

The analysis of large-scale gene expression correlated to the phase changes of the migratory locust

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Communicated by Zhu Chen, Shanghai Second Medical University, Shanghai, China, October 19, 2004 (received for review July 20, 2004)

The migratory locust is one of the most notorious agricultural pests that undergo a well known reversible, density-dependent phase transition from the solitary to the gregarious. To demonstrate the underlying molecular mechanisms of the phase change, we generated 76,012 ESTs from the whole body and dissected organs in the two phases. Comparing 12,161 unigene clusters, we identified 532 genes as phase-related ($P < 0.01$). Comprehensive assessment of the phase-related expression revealed that, whereas most of the genes in various categories from hind legs and the midgut are down-regulated in the gregarious phase, several gene classes in the head are impressively up-regulated, including those with peptidase, receptor, and oxygen-binding activities and those related to development, cell growth, and responses to external stimuli. Among them, a superfamily of proteins, the JHPH superfamily, which includes juvenile hormone-binding protein, hexamers, prophenoloxidase, and hemocyanins, were highly expressed in the heads of the gregarious hoppers and hind legs of the solitary hoppers. Quantitative PCR experiments confirmed in part the EST results. These differentially regulated genes have strong functional implications that numerous molecular activities are involved in phase plasticity. This study provides ample molecular markers and genomic information on hemimetabolous insects and insights into the genetic and molecular mechanisms of phase changes in locusts.

solitary phase | gregarious phase | EST | unigene

Locusts are a phylogenetically heterogeneous insect group within the family Acrididae, typically demonstrating a pronounced ability to change phases from the solitary to gregarious in response to population density (1–5). There are 10 or 11 grasshopper species that have the ability to demonstrate some density-dependent phase differences. A phylogenetic comparison would be a very useful way to gain information about key genes important for phase transition. One of these species, the migratory locust (*Locusta migratoria* L.), is highly prevalent in Africa, the Middle East, Australia, Europe, and Asia and exhibits remarkable phenotypic polymorphisms between the two phases. Only gregarious bands of nymphs and swarming adults result in migratory plagues, whereas the less harmful solitary locusts are cryptic and live at very low densities. The locust plagues have been a historical problem in China and worldwide. Such outbreaks have become more and more frequent in China during the past decade because of inadequate prevention and control techniques as well as environmental changes, such as warmer winters and droughts (6).

Phenotypic phase changes of locust species are linked with differences in morphology, behavior, reproduction, endocrine balance (3, 4, 7, 8), and disease resistance (9). The transition between the two phases is reversible before they reach the fourth or fifth instar (10). The gregarious traits and behavior are also regulated by pheromones (11, 12), neuropeptides (13, 14), and pigmentotrophin (15–18). Even artificial physical stimulation on the hind legs of the desert locust can trigger phase transformation (11). In addition, foam from reproductive glands of the crowded female encourages gregarious behavioral features, even affecting the color of young hoppers (19). Phase changes are not

unique to locusts; many other insect species also exhibit phenotypic plasticity in response to changes in rearing density, implying that environmental factors also contribute to phenotypic changes (3).

Despite intensive investigations focusing on phase transition over the past few decades, very little work has been done on the molecular changes associated with phase transitions (20–23). A study with PCR-based differential display of transcripts has identified two genes from desert locust, which showed significant differences in expression between the solitary and gregarious phases (24). Unfortunately, the migratory locust has an unusually large genome size of 8,500 Mb (25), >70 times that of the *Drosophila melanogaster* genome. Strategically, for such an enormous genome, a stepwise implementation of gene discovery processes, such as acquisition of EST and microarray-based studies, should be commenced and followed by molecular marker-based physical and genetic mapping. We have acquired high-coverage EST data to discover and identify genes involved in phase transition. By comparing gene expression profiles between the two phases in the migratory locust, based on 76,012 high-quality raw EST data and 12,161 clustered unigenes, we have embarked on an attempt to interrogate the molecular and genetic bases of phase polymorphism. Our data have been deposited in the GenBank database (accession nos. C0819675–C0832059 and C0832067–C0865130).

