in Relation to Senescence-Linked Proteins. Immunoblot analysis was used as a first step to ascertain whether longevity or delayed senescence in HV-grown plants is reflected in differential accumulation of proteins central to senescence. Leaf senescence is marked by disassembly and degradation of plastid

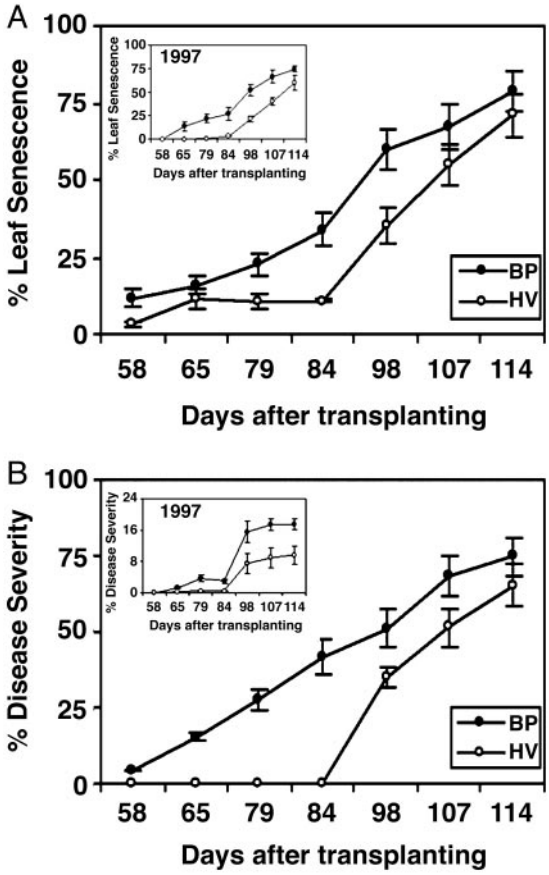

Fig. 1. Delayed leaf senescence and reduced disease severity are characteristic of tomato plants field-cultivated under HV mulch. On the indicated days, five leaflets from the lower, middle, and upper canopy levels of five plants were scored for the percentage of symptomatic area per leaflet (disease progress curve, *B*) and % leaf senescence (*A*). *Insets* are for the 1997 season, whereas the main panels are for the 2000 season. Values presented are average ± SE (n = 15).

proteins such as ribulose-1,5-biphosphate carboxylase/oxygenase (rubisco) (12), GS-1 (13), ATPases and other proteins associated with photosynthesis (14), and proteases (15). Each of these three protein markers of senescence, rubisco small subunit, rubisco large subunit, and GS-1 had markedly reduced levels at day 108 versus day 96 in leaves from plants grown in BP mulch as compared to those grown in HV mulch (Fig. 2*A*). The results paralleled the onset of rapid senescence in the BP-mulch-grown plants and the delayed senescence in the HV plants (Fig. 1). The specificity of these patterns is elaborated by the finding that other plastid (14) or cytosolic (15) proteins did not exhibit 108/96 ratio differences between the two cultivation practices (Fig. 2*B*). Some of these proteins are known to remain stable late into leaf senescence (14).

Identity of Differentially Expressed Transcripts. cDNA subtraction cloning, sequencing, and BLAST analysis (16) identified genes whose expression in tomato leaves is differentially regulated in BP versus HV cultivation practices. Genes thus identified included *rbcS*, *rbcL*, cytosolic *GS1*, *NiR*, osmotin, *chiB*, plastidic *G6PD*, and *SAG12*. The preponderance of the rubisco and GS-1 cDNA in HV-grown plants versus those cultivated on BP strengthened the immunoblot data (Fig. 2 *A* and *C*). The selectivity of *NiR*, *G6PD*, *chiB*, osmotin, and *SAG12* genes, which are associated with C/N signaling as well as plant defense against pests, reinforced our view that a distinct gene expression profile

