A comprehensive omics analysis and functional survey of cuticular proteins in the brown planthopper

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Cuticle, mainly composed of chitin and cuticular proteins (CPs), is a multifunctional structure of arthropods. CPs usually account for >1% of the total insect proteins. Why does an insect encode so many different CP genes in the genome? In this study, we use comprehensive large-scale technologies to study the full complement of CPs (i.e., the CP-ome) of the brown planthopper (BPH), Nilaparvata lugens, a major rice plant pest. Eight CP families (CPR, CPF, TWDL, CPLCP, CPG, CPAP1, CPAP3, and CPAPn) including 140 proteins in BPH, in which CPAPn is a CP family that we discovered. The CPG family that was considered to be restricted to the Lepidoptera has also been identified in BPH. As reported here, CPLCP family members are characterized by three conserved sequence motifs. In addition, we identified a testis protein family with a peritrophin A domain that we named TPAP. We authenticated the real existence of 106 proteins among the 140 CPs. RNA interference (RNAi) experiments were conducted against 135 CPs; no Derivatives License 4.0 (CC BY-NC-ND). This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1716951115/-/DCSupplemental.

Significance

The cuticle, mainly composed of chitin and cuticular proteins (CPs), is a multifunctional structure of arthropods. CPs usually account for >1% of the total insect proteins encoded in the genome. Why does an insect need so many different CPs? In this study, we use comprehensive large-scale technologies to study the full complement of CPs and their functions in the brown planthopper (BPH). A total of 32 of the 140 BPH CP genes are found to be essential for nymph/adult development, egg production, or embryo development; in addition, redundant and complementary functions of CPs are revealed.

Author contributions: C.-X.Z. designed research; P.-L.P. and Y.-X.Y. analyzed data; and P.-L.P., B.M., and C.-X.Z. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession nos. MF942728–MF942869).

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sequences, first, 135 of the above 140 N. lugens CP gene sequences were successfully verified and revised through PCR, RACE, and gene clone sequencing (Dataset S1). To further validate and authenticate the CPs of N. lugens at the protein level, proteins from four tissue samples were collected and digested with trypsin. The resulting peptides were analyzed by shotgun ultra-performance liquid chromatography (UPLC)-tandem MS/MS. The four protein samples were cast first-instar nymph cuticles (CC1), cast third- to fifth-instar nymph cuticles (CC3-5), adult long wings (LW), and fifth-instar nymph abdominal cuticles (AC).

Only proteins with at least two peptide-spectrum matches and a score of >4.0 were considered reliable. Under these conditions, we identified a total of 519; 686; 1,341; and 710 proteins from CC1, CC3-5, LW, and AC protein samples, respectively (Dataset S2, section S2). It is possible that not all peptides could be detected because they were too long for detection or because cuticle components were cross-linked to each other (7). Different CPs might have different solubility (8), and sclerotization could also make some CPs unextractable (9).

To confirm the existence of the 140 BPH putative CPs, manual annotation of the peptide sequences was carried out to verify or correct the predictions from the National Center for Biotechnology Information (NCBI). Together, in four MS experiments, 106 CPs (75.71%) were detected in at least one MS result, 93 CPs (66.43%) were detected in at least two MS results, and 48 CPs (34.29%) were detected in all four MS results (Fig. 1 and SI Appendix, Table S1). For each MS result, 76; 92; 57; and 97 CPs were identified in CC1, CC3-5, LW, and AC samples, respectively. For CPF, CPG, and CPA3 family proteins, all proteins were detected in at least one of the four MS results. The five CP genes that failed to be authenticated through PCR or RACE (NlugCpr41, NlugCpr43, NlugCpr56, NlugCpr61, NlugCpr62, NlugCpr64, NlugCpr69, NlugCpr73, and NlugCpr83 from the CPR family; NlugCPLCP2 from the CPLCP family; NlugCPAP1-E, -H, -K, and -N from the CPAP1 family; and NlugCPAPn-7 from the CPAPn family) led to lethal phenotypes with high mortality, indicating their essential roles in BPH integument structure and normal development. The survival rate of the treated BPHs was >90% survival rate of BPHs treated with dsRNA targeting the mock gene encoding green fluorescent protein (dsGFP) (Fig. 2). Intriguingly, the lethal phenotypes after injection of these 15 dsRNAs could be divided into four distinct classes, phenotypes I–IV: phenotype I, treated BPHs died before ecdysis; phenotype II, molting difficulties and adults with thin body shape; phenotype III, treated BPHs died during ecdysis or had abnormal organs after adult emergence; and phenotype IV, adults died with thin body shape (Fig. 3 and SI Appendix, section 3). The body weight as well as the triglyceride content of the dsRNA-treated female adults showing lethal phenotype IV was significantly decreased compared with the dsGFP-treated group; and honeydew excretion measurement suggested that the decreased feeding activity could have been an important reason for lethal phenotype IV (SI Appendix, section 3). In addition, RNAi against one gene (NlugCPAP3-D1) led to a much thinner body shape similar to phenotype IV, but only ~27.3% lethality 9 days after injection. The knockdown of other CP genes did not cause any distinguishable physiological malfunction or high death rate in BPHs, compared with insects that were treated with dsGFP.