Materials and Methods

Insects. Locusts were collected from a gregarious population in Huanghua, Hebei Province, China. Field populations were raised under experimental conditions as stock cultures. The gregarious and solitary cultures were harvested by following a previously reported procedure (5) and started with fifth-instar hoppers derived from second and third generations of gregarious and solitary stocks, respectively. The gregarious cultures were reared in large, well ventilated plastic bins (56 cm × 76 cm × 60 cm) at densities of 500–1,000 insects per container until the hoppers grew to the fifth-instar stage. The solitary culture consisted of individual insects that originated from the solitary stocks and were reared in isolation to the fifth-instar stage; individual eggs that were separated from the pod several days before hatching were used to start the culture. Newly emerged hoppers were placed in small containers (8 cm × 4.5 cm × 2 cm). The solitarily reared insects were kept in physical, visual, and olfactory isolation that was achieved by ventilating each cage with charcoal-filtered compressed air. The rearing conditions in crowded and solitary cultures were otherwise similar with regards to temperature, lighting regimes, relative humidity, and

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Abbreviations: GO, GENE ONTOLOGY; JHPH, juvenile hormone-binding protein, hexamers, prophenoloxidase, and hemocyanins; Q-PCR, quantitative PCR.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. C0819675–C0832059 and C0832067–C0865130).

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Table 1. EST datasets and their distributions among cDNA libraries

Data	Total	Gregarious				Solitary		
		Body	Head	Midgut	H-leg	Head	Midgut	H-leg
Acquired	76,012	18,480	10,996	10,291	8,981	10,675	9,628	6,961
Analyzed	45,449	12,385	5,724	6,831	4,620	5,361	6,062	4,466
Mit-related	18,545	2,677	3,763	3,317	2,976	2,796	1,834	1,182
Others	12,018	3,418	1,509	143	1,385	2,796	1,732	1,313

Genes related to mitochondria (Mit) and other housekeeping functions, such as certain ribosomal proteins and histones, were not used for the analysis. H-leg, hind leg.

food. The locusts that were produced exhibited gregarious and solitary physical characteristics.

cDNA Libraries. Seven nonnormalized cDNA libraries were constructed from mRNAs isolated from different locust organs: the head, hind legs, and midgut, with an equal number from male and female individuals. The whole body of female fifth-instar insects reared under gregarious conditions was used for the construction of a whole-body library. Total RNAs were isolated with TRIzol reagent (Invitrogen), and mRNAs were purified with Oligotex mRNA Isolation Kits (Qiagen, Valencia, CA). The cDNAs were reverse-transcribed (SuperScript II-RT, Invitrogen) and ligated to a plasmid pBluescript II SK (+) vector.

EST Assembly and Gene Identification. High-quality ESTs were first grouped into clusters by using the single-linkage clustering method (26). Two ESTs with >90% identity over >100-bp length were grouped into the same cluster. The sequence assembly software package, PHRED-PHAP-CONSED (27), was used to build consensus sequences and to view the result for each cluster.

Genes were identified by sequence similarity comparison against the SWISS-PROT and TrEMBL protein databases (28, 29) [SWISS-PROT 41.13 and TrEMBL 24 were downloaded from European Bioinformatics Institute; GENE ONTOLOGY (GO) 1.320] with BLAST at E values $<1e-10$. If one or more protein sequences in the integrated protein database had high sequence similarity to a unigene or a cluster, GO annotations of the most similar one were assigned to the query.

EST Expression Profiling and Quantitative PCR (Q-PCR). A statistical method (30) for comparing gene expression profiles in multiple cDNA libraries was used for the data analysis. To assess the significance of the differences between different expression profiles, R values were calculated for each unigene. A gene (or unigene) is said to have a different expression profile between the two phases when different from a random distribution model at $P < 0.01$.

Q-PCR experiments were conducted according to standard protocol (Real-Time Detection system, Bio-Rad). Briefly, mRNAs from a dissected tissue were isolated and quality checked by using electrophoresis and treated with RNase-free DNase (Roche Diagnostics, Mannheim, Germany). The cDNA was synthesized from 2 μ g of total RNA with the SuperScript II system (Invitrogen). Primers were designed based on PRIMER3.0 (<http://frodo.wi.mit.edu>) (also see Table 4). The 18S rRNA sequence of *L. migratoria* (GenBank accession no. AF370793) and a cloned Hsp90 sequence were used for normalization and to calibrate standard curves, respectively. The experiment was performed in three parallel replicates. Data were analyzed by using ANOVA and the Tukey honest significant difference test.