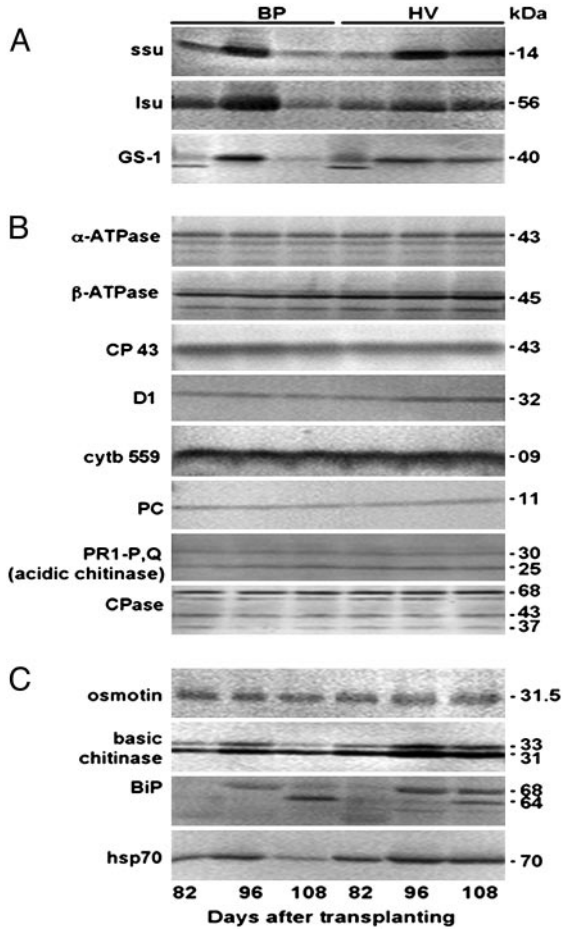

Fig. 2. Leaf protein expression profiling of tomato plants cultivated on either BP or HV mulch by using immunoblot analysis. (*A*) Immunological assay with rubisco small subunit (ssu), rubisco large subunit (lsu), and cytosolic GS1 antibodies. (*B*) Immunological assay with α-ATPase, β-ATPase, chlorophyll-binding protein 43 (CP 43), PS II D1, cytochrome b_{559} (cytb 559), plastocyanin (PC), pathogenesis-related protein (PR1-P,Q), and carboxypeptidase (CPase) antibodies. (*C*) Immunological assay with osmotin, basic chitinase-binding protein (BiP), and heat shock protein 70 (hsp70) antibodies. Each lane was loaded on an equal protein/chlorophyll basis (10 μg of protein or 4 μg of Chl per well).

might delineate growth and longevity of tomato plants on HV mulch (Figs. 1 and 6).

Gene-specific probes were used to study accumulation patterns of the corresponding transcripts. The accumulation patterns of *rbcS*, *rbcL*, and *GS1* transcripts mirrored their protein patterns (compare Fig. 3 with Fig. 2). For instance, *rbcL* transcript levels peaked on day 96 in BP-grown foliage, followed by ≈43% decrease by day 108. In contrast, the *rbcL* transcripts remained at the same or a little higher steady state level between days 96 and 108 in HV samples (Fig. 3*A* and Table 3, which is published as supporting information on the PNAS web site). Likewise, transcripts for *NiR*, *G6PD*, *chiB*, and osmotin accumulated progressively in leaves from HV plants, whereas in those from BP mulch, their levels on the corresponding 108-day samples were significantly reduced (Fig. 3). However, the expression profile for *SAG12*, identified in reverse subtraction experiments, was opposite to those described above. It was more robust in leaves from the BP-grown plants, increasing progressively from 82 to 108 days. Overall accumulation was ≈27% more at day 108 in BP-grown leaves as compared to HV (Fig. 3*D* and Table 3).