Injection of all 135 CP dsRNAs against second-instar nymphs showed that all genes that had important functions in late nymphs or adults also played important roles during early nymph developmental stages. However, the respective phenotypes were not necessarily the same after injection with the same dsRNA into second-instar nymphs (Table 1). For example, knockdown of off-target effects had happened in the dsRNA injection experiments. Quantitative RT-PCR (RT-qPCR) assays confirmed that all target genes were efficiently suppressed by the dsRNA molecules (Dataset S3).

All 135 CP dsRNAs were injected into the thorax of fifth-instar nymphs. In general, the injection of two different dsRNAs targeting one gene caused the same phenotype. RNAi against 15 CP genes (NlugCpr6, NlugCpr47, NlugCpr56, NlugCpr61, NlugCpr62, NlugCpr64, NlugCpr69, NlugCpr73, and NlugCpr83 from the CPR family; NlugCPLCP2 from the CPLCP family; NlugCPAP1-E, -H, -K, and -N from the CPAP1 family; and NlugCPAPn-7 from the CPAPn family) led to lethal phenotypes with high mortality, indicating their essential roles in BPH integument structure and normal development. The survival rate of the treated BPHs was <20% 9 days after dsRNA treatments, compared with ~90% survival rate of BPHs treated with dsRNA targeting the mock gene encoding green fluorescent protein (dsGFP) (Fig. 2). Intriguingly, the lethal phenotypes after injection of these 15 dsRNAs could be divided into four distinct classes, phenotypes I–IV: phenotype I, treated BPHs died before ecdysis; phenotype II, molting difficulties and adults with thin body shape; phenotype III, treated BPHs died during ecdysis or had abnormal organs after adult emergence; and phenotype IV, adults died with thin body shape (Fig. 3 and SI Appendix, section 3). The body weight as well as the triglyceride content of the dsRNA-treated female adults showing lethal phenotype IV was significantly decreased compared with the dsGFP-treated group; and honeydew excretion measurement suggested that the decreased feeding activity could have been an important reason for lethal phenotype IV (SI Appendix, section 3). In addition, RNAi against one gene (NlugCPAP3-D1) led to a much thinner body shape similar to phenotype IV, but only ~27.3% lethality 9 days after injection. The knockdown of other CP genes did not cause any distinguishable physiological malfunction or high death rate in BPHs, compared with insects that were treated with dsGFP.

Injection of all 135 CP dsRNAs against second-instar nymphs showed that all genes that had important functions in late nymphs or adults also played important roles during early nymph developmental stages. However, the respective phenotypes were not necessarily the same after injection with the same dsRNA into second-instar nymphs (Table 1). For example, knockdown of
number of total eggs produced by one female adult and the average egg hatchability after RNAi were used to assess the function of each corresponding CP. The results showed that 20 CP genes were indispensable. The reproductive ability (total number of eggs) significantly decreased after knockdown of three genes (NlugCpr15, NlugCpr47, and NlugCPAP1-H), and the egg hatchability significantly decreased after knockdown of 19 different CP genes (Table 1). Notably, after RNAi against the 19 essential CP genes, no first-instar nymph hatched after knockdown of NlugCPAP3-B, and the egg hatchability was <10% after knockdown of NlugCpr3, NlugCpr51, NlugCpr52, NlugCpr58, and NlugCPAP3-D1. Knockdown of two CP genes, NlugCpr47 and NlugCPAP1-H, led to both low reproductive ability and low egg hatchability. Interestingly, among the total number of 20 indispensable CP genes for embryogenesis, only five (NlugCpr47, NlugCpr73, NlugCPAP1-E, -H, and NlugCPAP3-D1) also played important roles in nymphs and adults.