Supporting Information. For full experimental details, refer to Fig. 4 and Tables 3–5, which are published as supporting information on the PNAS web site.

Results and Discussion

EST Coverage and Annotation. We chose three relevant organs from the migratory locust in the two phenotypic phases for this study: the head (for neuronal and hormonal relevance), hind legs (for migratory characteristics), and midgut (for dietary and digestive traits) (Table 1). To facilitate sequence assembly and to provide adequate gene coverage, we also sequenced a whole-body cDNA library in which different organs were pooled from the gregarious hopper. We obtained 45,474 EST contigs (cumulative length of 21,760,812 bp) and 12,161 unigene clusters (7,611 single ESTs and 4,550 multiple EST clusters; Table 3) after removal of low-quality ends of the sequence traces and other noninformative data, such as short sequences and contaminants.

To annotate the unigene clusters, we first compared the consensus sequences to gene sequences of all public nucleotide and protein databases by using BLAST-based tools with two cutoff E values, $<1e-10$ and $<1e-5$, after careful parameter assessment on the average homology between locusts and the fruit fly. We then used GO annotation tools to look for protein domains and relied on the *D. melanogaster* database, Flybase (www.fruitfly.org/DGC/index.html; Berkeley *Drosophila* Genome Project BDGP 3.1), for putative functional annotations. At the two E values, we were able to identify 35.9% and 44.0% of the unigene clusters by sequence homology to genes from *D. melanogaster* and *Bombyx mori* (33.5% and 38.1%), respectively (Fig. 1), as well as other known genes. At present, we are unable to annotate most of the *L. migratoria* genes because of compound reasons, both the fragmented nature of the EST clusters and the tremendous evolutionary distances between the Orthoptera, the Lepidoptera, and the Diptera, which are believed to have diverged 250–300 million years ago during the Carboniferous and Permian periods (31). However, it is highly conceivable that, together with the acquisition of their full-length cDNA and rapid data accumulation in the public databases, we will have the ability to functionally characterize most of the genes in the near future.

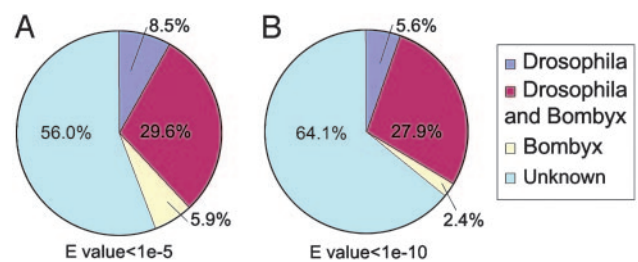


Fig. 1. The distribution of annotated unigenes based on publicly available databases (*D. melanogaster* and *B. mori*) by BLASTX. Homology was estimated based on different E values, $<1e-5$ (A) and $<1e-10$ (B). Genomic data from *B. mori* are also included (Q. Xia and J.Y., unpublished data).

Gene Discovery from a cDNA Library Made from the Whole Body. As a gene discovery exercise, we sequenced twice as many ESTs from the whole-body library made from the gregarious hopper as opposed to the organ-specific libraries. It yielded 12,385 ESTs, which were clustered into 4,527 unigenes. From this total, 2,482 unigenes were absent in the organ-specific libraries. To evaluate biological significance, we choose several gene categories for our initial analysis from the pooled unigene collection of all libraries, which includes genes that are related to hormonal, wing patterning, and developmental regulation as well as their network components. A significant number of the genes (nearly 30% of what have been annotated in the fruit fly) are known. We listed 71 (578 ESTs), 68 (490 ESTs), and 102 (2,885 ESTs) genes in these three categories, respectively, in Table 4. Because a large number of *L. migratoria* genes are highly diverged from those of better characterized insect species, heroic attempts in homology-based gene identification have not yet been completed. Nevertheless, we verified the expression pattern of a previously discovered gene (25), named gregarious-specific gene (GSG), a member of the *Drosophila* SPARC (CG6378-PA) family. The SPARC homolog in the migratory locust is $\approx 49\%$ (160 of 327 amino acids, LMC_001275), identical to that of desert locust in our partial alignment. A total of 17 ESTs were found in six of our seven libraries. Aside from three ESTs in the gregarious whole-body library, eight ESTs were identified in the head libraries, seven from the gregarious library, and one from the solitary form. Three ESTs were observed in the hind-leg libraries; all are from the gregarious phase. One and two ESTs were also seen in the gregarious and the solitary locust midguts, respectively.