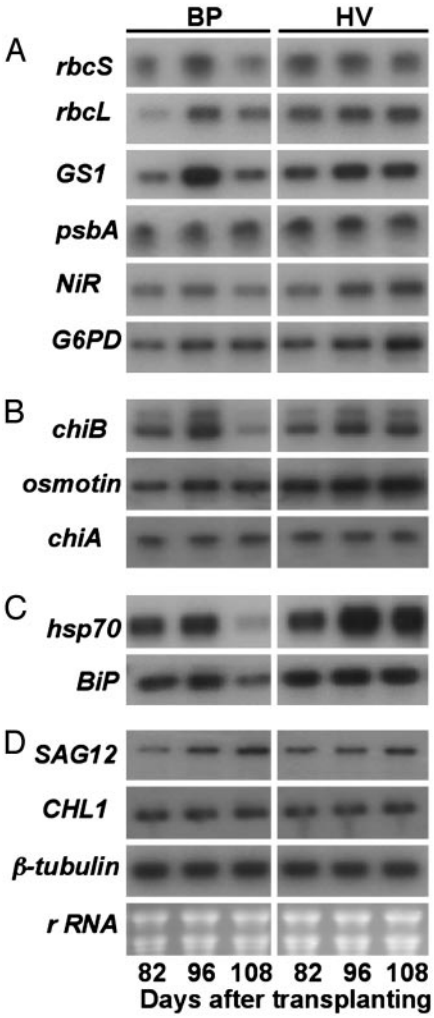

Fig. 3. Leaf RNA expression profiling of tomato plants cultivated on either BP or HV mulch by using RNA–DNA hybridization blot analysis. Each lane was loaded with 25 μg of RNA. (*A*) Hybridization was carried out with gene-specific probes for nuclear- or chloroplast-encoded genes as listed (see Table 2). (*A*) *rbcS* (GenBank accession no. AW039874), *rbcL* (GenBank accession no. AI486088), *GS1* (GenBank accession no. AW626325), *psbA*, *NiR*, and *G6PD*. (*B*) *chiB* (GenBank accession no. AI776962), *osmotin*, and *chiA* (GenBank accession no. BG135176). (*C*) *hsp70* (GenBank accession no. AW617489) and *BiP* (GenBank accession no. AW931570). (*D*) *SAG12* (GenBank accession no. AI776170), *CHL1* (GenBank accession no. BF050343), and *β-tubulin*. PCRs were performed in a 25-μl reaction containing 25 ng of template DNA, 200 nM each primer, 200 μM each dNTP, and 2.5 units of *Taq*DNA polymerase (Perkin–Elmer Cetus). A program consisting of 35 cycles of 50-sec denaturation at 94°C, cooling to 55°C for 1 min to anneal, and extension at 72°C for 2 min was used. All PCR products were gel-purified and radiolabeled with [α-^{32}P]dCTP by primer extension from random 9-mer primers by using a Random Primer Labeling kit (Stratagene). Hybridization was performed in 5× Denhardt's solution, 4× SETS, 0.1% SDS, 10% dextran sulfate, and 50 μg/ml herring testes DNA at 65°C for 16 h. Filters were washed twice at 65°C in 2× SSC/0.1% SDS for 25 min followed by a single wash in 0.2× SSC/0.1% SDS for 30 min. The membranes were reprobed by stripping the membranes with boiled 0.5% (wt/vol) SDS solution and allowing them to cool at room temperature. Dried filters were exposed to Kodak x-ray films at −80°C with intensifying screens.

To ascertain the specificity of the differential gene expression profiles, we randomly chose a few key genes to perform DNA–RNA hybridization analysis using probes for an acidic chitinase (*chiA*) (17), chloroplast-encoded D1 (*psbA*) (18), a nitrogen transporter *CHL1* (*NRT1.1*; ref. 19), and chaperones [*hsp70* (20) and luminal BiP (21)]. Both *hsp70* and *BiP* transcripts displayed

Table 1. Promoter analysis of some genes whose transcripts are up-regulated in HV-grown leaves shows that they share the N-responsive element TATCTA, a NIT2 protein-binding site

Gene name	GenBank accession no.	Promoter size, kb	TATCTA location
rbcS	X66072	0.740	−322
rbcL	AF397080	0.769	−706
GS-1	AJ011009	3.437	−412
NiR	U10419	2.165	−700
G6PD	AF231351	0.934	−844 and −899

longer duration in HV-grown plants compared to those grown in BP mulch (compare day 108 samples, Fig. 3). In contrast, the transcript levels of *chiA*, *CHL1* and *psbA* remained more or less the same throughout the sampling period under both cultivation conditions (Fig. 3). The fundamentally distinct expression profiles seen for the above described genes in leaves from the two cultivation practices, irrespective of the diversity of their cellular location, attests to the highly regulated metabolism and signaling in plants cultivated in HV mulch.