Table 1. Summary of phenotypes after RNAi for 32 essential CP genes at three developmental stages in *N. lugens*

| Gene             | dsRNA injection time and phenotypes |
|------------------|-----------------------------------|
| NlugCpr3         | IV² (T-en) DR                     |
| NlugCpr6         | IV² (T-en) DR                     |
| NlugCpr8         | IV² (T-en) DR                     |
| NlugCpr10        | IV² (T-en) DR                     |
| NlugCpr15        | DR                                |
| NlugCpr24        | DR                                |
| NlugCpr36        | DR                                |
| NlugCpr47        | III² (L-en) ME, LEP, LEH          |
| NlugCpr51        | LEH                               |
| NlugCpr52        | LEH                               |
| NlugCpr54        | LEH                               |
| NlugCpr56        | IV² (T-en, T-ex) DR               |
| NlugCpr58        | IV² (T-en, T-ex) DR               |
| NlugCpr61        | IV² (T-en) DR                     |
| NlugCpr62        | IV² (T-en, T-ex) DR               |
| NlugCpr64        | IV² (T-en, T-ex) DR               |
| NlugCpr69        | IV² (T-en, T-ex) DR               |
| NlugCpr73        | IV² (T-en, T-ex) DR               |
| NlugCpr83        | IV² (T-en, T-ex) DR               |
| NlugCpr90        | LEH                               |
| NlugCpr94        | LEH                               |
| NlugTwedlE3      | LEH                               |
| NlugCPAP3-C      | II² (T-en, T-ex) DR               |
| NlugCPAP1-E      | IV² (T-en) NM², LEH               |
| NlugCPAP1-H      | II² (T-en) NM², LEH               |
| NlugCPAP1-I      | II² (T-en) LEH                    |
| NlugCPAP3-K      | IV² (T-en, T-ex) DR               |
| NlugCPAP3-N      | IV² (T-en, T-ex) DR               |
| NlugCPAP3-B      | IV² (T-en, T-ex) DR               |
| NlugCPAP3-C5b    | IV² (T-en, T-ex) DR               |
| NlugCPAP3-D1     | IV² (T-en, T-ex) DR               |
| NlugCPAPn-7      | II² (C-en, T-ex) NM¹              |

The insects were treated as described in Materials and Methods. DR, developmental retardation; LEH, low egg hatchability; LEP, low egg production ability; ME, malformed eyes; NM, second-third nymph molting. Phenotypes in the brackets indicate TEM observation results: C-en, curved and disordered endocuticle; L-en, loose and thick endocuticle; T-en, thin and disordered endocuticle; T-ex, thin exocuticle.

The phenotypes listed were significantly decreased according to statistical test (Dataset S4). Phenotypes I–IV were classified as shown in Fig. 3.

*Egg hatchability was <10%.

*The phenotypes were lethal.

Since parental RNAi has been demonstrated to occur in BPHs (10), we conducted the RNAi experiments on the newly emerged female adults before their ovaries became mature to observe the phenotypes of egg production and embryos from the next generation. A total of 68 CP genes (either relatively highly expressed in the ovary or expressed obviously during embryogenesis) were selected for RNAi experiments (Dataset S4). The average

NlugCPAP1-E in fifth-instar nymphs resulted in lethal phenotype IV, while knockdown of NlugCPAP1-E in second-instar nymphs resulted in second-third nymph molting difficulties and high lethality. Only one gene, NlugCPAP3-C (encoding two alternative splice transcripts, NlugCPAP3-C5a and -C5b), which did not lead to distinguishable physiological malfunction or high death rate after RNAi treatment at fifth-instar nymph, showed lethal phenotype after injection of dsRNA in second-instar nymphs. Knockdown of the common region of NlugCPAP3-C5a and -C5b or of the specific region of NlugCPAP3-C5b resulted in very thin body shape and ~90% death rate after adult emergence, but knockdown directed against the specific region of NlugCPAP3-C5a showed no obvious phenotype (see more information in SI Appendix, section 3).

