Transcriptome Comparative Profiling of Barley eibi1 Mutant Reveals Pleiotropic Effects of HvABCG31 Gene on Cuticle Biogenesis and Stress Responsive Pathways

Zujun Yang 1*, Tao Zhang 1, Tao Lang 1, Guangrong Li 1, Guoxiong Chen 2 and Eviatar Nevo 3

1 School of Life Science and Technology, University of Electronic Science and Technology of China, Chengdu 610054, Sichuan, China; E-Mails: zhangtao@uestc.edu.cn (T.Z.); langtao123xxx@126.com (T.L.); ligr28@uestc.edu.cn (G.L.)
2 Extreme Stress Resistance and Biotechnology Laboratory, Cold and Arid Regions Environmental and Engineering Institute, Chinese Academy of Sciences, Lanzhou 730000, Gansu, China; E-Mail: guoxiong@hotmail.com
3 Institute of Evolution, University of Haifa, Mount Carmel, Haifa 31905, Israel; E-Mail: nevo@research.haifa.ac.il

* Author to whom correspondence should be addressed; E-Mail: yangzujun@uestc.edu.cn; Tel.: +86-28-8320-6556; Fax: +86-28-8320-1018.

Received: 2 September 2013; in revised form: 26 September 2013 / Accepted: 26 September 2013 / Published: 14 October 2013

Abstract: Wild barley eibi1 mutant with HvABCG31 gene mutation has low capacity to retain leaf water, a phenotype associated with reduced cutin deposition and a thin cuticle. To better understand how such a mutant plant survives, we performed a genome-wide gene expression analysis. The leaf transcriptomes between the near-isogenic lines eibi1 and the wild type were compared using the 22-k Barley1 Affymetrix microarray. We found that the pleiotropic effect of the single gene HvABCG31 mutation was linked to the co-regulation of metabolic processes and stress-related system. The cuticle development involved cytochrome P450 family members and fatty acid metabolism pathways were significantly up-regulated by the HvABCG31 mutation, which might be anticipated to reduce the levels of cutin monomers or wax and display conspicuous cuticle defects. The candidate genes for responses to stress were induced by eibi1 mutant through activating the jasmonate pathway. The down-regulation of co-expressed enzyme genes responsible for DNA methylation and histone deacetylation also suggested that HvABCG31 mutation may affect the epigenetic regulation for barley development. Comparison of transcriptomic profiling of barley under biotic and abiotic stresses revealed that the functions of HvABCG31 gene to high-water
loss rate might be different from other osmotic stresses of gene mutations in barley. The transcriptional profiling of the \( \text{HvABCG31} \) mutation provided candidate genes for further investigation of the physiological and developmental changes caused by the mutant.

**Keywords:** Affymetrix GeneChip; barley; \( eibi1/\text{HvABCG31} \) mutant; transcriptome analysis

### 1. Introduction

The drought-hypersensitive mutant \( eibi1 \) was obtained from a wild barley (\( \textit{Hordeum spontaneum} \) Koch) accession in Israel [1]. The excessive water loss of the \( eibi1 \) mutant plant was related to a recessive mutation localized in a pericentromeric region of chromosome 3H [2]. Studies also revealed severe effects on plant morphology, in particular, the reduced leaf cuticle development, which was associated to the high-water loss rate. Recently, a candidate gene for \( eibi1 \), based on high resolution genetic mapping was also reported [3]. A mutation on \( \text{HvABCG31} \), an ATP-binding cassette (ABC) subfamily G (ABCG) full transporter, was associated with the \( eibi1 \) phenotype [4].

The map-based cloning offers a promising relationship between candidate genes and a corresponding phenotypic trait [5]. However, the difficulty of barley transformation could not easily allow the functional analysis of the candidate gene with respect to its phenotypes. Recently, the rapid development of genomics based on high-throughput sequencing technologies, has facilitated the establishment of the function of target genes [6]. Availability of microarray platforms representing a large proportion of barley genes has enabled the application of transcriptomic analysis to several known mutations in barley including biotic and abiotic stress-related genes [7–11].