Coordination Between Gene Expression and Protein Profiles. Immunoblot analysis was also performed with antibodies against osmotin, basic chitinase, acidic chitinase (PR1-P,Q), hsp70, and BiP. Osmotin, identified as a 31.5-kDa protein, and two basic chitinase immunoreactive proteins, identified as 31- and 33-kDa protein bands (Fig. 2*C*), had a higher steady-state level in leaves from HV plants compared to the BP-grown plants. Basic chitinase levels were >2-fold higher in day 96 and day 108 HV samples as compared to those in BP samples. In comparison, acidic chitinase protein (PR1-P,Q) levels were no different between the two cultivation practices (Fig. 2*B*), which is also consistent with the profiles seen of their transcripts (compare Fig. 2*B* and Fig. 3). The antibodies against yeast binding protein (BiP) immunoreacted with 64- and 68-kDa protein bands; the 64-kDa band was likely a degradation product (Fig. 2*C*). The 68-kDa BiP showed more degradation in the BP-grown samples relative to the HV-grown plants; moreover, at day 108, the level of the parent protein remained more stable in samples from HV-grown plants than in those grown in BP. Antibodies to hsp70 detected a 70-kDa immunoreactive band that was also more elevated in leaves from HV-grown plants than those from BP mulch-grown plants. These patterns mimic profiles of their transcripts (Fig. 3). Together, these data indicate a consistent and coordinated relationship between the transcript expression profiles and their corresponding proteins.

Differential Hormonal Signaling: A Hallmark of HV-Grown Tomatoes. Plant hormones play a major role in regulating growth and development of plants. We approached the subject of hormonal involvement by looking for known hormone-responsive *cis*-elements in the 5′ flanking regions of the genes whose transcripts we found to be differentially expressed between the two cultivation practices. From our analysis, two results were evident: one, the promoters of *rbcS*, *rbcL*, *GS-1*, *NiR*, and *G6PD* share a copy of the NIT2 element implicated in nitrogen regulation (22) (Table 1). Second, promoter regions of most of the differentially expressed genes identified here contain putative *cis*-elements for responsiveness to hormones such as ABA (ABRE), auxin (AuxRR-core), ethylene (ERE), and gibberellic acid (GA) (P-box). We reasoned that differential hormonal signaling could contribute interactively with C/N signaling to explain the beneficial responses of tomatoes grown in HV mulch. We approached this scenario by synthesizing gene-specific probes for key genes involved in either biosynthesis of or responsiveness to

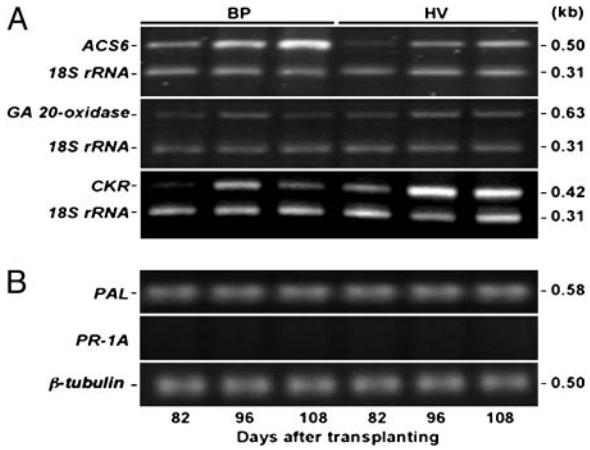

Fig. 4. Expression profiles of key hormone- and NO-responsive transcripts in tomato leaves from plants grown in either BP or HV mulch. Shown is RT-PCR amplification of *LeACS6*, GA_{20} oxidase, *CKR*, and *18S rRNA* (*A*) and *PAL*, *PR-1A*, and *β-tubulin* (*B*). RT-PCR detection was carried out on leaf cDNA templates by using primers specific to each gene (Table 2). Other methods used were as described in Fig. 3. RT-PCRs for *ACS6*, *CKR*, and *GA20-oxidase* were also spiked with 1 μM primers specifically designed to amplify an internal 315-bp fragment of 18S rRNA as invariant endogenous control (Ambion). Cycle numbers were optimized (between 19 and 24 cycles) for each sample to obtain data in the linear range for quantification. Leaf samples from 82-, 96-, and 108-day-old plants grown on either BP mulch (lanes 1–3) or HV mulch (lanes 4–6).

auxin (*ACS6*) (23), GA (GA_{20} oxidase) (24), or cytokinin (CK) (*CKR*) (25), and quantifying levels of the corresponding transcripts by RT-PCR.

In the leaves from BP-grown plants, *ACS6* transcripts appeared earlier and accumulated to higher levels as compared to the leaves from the HV-grown plants (Fig. 4*A*). These results are consistent with the delayed senescence in the latter cultivation practice, because activation of *ACS*6 in BP-mulch-grown tomatoes is an indication that the tissue is primed for ethylene production and thereby for senescence. These data further attest that the BP-grown plants senesce earlier than the HV-grown plants and that prosenescence hormonal signaling caused by ethylene is less active in the HV-grown foliage than in that grown on BP.