Fig. 3. Lethal phenotypes of BPHs injected with dsRNA for 15 essential CP genes at late-instar nymphs. dsRNAs against the CP genes were injected at early stage fifth-instar BPH nymphs (0–12 h). dsGFP was injected as negative control for the nonspecific effects of dsRNA. The control group (female adult BPH injected with dsGFP) used for phenotypes II and IV was the same as used for phenotype III in the figure.
These results indicate that the functions of CPs during nymphal stages through the adult stage were relatively consistent. However, the functions of CPs at egg/embryo developmental stage were dramatically different from that of nymphal or adult stages. All above RNAi phenotypes for the total 32 indispensable CP genes in BPH are summarized in Table 1.

**CP Functions Revealed by Transmission Electron Microscopy.** Because of the diversity of BPH lethal phenotypes upon knockdown of the 17 indispensable CP genes (NlugCpr6, NlugCpr47, NlugCpr56, NlugCpr61, NlugCpr62, NlugCpr64, NlugCpr69, NlugCpr73, NlugCpr83, NlugCPLCP2, NlugCPAP1-E, -H, -K, -N, NlugCPAP3-C5b, -D1, and NlugCPAp7) for normal nymph/adult development, we were interested in their integument ultrastructure defects. By transmission electron microscopy (TEM) observation, we found that BPHs treated with dsGFP displayed a normal structure of integument. However, BPHs treated with dsRNA for each of the above 17 CP genes had distinctly different integument structure compared with the control group (Fig. 4).

In BPHs that died with a thin body shape after adult emergence after knockdown of 14 different CP genes (NlugCpr6, NlugCpr56, NlugCpr61, NlugCpr62, NlugCpr64, NlugCpr69, NlugCpr73, NlugCpr83, NlugCPLCP2, NlugCPAP1-E, -H, -K, NlugCPAP3-C5b, and -D1), the endocuticles were significantly thinner and disordered compared with that of dsGFP. The well-ordered lamellar structure of the endocuticle almost did not exist in the treated BPHs, suggesting that these 14 CPs might be involved in the formation of the endocuticle. Note that in the BPHs treated with dsNlugCpr56, dsNlugCpr62, dsNlugCpr64, dsNlugCpr69, dsNlugCpr73, dsNlugCpr83, dsNlugCPLCP2, and dsNlugCPAP1-K, the exocuticle was also thinner than in dsGFP-treated BPHs, suggesting that these eight proteins might also be involved in the formation of BPH exocuticle.

In the BPHs that died before or during ecdisis after RNAi treatment (phenotypes I, II, and III), the malformation of the integument structure was different. After dsNlugCPAp1-N treatment, the well-ordered endocuticle structure almost disappeared. After dsNlugCPApn7 treatment, the exocuticle became thinner, and the lamellae of the endocuticle became curved and disordered. Following dsNlugCpr47 treatment, the procuticle structure seemed to be loose and became thicker than the control group (Fig. 4). TEM observation clearly indicated that these CPs are indispensable for the formation and maintenance of structure stability in the BPH procuticle.

**Complementary Functions of BPH CPs Revealed by Combined RNAi Experiments.** The large number of proteins in the BPH CPR family was intriguing. Apart from the nine CPR genes that provoke lethal phenotypes after RNAi, we also carried out combined RNAi experiments on the remaining of the CPR genes. Fifteen groups were divided according to their homology in the phylogenetic tree (SI Appendix, Fig. S1). The grouped dsRNAs were injected into fifth-instar nymphs (0–12 h). Surprisingly, no high lethal rate was observed 9 d after injection. Only BPHs treated with dsRNAs for Group13 had obvious wing malformations (SI Appendix, Fig. S2). This result suggests that CPR family proteins might have compensatory effects on each other.