Differentially Expressed Unigenes in the Phase- and Organ-Specific Libraries. We acquired a similar number of ESTs from each of the organ- and phase-specific libraries made from the fifth-instar hoppers at the two phases, thus allowing us to do pairwise comparisons. Comprehensive assessment of gene expression patterns revealed striking differences between the two phases. Most of the annotated gene classes were down-regulated in the gregarious phase in comparison to the solitary form, and some correlate well to the function of the selected organs. For instance, metabolic, structural, and muscle-specific genes are all down-regulated in hind legs of the gregarious hoppers (Fig. 2). A similar pattern was observed in the midgut, where cytoskeleton components, catalytic, and peptidase activities are abundant. A complex feature was seen in the head. Whereas several housekeeping gene categories, such as metabolism and biosynthesis, were down-regulated, others, including activities of peptidase, receptors, and oxygen binding, as well as development, growth, external stimulus responses, and apoptosis, were up-regulated. These results suggest that there are specific regulatory activities in nerve cells during phase transition, most likely controlled through hormonal signals that govern the transition of morphological plasticity. Correlation study is certainly the next step to relate the differentially regulated genes between the two phases to particular molecular mechanisms and cellular processes categorized in the analysis.

On closer examination of the head libraries, we found 153 unigenes that showed differential expression between the libraries at a confidence interval $P < 0.05$. Among them, 71 unigenes showed differential or specific expression at a high confidence level ($P < 0.01$), and 40 of them yielded functional assignments based on GO databases. The most outstanding difference is the expression of a group of unigenes that are related to a superfamily of genes that include the larval serum protein complex and juvenile hormone-binding protein, hexamerins, prophenoloxidase, and hemocyanins (JHPH) (32–34). We refer to this superfamily of genes as the JHPH superfamily. At least three major families of genes were discovered in our data set, based on sequence similarities (Table 2). The sequence alignment of