Contrary to the *ACS6* transcript data, the picture for GA_{20} oxidase and, especially, *CKR* was just the opposite. RT-PCR for GA_{20} oxidase transcripts was not robust. However, the transcripts were more prominent and long-lived in the later stages of leaf growth in the HV plants (Fig. 4*A*). RT-PCR analysis of *CKR*, CK receptor kinase, revealed a dramatic accumulation of the transcripts in the leaves from HV-grown tomato plants (Fig. 4*A*). In the BP-grown plants, the transcripts decreased considerably on day 108. These data support the involvement of differential hormonal signaling, CK in particular, in the HV-grown crops.

Discussion

Interactions between HV mulch and tomato plant initiate signals that regulate expression of specific classes of genes that make up the fingerprint of the HV based alternative agriculture. Among others, these gene classes comprise: N-responsive genes such as *NiR*, *GS1*, *rbcL*, *rbcS*, and *G6PD*; chaperone genes such as *hsp70* and *BiP*; defense genes such as chitinase and osmotin; a CK-responsive gene, *CKR*; and GA_{20} oxidase. The transcripts of these genes are at a higher steady-state level in the HV-grown tomato leaves, which is an indication of efficient utilization and mobilization of N, higher photosynthetic rates and, thus, of carbon mobilization, sustained reducing power generation, and defense promotion. The net result is that these tomato plants live

longer, have delayed leaf senescence, and are more tolerant to diseases. In this context, it is noted that overexpression of cytosolic GS-1 in transgenic tobacco leads to an improved growth phenotype (26). The nature of the initial signal(s) that dictates the gene/protein expression profiles shown here remains to be determined and is fundamental to future investigations. Nitric oxide (NO) is one of the many signaling messengers shown to participate in plant disease resistance (27). However, NO signaling is likely not involved in delayed disease characteristic of tomatoes cultivated in the HV-grown plants because no differences between HV and BP-grown tomatoes were found in NO-responsive (27) phenylalanine ammonia lyase (*PAL*) transcripts, whereas pathogenesis-related *PR-1A* transcripts were undetectable (Fig. 4*B*).

The HV system provides a medium by which bioactive nitrate is "slow" released to the leaves; this release depends on the rainfall/irrigation regimen and favors NO_2^- reduction to NH_4^+ by *NiR*. The resulting NH_4^+ is assimilated into glutamine by GS-1. Glucose-6-phosphate dehydrogenase (G6PD) enables NADPH regeneration, and photosynthesis is prolonged. Consistent with this scenario, at least one copy of the NIT2 element, which is implicated in nitrogen regulation (22), is present in the promoters of *rbcS*, *rbcL*, *GS1*, *NiR*, and *G6PD* (Table 1). Therefore, it is not surprising to find these genes among the many that respond to short-term exposure to nitrate levels (28). Glutamine (19) and/or other amino acids (29) have been suggested to sense the threshold N status in plants, but definitive evidence for a particular sensor has yet to be presented. A deeper look into the gene expression profiles presented here revealed that the HV system encompasses a unique regulation in addition to sharing features common to the C/N ratio signaling. For instance, in our study, we did not find up-regulation of senescence-associated protein (*SAG12*) or the nitrate transporter *CHL1*, whose transcripts respond to short-term exposure to external nitrate concentrations (30). Furthermore, we found significant increase in the osmotin transcripts (Fig. 3). In contrast, osmotin gene expression actually decreases by 2.5-fold in *Arabidopsis* exposed to high nitrate concentrations (30). In the HV-cultivated tomato, two other important chaperone genes, *hsp70* and *BiP*, are up-regulated (Figs. 2 and 3); these genes do not figure among those shown to be under N regulation. Higher and durable accumulation of *hsp70* and *BiP* transcripts in HV-cultivated tomato is an indication of their recruitment in keeping the anabolic machinery functional (31). Interestingly, BiP overexpression in transgenic tobacco has been related also to alleviation of endogenous oxidative stress (32). Furthermore, we did not find a consistent trend between leaf N content and the differences in disease severity and senescence observed between the mulches.

Delayed leaf senescence in the HV cultivation is coordinated with delayed disease development (Figs. 1 and 6). Thus, it was not surprising to find in the HV-grown leaves marked elevation in the transcript and protein levels of two antifungal defense proteins, basic chitinase (33) and osmotin (34). In contrast, these two defense proteins are independently induced during leaf senescence: chitinase in *Brassica napus* (35) and osmotin in *Arabidopsis* (36). These seemingly contradictory findings, in fact, corroborate our contention that the HV-based alternative agriculture encompasses a unique mechanism for metabolic and gene regulation.