**BPH CP Genes Are Highly Tissue-Specific and Have Four Distinct Developmental Expression Patterns.** We estimated the expression level of each transcript using FPKM (fragments per kilobase of exons per million fragments mapped) values (Dataset S5) and found that BPH CP genes had highly tissue-specific expression patterns (FPKM). A total of 126 genes (90.0%) were highly expressed in the integument (Fig. 5A, branch d). The remaining 14 genes (10.0%) had their highest expression level in other tissues: 7 in the ovaries, 4 in the testis, and 3 in the gut (Fig. 5A, branches a, b, and c). Intriguingly, knockdown of four genes (NlugCpr3, NlugCpr8, NlugCpr10, and NlugCpr90) highly expressed in the ovaries significantly decreased egg hatchability.

To study the developmental expression patterns of *N. lugens* CP genes, we selected four time periods (e.g., fourth-instar, fifth-instar, and adult stages) covering three developmental stages (egg, nymph, and adult). Analysis of the FPKM transcript data among the four time periods revealed four distinct expression patterns from four clusters of CP genes (clusters I–IV) (Fig. 5B and SI Appendix, Fig. S3). The results showed that transcripts of most BPH CP genes were present in all three developmental stages.

Cluster I genes were highly expressed in early to midstage embryos. Only eight genes (5.7%) belong to cluster I. Among these eight genes, six of them (NlugCpr1, NlugCpr2, NlugCpr3, NlugCpr8, NlugCpr5, and NlugCpr55) came from the same branch in Fig. 5A (branch a). Cluster III contains 98 genes and is thereby the largest cluster (70.0%). Among the genes that caused lethal phenotypes after RNAi experiments, most of them belong to this cluster, including NlugCpr56, NlugCpr61, NlugCpr62, NlugCpr64, NlugCPAP1-E, -H, etc. The expression pattern of cluster III genes had two features. First, they had their expression levels peaking near the end of each nymph stage and reached the minimum near the middle of each nymph stage. Secondly, genes from cluster III were highly expressed at the end of embryogenesis, indicating that these genes were also important in late embryonic integument formation. Their gene expression levels during adult stage remained low.

The expression peak of cluster II genes occurred earlier than the peak of cluster III genes. Ten genes in cluster II (7.1%) reached their highest expression level at early to midstage of each nymphal stage. Expression of cluster IV genes peaked later than cluster III just at ecdisis. A total of 24 genes (17.1%) belong to
N. lugens was also dramatically increased. Tissue specificity and developmental expression patterns of all genes did not affect BPH. TcCPR4 resulted in an amorphous material in the lumen of pore canals instead of fibers in the elytral cuticle, which is massive. Further studies are needed to elucidate whether the knockdown of NlugCpr22 and other CP genes which did not lead to distinguishable phenotypes or high death rate in BPHs would lead to minor changes in ultrastructure and weaken their acceptability to environmental hazards.

The expression patterns of 140 genes from eight N. lugens CP families underscored the complicated relationship between timing and the type of cuticle being formed. In insects, if a transcript does not appear until after ecdysis, the respective protein will be used in forming the endocuticle. Conversely, if a transcript appears before ecdysis, the corresponding protein probably will be used in forming the exocuticle. Based on this definition, cluster III proteins might be involved in forming the exocuticle, while cluster II and IV proteins might be involved in forming the endocuticle (4). This simple view contradicts our findings that many genes from cluster III (such as NlugCpr56, NlugCpr61, NlugCpr64, NlugCpr69, NlugCpr73, and NlugCpr83) (all from the RR-2 subgroup) led to thin endocuticles and thin exocuticles. Combined with their respective mRNA expression patterns, we could deduce that they may contribute to the structure of both exocuticle and endocuticle. However, EM immunodetection experiments are needed to study the precise localization and function of the individual proteins (14, 15).

This comprehensive CP-ome study unifying genomic, transcriptomic, and proteomic aspects in N. lugens, together with analyses of gene function and expression profiles, contributes...
substantially to the insect cuticle research field. These findings may furthermore stimulate the design and development of insecticides specifically targeting cuticle proteins.

Materials and Methods
Insects. BPHs used in this study were originally obtained from Hangzhou (30°16’N, 120°11’E), China, in 2008. The insects were reared on fresh rice seedlings (strain: Xiushui 134) in a walk-in chamber at 26 ± 0.5 °C and 50 ± 5% relative humidity under a photoperiod of 16:8 h (light:dark).