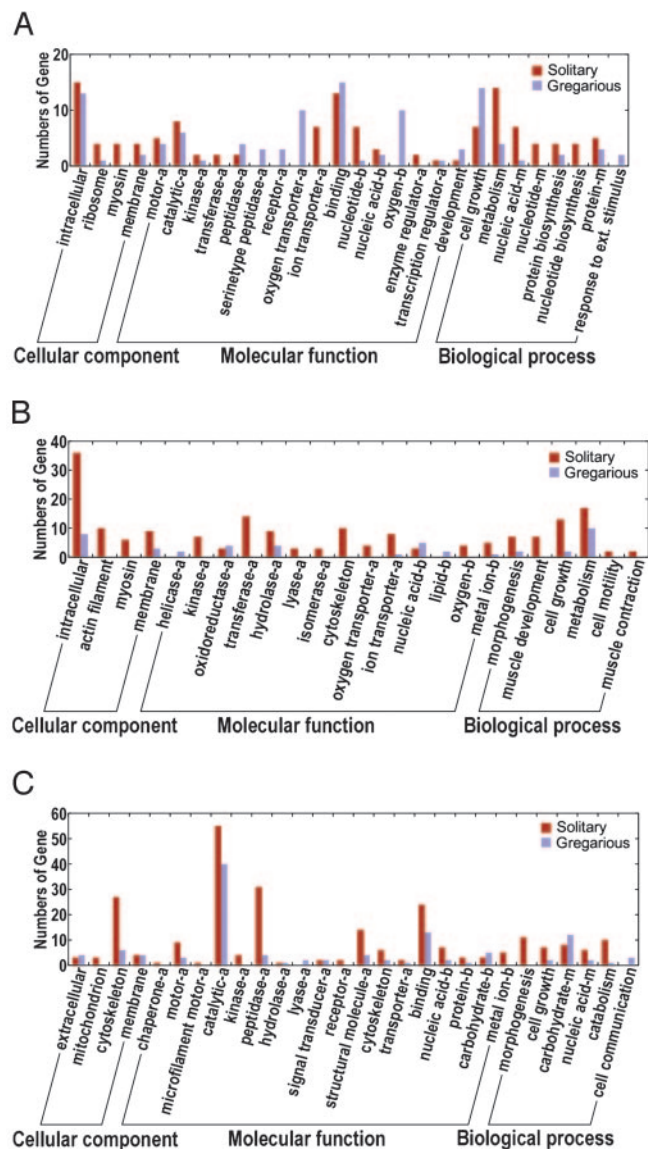


Fig. 2. GO classification of genes differentially expressed ($P < 0.01$) in the head (A), hind legs (B) and midgut (C) of solitary and gregarious locusts. Categories having fewer than three unigene clusters are not included. In the go functional categories, -a, -b, and -m stand for activity, binding, and metabolism, respectively.

representative sequences from our data set and several insect proteins is shown in Fig. 4. The sequence identities among the ESTs in a given gene family and between the families are 92–100% and 80–90%, respectively. All three families, JHPH1, JHPH2, and JHPH3, were found highly expressed in the heads of gregarious locusts and in hind legs of solitary locusts. As a comparative note, we found only five ESTs, four from the JHPH1 family (LMC_001182) and one from the JHPH3 family (LMC_003031) in the midgut of the solitary hoppers. Only one EST was found from the JHPH2 family (LMC_003496), which was expressed in the gregarious phase of the midgut. Therefore, the midgut is similar to the hind legs with regard to the JHPH gene expression, i.e., they are predominantly expressed in the solitary phase. Although this collective number may not be highly significant, it supports the notion of both tissue and phase specificity. In addition to these overall features, we note that individual members of the JHPH superfamily also show gene-

Table 2. Sequence identities (%) of the JPHH superfamily of genes in *L. migratoria*

	JPHH family 1	JPHH family 2	JPHH family 3
JPHH family 1	96–99	80–84	80–85
JPHH family 2	—	92–100	80–90
JPHH family 3	—	—	96–100

The JPHH superfamily genes were grouped into three families based on nucleotide homology by BLASTN. Homology ranked from 92% to 100% is likely variation among the same gene family, including population heterogeneity among the same genes. Unigenes with identity <90% presumably represent different genes of the superfamily. JPHH1 is composed of LMC_001181/1182 and LMS_000264. JPHH2 contains most of the unigenes clusters, including LMC_003013/3986/3550/3866/3981/3802/3227/3142/3015/3951/4016/3975/3902/4006/3999/3697/3718/3424/3420/3459/3165/3978/3284/3496/3333/3044/3345/3645/3492, and LMS_001278/0919/1198/1218/1473. JPHH3 contains LMC_003019/3031/3204/3994/3797/3880/3816/3579/3344 and LMS_000655/0779/0748/1204.

based variations in tissue and phase specificity, suggesting that they are encoded by different genes. For instance, the JPHH1 and JPHH3 families are ubiquitous and the same set of genes were all expressed in both the head and hind legs, but the JPHH1 family was up-regulated in the gregarious phase, whereas the JPHH3 family was up-regulated in the solitary phase. We believe that the JPHH1 proteins are encoded by a limited number of genes because the identities among the high-quality ESTs are between 97% and 100%. The JPHH2 and JPHH3 families are believed to contain multiple genes as paralogs or homologs, especially the JPHH2 family, which contains 33 unigenes and 548 ESTs. A few genes of the JPHH2 family seem either uniquely expressed in the gregarious head (a cluster, LMC_003978, of 30 ESTs) or in the solitary head (a cluster, LMC_003645, of 6 ESTs), manifesting very stringent tissue and phase specificity. These characteristics and pronounced differences will help to unveil specific functions of the two phases when detailed gene structures and gene-specific expression patterns are further investigated.