The expression profiles of auxin-responsive *ACS6*, GA_{20} oxidase, and CK receptor kinase (*CKR*) suggest a role for GA and CK signaling in delayed leaf senescence and enhanced disease tolerance in the HV-grown plants. CK regulates a myriad of processes in plant growth and development, but the role as an antisenescence hormone (37) and an N signaling molecule (38) is more pertinent to the discussion here. From the *CKR* expression profile, we assume that the HV-cultivated tomato has a relatively sustained transport of CK from roots to shoots. A decrease in CK signaling is generally associated with initiation of senescence in plant organs (25). Strong support for a role for CK in delaying leaf senescence was provided by studies that showed a direct correlation between the elevation of CK level and retention of greenness in tobacco leaf genetically altered to express the *Agrobacterium* CK biosynthesis gene, *tmr* (39). In *Arabidopsis*, *CKI1* and *ARR2* act as positive regulators in diverse CK responses, including delayed leaf senescence (25). Expression of bacterial CK biosynthesis genes in tobacco plants enabled CK accumulation, which, in turn, induced several defense-related genes, including chitinase (40) and osmotin (41). In this context, it is interesting to note that, very recently, a role for the CK-signaling pathways has been suggested in plant–microbe interactions involving rhizobacteria (42).

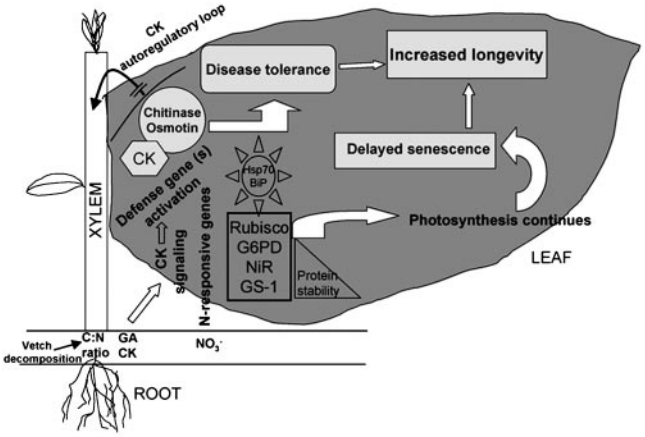

Fig. 5. A schematic model showing factors that may be responsible for increased disease tolerance and delayed senescence in tomatoes grown under HV-based alternative agriculture system. In this model, it is hypothesized that HV decomposition causes regulated release of C and N metabolites, which results in differential sensing via CKs and/or gibberellins and whose transport to shoots up-regulates expression of specific defense genes such as basic chitinase and osmotin. The accumulation, and perhaps higher stability, of specific gene products under HV is coordinated with a delay in senescence and up-regulation of key photosynthesis genes.

Our results suggest a model (Fig. 5), a part of which is in consonance with the scheme proposed by Sugiyama and Sakakibara (43), related to nitrogen signal transduction from root to shoot. Our scheme proposes a select and unique relationship between leaf senescence, disease tolerance, and specific gene expression in HV-grown tomato plant. The root system in HV-grown tomato plant is more robust, having a larger spread of roots compared to tomato plants grown on BP mulch (44); maintenance of root development is beneficial for higher yields, particularly under water-deficit conditions (45). Robust root growth habit also favors CK synthesis, thereby enabling more CK available from the root to the shoot. How may CK signal leaf longevity as well as defense against pests in HV-grown tomatoes? A likely connection may be related to coordinated accumulation of *CKR* with basic chitinase and osmotin transcripts, because these were both shown to bind CKs in tobacco callus (46). Also, these two proteins bind actin, causing cytoplasmic aggregation, and thereby likely participate in pathogen defense in potato cell suspensions (47). We suggest that, as a result of the tripartite combination of CK, basic chitinase, and osmotin, the two defense proteins remain stable for a longer duration, which, in turn, keeps the level of free CK to a minimum once it is perceived at the leaf membranes and enters the cellular pool (Fig. 5). We speculate that this sequestration of CK can achieve the objective of delaying feedback (autoregulatory loop) signaling from the

shoot to the root by high free CK levels, and thereby allow for continued flow of CK from the root to the shoot. The continued influx of CK into the leaf, indirectly indicated by higher *CKR* transcript levels (Fig. 4*A*) (48), signals the processes that keep the leaf from senescing by inhibiting the accumulation of senescence-enhanced genes, for instance, *SAG12* (Fig. 3*D* and ref. 49).