Gene Identification. Insect CP amino acids and gene sequences were obtained from NCBI (https://www.ncbi.nlm.nih.gov/), EnsemblMetazoa (metazoa.ensembl.org), CutProtFam (alias:biol.uoa.gr/CutProtFam-Pred/home.php) (16), and CuticleDB (bioinformatics2.biolo.uoa.gr/cuticleDB) (17). The common motifs of CPs from D. melanogaster, Bombyx mori, A. gambiae, Tribolium castaneum, Apis mellifera, and Locusta migratoria were used as queries against the BPH genome (https://www.ncbi.nlm.nih.gov/), BioProject PRJNA177647; 27,571 coding protein sequences) and transcriptomic databases (https://www.ncbi.nlm.nih.gov/gov/sra, accession no. SRR023419; 21,908 coding protein sequences). The full-length cDNA sequences were obtained from transcriptomic databases, and most of them were confirmed by RT-PCR. The full cDNA sequences of some CP genes were cloned by using RACE core sets (catalog nos. 6107 and 6106; TaKaRa) according to the manufacturer’s instructions. The primers used are shown in Dataset S1.

Sequence Analysis. The ORF prediction was performed on the Softberry website. The SMART program (smart.embl-heidelberg.de) was used for the identification of modular domains. The signal peptide prediction program SignalP 4.1 server (www.cbs.dtu.dk/services/SignalP) was used to predict N-terminal signal peptides. The multiple sequence alignment of the CPLEP family proteins was carried out by using ClustalX software (18). The phylogenetic trees were constructed via the neighbor-joining method by using the MEGA6.06 program (19). Homologous relationships were determined by boot-strap analysis based on 1,000 or 5,000 replications, as each legend describes.

Cuticle Protein Sample Preparation for UPLC-MS/MS. Four different BPH cuticle tissue samples were prepared for UPLC-MS/MS analysis. Cast first-instar nymph cuticles (CC1) were collected from ~12,000 first-instar nymphs. Cast third- to fifth-instar nymph cuticles (CC3–5) were collected from ~700 fifth-instar BPHs, 200 fourth-instar BPHs, and 200 third-instar BPHs. The BPHs were maintained in a polycarbonate box of 20 cm in length, 14 cm in width, and 9 cm in height with fresh rice seedlings. The cast cuticles were collected with forceps, and the cDNA sequences of some CP genes were cloned by using RACE core sets (catalog nos. 6107 and 6106; TaKaRa) according to the manufacturer’s instructions. The primers used are shown in Dataset S1.

RNAi Experiments. There were two regions of each gene selected for dsRNA synthesis to overcome possible off-target effects. The regions were designed to have no other similar sequences in the genome (SI Appendix, section 3). The purified DNA template was used to produce dsRNAs, and the primers were designed to contain the T7 RNA polymerase promoter at both ends (Dataset S1). The dsRNA was then synthesized through RT-PCR amplification by using the MEGA script T7 High Yield Transcription Kit (catalog no. AM1334; Ambion), according to the manufacturer’s instructions. The concentration and quality of dsRNAs were measured with NanoDrop 2000 (Thermo Fisher Scientific), and the size of the dsRNAs was verified via electrophoresis in a 1% agarose gel. The quantities of dsRNA injected into second- and fifth-instar nymphs and adults were ~25, 200, and 250 ng, respectively.

Female adult BPHs treated with dsRNAs were crossed to healthy male BPHs for 3 d and then transferred onto fresh rice seedlings in glass tubes. One female together with two males were in one tube to produce offspring for 3 d. Subsequently, the females were removed, and the eggs/seedlings were maintained for 10 d for counting the number of hatched offspring. The leaves and stems of the rice seedlings were dissected under the microscope to count the number of eggs failing to hatch. Each target gene was carried out for 10 biological replicates. For the method of microinjection with dsRNA, see SI Appendix, section 5.

TEM Observation. The dorsal abdominal integument of BPH for examining the ultrastructure was separated carefully under the microscope, and the integument between the fourth and eighth segments was dissected for sample preparation and final observation. The time for insect collecting was described in the previous sections. The sample preparation was performed as reported (20), and specimen sections were observed on a Hitachi Model H-7650 TEM.

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