Although it seems less impressive than the head library, the hind-leg library holds more differentially expressed genes (189 unigenes, $P < 0.05$); 115 differentially expressed unigenes were identified with high confidence ($P < 0.01$) (Table 5). The few highly expressed genes in the gregarious hoppers include nucleic acid (including ATP and GTP binding), calcium-dependent phospholipid-binding proteins, and carbohydrate metabolism-related genes, suggesting that gene regulation in the leg correlates with that observed in the head. As expected, many muscle-related genes, such as actin, myosin, and those involved in muscle development and contraction, are up-regulated in the solitary as opposed to gregarious hoppers (an average ratio of 4.0) (Fig. 2B). This observation is consistent with the notion that the solitary locust has relatively longer hind legs and stronger leaping ability (1).

The number of phase-specific unigenes in the midgut ranks the highest among the three tissues involved in this study. There are 290 specific unigenes in the midgut that are related to phase transition ($P < 0.05$); most of these genes are metabolism-related (Fig. 2C). At the highest confidence level ($P < 0.01$), two-thirds of these genes (100 of 157 unigenes) are uniquely expressed in the solitary phase (Table 5). In other words, they may be silenced or significantly down-regulated in the gregarious locusts. It is unclear why the gregarious hopper down-regulates so many metabolism-related genes as opposed to the solitary ones, other than to balance energy consumption for the transition between phenotypic types. Previous studies showed that genes associated with hydrocarbon metabolism are involved in pheromone metabolisms (35, 36). One annotated gene (5 ESTs in the gregarious

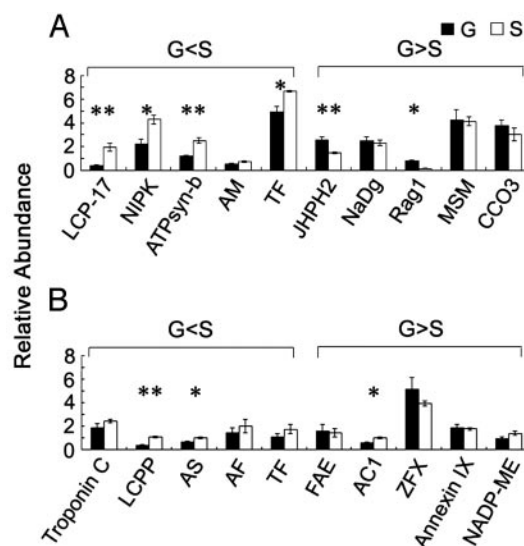


Fig. 3. Representative Q-PCR results from selected unigenes. Differentially expressed unigenes discovered in EST survey in solitary and gregarious phases from the head (A) and hind-leg (B) libraries were validated experimentally. Expression levels were normalized as relative abundance (y axis). Scales over the bar graph indicate SEs. Asterisks on the top of each bar highlight the significance levels of the individual experiments (*, $P < 0.05$; **, $P < 0.01$). Unfilled bars and filled bars denote the expression levels of the solitary (S) and gregarious (G) phases, respectively. The abbreviations in the chart and unigenes used in the experiment are as follows: LCP-17, larval cuticle protein LCP-17 precursor (LMC.004183); NIPK, neuronal cell death inducible putative kinase (LMC.001295); ATPsyn-b, F-type ATPase complex β subunit (LMC.002337); AM, ATP-binding myosin (LMC.002592); TF, transcription factor (LMC.004015); JPHH2, member of JPHH2 family (LMC.002189); NaDg, β -N-acetylhexosaminidase activity (LMC.003978); Rag1, recombination activating gene 1 (LMC.000750); MSM, microtubule structural molecule activity (LMC.004292); CCO3, cytochrome c oxidase chain 3 (LMC.001579); troponin C (LMC.001149); LCPP, larval cuticle protein precursor (LMC.002346); AS, similar to asparagine synthetase (LMC.002788); AF, actin filament components (LMC.000202); FAE, putative fatty acid elongase (LMC.000202); AC1, similar to brain adenylate cyclase 1 (LMC.001410); ZFX, similar to sex-determining protein Zfx (LMC.001624); annexin IX (LMC.002984); and NADP-ME, NADP-dependent malic enzyme (LMC.003913).