Evidence for differential hormonal signaling in tomato plants cultivated in the two mulch systems is further exemplified by up-regulation of *ACS6* in the BP-grown plants, a key gene in the biosynthesis of the senescence hormone ethylene (50) and an auxin-inducible enzyme (23). Coincident with early and higher accumulation of *ACS6* transcripts in the BP-grown tomato leaves is the accumulation of cysteine protease transcripts, an additional senescence-associated gene. The simultaneous increase in cysteine protease and *ACS6* transcripts does not appear to be a mere coincidence. A. Avni and coworkers (personal communication) have found that a cysteine protease-like protein binds to the promoter region of a related tomato ACC synthase gene, *ACS2* gene. They further found that overexpression of the cysteine protease-like protein in transgenic plants harboring the GUS reporter gene under the control of the *ACS2* promoter results in the activation of the GUS gene. Thus, higher accumulation of cysteine protease transcript in tomato plants grown under BP mulch could activate ethylene biosynthesis by induction of ACC synthase transcripts, thereby promoting senescence in these plants earlier than in the HV-grown ones.

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1. Trewavas, A. (2001) *Nature* **410,** 409–410.
2. National Research Council, Board on Agriculture (1989) *Alternative Agriculture* (Natl. Acad. Sci. Press, Washington, DC).
3. Lewis, W. J., van Lenteren, J. C., Phatak, S. C. & Tumlinson, J. H., III (1997) *Proc. Natl. Acad. Sci. USA* **94,** 12243–12248.
4. Abdul-Baki, A. A., Teasdale, J. R., Korcak, R., Chitwood, D. J. & Huettel, R. N. (1996) *HortScience* **31,** 65–69.
5. McVay, K. A., Radcliffe, D. E. & Hargrove, W. L. (1989) *Soil Sci. Soc. Am. J.* **53,** 1856–1862.
6. Flach, K. W. (1990) *J. Soil Water Conserv.* **45,** 42–47.
7. Mills, D. J., Coffman, C. B., Teasdale, J. R., Everts, K. L. & Anderson, J. D. (2002) *Plant Dis.* **86,** 356–361.
8. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
9. Prescott, A. & Martin, C. (1987) *Plant Mol. Biol. Rep.* **4,** 219–224.
10. Mattoo, A. K., Pick, U., Hoffman-Falk, H. & Edelman, M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1572–1576.
11. Callahan, F. E., Wergin, W. P., Nathan, N., Edelman, M. & Mattoo, A. K. (1989) *Plant Physiol.* **91,** 629–635.
12. Mae, T., Makino, A. & Ohira, K. (1983) *Plant Cell Physiol.* **24,** 1079–1086.
13. Feller, U. & Fischer, A. (1994) *Crit. Rev. Plant Sci.* **13,** 241–273.
14. Roberts, D. R., Thompson, J. E., Dumbroff, E. B., Gepstein, G. & Mattoo, A. K. (1987) *Plant Mol. Biol.* **9,** 343–353.
15. Mehta, R. A., Warmbardt, R. D. & Mattoo, A. K. (1996) *Plant Physiol.* **110,** 883–892.
16. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
17. Samac, D. A., Hironaka, C. M., Yallaly, P. E. & Shah, D. M. (1990) *Plant Physiol.* **93,** 907–914.
18. Avni, A., Mehta, R. A., Mattoo, A. K., Greenberg, B. M., Chattoo, B. B., Heller, D. & Edelman, M. (1991) *Plant Mol. Biol.* **17,** 919–921.
19. Glass, A. D. M., Britto, D. T., Kaiser, B. N., Kinghorn, J. R., Kronzucker, H. J., Kumar, A., Okamoto, M., Rawat, S., Siddiqi, M. Y., Unkles, S. E. & Vidmar, J. J. (2002) *J. Exp. Bot.* **53,** 855–864.
20. Li, Q. B., Haskell, D. W. & Guy, C. L. (1999) *Plant Mol. Biol.* **39,** 21–34.
21. Kalinski, A., Rowley, D. L., Loer, D. S., Foley, C., Buta, G. & Herman, E. M. (1995) *Planta* **195,** 611–621.
22. Fu, Y.-H. & Marzluf, C. A. (1990) *Proc. Natl. Acad. Sci. USA.* **87,** 5331–5335.
23. Yoon, I. S., Park, D. H., Mori, H., Imaseki, H. & Kang, B. G. (1999) *Plant Cell Physiol.* **40,** 431–438.
24. Carrera, E., Bou, J., Garcia-Martinez, J. L. & Prat, S. (2000) *Plant J.* **22,** 247–256.
25. Hwang, I. & Sheen, J. (2001) *Nature* **413,** 383–389.
26. Oliverira, I C., Brears, T., Knight, T J., Clark, A. & Coruzzi, G. M. (2002) *Plant Physiol.* **129,** 1170–1180.
27. Durner, J. & Klessig, D. F. (1999) *Curr. Opin. Plant Biol.* **2,** 369–374.
28. Foyer, C. H., Parry, M. & Noctor, G. (2003) *J. Exp. Bot.* **54,** 585–593.
29. Stitt, M., Muller, C., Matt, P., Gibon, Y., Carillo, P., Morcuende, R., Scheible, W. R. & Krapp, A. (2002) *J. Exp. Bot.* **53,** 959–970.
30. Wang, R., Guegler, K., LaBrie, S. T. & Crawford, N. M. (2000) *Plant Cell* **12,** 1491–1509.
31. Hammond, C. & Helenius, A. (1995) *Curr. Opin. Cell Biol.* **7,** 523–529.
32. Alvim, F. C., Carolino, S. M., Cascardo, J. C., Nunes, C. C., Martinez, C. A., Otoni, W. C. & Fontes, E. P. (2001) *Plant Physiol.* **126,** 1042–1054.
33. Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J. & Broglie, R. (1991) *Science* **254,** 1194–1197.
34. Liu, D., Raghothama, K. G., Hasegawa, P. M. & Bressan, R. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1888–1892.
35. Hanfrey, C., Fife, M. & Buchanan-Wollaston, V. (1996) *Plant Mol. Biol.* **30,** 597–609.
36. Capelli, N., Diogon, T., Greppin, H. & Simon, P. (1997) *Gene* **191,** 51–56.
37. Noodén, L. D., Kahanak, G. M. & Okatan, Y. (1979) *Science* **206,** 841–843.
38. Sakakibara, H., Suzuki, M., Takei, K., Deji, A., Taniguchi, M. & Sugiyama, T. (1998) *Plant J.* **14,** 337–344.
39. Smart, C. M., Scofield, S. R., Bevan, M. W. & Dyer, T. A. (1991) *Plant Cell* **3,** 647–656.
40. Memelink, J., Hoge, J. H. C. & Schilperoort, R. A. (1987) *EMBO J.* **6,** 3579–3583.
41. Thomas, J. C., Smigocki, A. C. & Bohnert, H. J. (1995) *Plant Mol. Biol.* **27,** 225–235.
42. Ryu, C.-M., Farag, M. A., Hu, C.-H., Reddy, M. S., Wei, H.-X., Pare, P. W. & Kloepper, J. W. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 4927–4932.
43. Sugiyama, T. & Sakakibara, H. (2002) in *Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism*, eds. Foyer, C. H. & Noctor, G. (Kluwer, Dordrecht, The Netherlands), pp. 227–238.
44. Sainju, U. M., Singh, B. P. & Whitehead, W. F. (2000) *Can. J. Soil Sci.* **80,** 523–532.
45. Serraj, R. & Sinclair, T. R. (2002) *Plant Cell Environ.* **25,** 333–341.
46. Kobayashi, K., Fukuda, M., Igarashi, D. & Sunaoshi, M. (2000) *Plant Cell Physiol.* **41,** 148–157.
47. Takemoto, D., Furuse, K., Doke, N. & Kawakita, K. (1997) *Plant Cell Physiol.* **38,** 441–448.
48. Papon, N., Clastre, M., Andreu, F., Gantet, P., Rideau, M. & Creche, J. (2002) *J. Exp. Bot.* **53,** 1989–1990.
49. Noh, Y. S. & Amasino, R. M. (1999) *Plant Mol. Biol.* **41,** 195–206.
50. Fluhr, R. & Mattoo, A. K. (1996) *Crit. Rev. Plant Sci.* **15,** 479–524.