head but absent in other organs) function is phenylalanine catabolism, which may be involved in JPHH protein synthesis because these proteins are phenylalanine- and tyrosine-rich proteins (37). Among the midgut genes, one chaperone gene (LMC_001326) stands out as preferentially expressed in solitary hoppers. The solitary population is the dominant surviving form until its density reaches a critical threshold that leads to a local outbreak or clumping (1, 2). Solitary populations exhibit relatively higher fecundity and extended lifespan (4) and, therefore, unique chaperones, often involved in cell protection and stability (38), may be associated with the solitary phase.

Validation of EST Data by Q-PCR. To validate our EST sampling results, we performed Q-PCR experiments with selected EST clusters that show differential expression in the two phases from both the head and hind-leg libraries. Representative results are shown in Fig. 3. All of the Q-PCR results from the differentially expressed genes in the head have confirmed the EST classifications; 6 of 10 unigenes displayed significant differential expression patterns ($P < 0.05$). In the hind-leg samples, we show two confirmed cases with high confidence and one exception, where an adenyl cyclase was found up-regulated in solitary hoppers instead of being down-regulated in gregarious hoppers as suggested by EST sampling. Such observations provide an important clue in the gene

regulatory cascade of phase transition, i.e., a high confirmation rate among differentially expressed, head-specific unigenes may be significant as opposed to that among unigenes in hind legs. The head, which includes the brain and endocrine centers, shows no morphologic change between the two phases, but it can be expected to express specific genes in upstream regulatory cascades, whereas gene expression patterns in the hind leg are likely to show more variations between samples. We paid particular attention to this point when we prepared mRNAs for our Q-PCR experiments. Limited numbers of individual hoppers were used for each batch, and multiple samples were tested. There are several other reasons why slight variations between EST sampling and Q-PCR experiments were observed, based on technological details. First, the RNA samples were prepared separately from different batches of phase-reared insects in which the transition from solitary to gregarious may not have been uniform. In addition, the physiological state of the solitary phase is considered to be relatively stable, compared with gregarious hoppers, because phenotypic changes involved are controlled by endocrine pathways in a complex cascade (39, 40). Second, PCR-based quantitation relies on logarithmic amplification of starting material (such as concentration of PCR templates), whereas EST sampling is more or less linear, depending very much on the quality of the clone library. Third, cloning efficiency specific to the EST-based method (such as the quality of mRNAs, the efficiency and completion of reverse transcription, and insert clonability) and primer specificity unique to the PCR-based method (such as uniqueness of the primer sequence, optimization of experimental conditions, and control samples) may result in some variations in the results.

Perspectives. This study provides an overview of the major genes in a hemimetabolous insect and provides basic information for comparative genomics; all other genomics-scale projects have focused on holometabolous insects, such as Diptera and Hymenoptera (20–23). We have demonstrated major differences in gene expression between the solitary and gregarious phases of the migratory locust, both in the whole body and in major organs. Functional classification of these genes reveals that many of the phase-specific genes are associated with physiological adjustments specific to the two phases. About 60–70% of the genes discovered are not annotated because of the lack of sequence homology to the publicly available data and divergence between locust and other insects whose genomes have been fully sequenced. Two lines of experimentation will be important for future investigations: microarray-based gene expression studies to reveal coordinated gene expression at multiple time points and full-length sequencing of the genes that have been identified as being differentially expressed in the two phenotypic phases. The phase-specific expression patterns, coupled with cloning experiments and genomic sequencing, will provide valuable insight and a starting point for understanding the molecular mechanisms controlling the phase transition and, eventually, to develop efficient approaches to control these globally important pest species.

We thank Dr. Wen Wang for suggestions and for a critical reading of the manuscript. The research was supported by the Ministry of Science and Technology, the People's Republic of China (project nos. 2001AA222141 and 2001CCA00400), and the Chinese Academy of Sciences (project no. KSCX2-SW-317).

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