To better understand how \( eibi1/\text{HvABCG31} \) mutant displayed the defective physiological and growth phenotypes, we performed a genome-wide gene expression analysis by using Affymetrix Barley1 GeneChip microarray. We found that apparent compensatory transcriptional responses in the mutant involved metabolic processes and stress-related pathways. The comparative analysis of \( eibi1 \) to other barley transcriptome components under various stress response signals was also revealed.

### 2. Results and Discussion

#### 2.1. Differential Transcriptomes of \( eibi1 \) Compared with the Wild Type

To investigate the \( eibi1 \) effects on downstream barley genes, we performed a microarray analysis using Affymetrix barley genome array chips. Out of 22,810 contigs on the chip, 488 (2.2%) contigs were up-regulated more than 2.0-fold, and 717 (3.7%) contigs were down-regulated to less than 0.5-fold in \( eibi1 \) compared to the wild type (Figure 1).

The selected contigs from the Affymetrix genome array chip results involved in biological processes, cellular components, and molecular functions were analyzed by a GO term enrichment tool (Figure 1). Since several different contigs represent a single gene, a total of 164 genes were differentially (both up and down-regulated) expressed in \( eibi1 \) compared with the wild type, involved in secondary metabolism, including cell skeleton, primary metabolism, signal transduction, cell growth and cell division, and defense responses. The secondary metabolism biosynthesis genes, some transcription factors and genes
belonging to different functional categories were down-regulated in eibi1 compared to the wild type. The genes involved in defense to stresses and lipid biosynthesis were up-regulated in eibi1 compared to the wild type.

**Figure 1.** Differentially expressed genes in eibi1 according to gene ontology. Stars indicate the up-regulated genes by eibi1, while others are down-regulated by eibi1.

Moreover, we also observed that a total of eight contigs involved in transport, including sugar transporters (Contig6706_at, HY05O16u_s_at) and iron transporters (HV_CEa0013E09r2_at, Contig6152_at) were up-regulated significantly in eibi1 compared to wild type. Similarly, six genes involved in transport, including two ABC transporters (Contig6016_s_at, Contig5296_at) and CorA-like Mg\(^{2+}\) transporters (Contig10637_s_at, Contig10636_at), were down-regulated in eibi1 compared to the wild type. The eibi1 mutant was caused by ABC transporter HvABCG31 gene mutation. The transcriptome profiling indicated that the HvABCG31 was not differentially expressed in leaves. Therefore, effect of eibi1 mutant on downstream genes was associated the defect of leaf phenotype.

### 2.2. Stress and Fatty Acid Metabolism Related Pathways Were Up-Regulated

As shown in Table 1, we found that four contigs (Contig1579_s_at, Contig1570_s_at, Contig1582_x_at, Contig1580_x_at) were up-regulated by 4–39-fold, as were all encoded Thionins, which are low-molecular-weight proteins (Mr ca. 5 kD) occurring in seeds, stems, roots, and leaves of a number of plant species. The different members of this plant protein family show both sequence and structural homology, and are toxic to bacteria, fungi, yeasts, and various naked cells *in vitro* [12].
Table 1. The up-regulated stress related marker/gene in *eibi1*.

| (Putative) gene function | Contig/gene designation | Regulation in *eibi1*/WT |
|--------------------------|-------------------------|-------------------------|
| Thionin                  | Contig1579_s_at         | 39.5                    |
|                          | Contig1570_s_at         | 18.5                    |
|                          | Contig1582_x_at         | 6.96                    |
|                          | Contig1580_x_at         | 4.07                    |
| Horcolin                 | Contig6157_s_at         | 9.42                    |
| Pathogen-related protein pir | Contig5607_s_at     | 4.87                    |
| Methyl jasmonate-inducible lipoxygenase 2 | Contig2305_at | 2.059                   |
| Lipoxygenase             | Contig23795_at          | 3.729                   |
| Metallophosphatase       | Contig2289_at           | 2.409                   |
| Carbonate dehydratase    | Contig897_s_at          | 2.363                   |
| Chitinase                | Contig2990_at           | 5.87                    |
| β-amylase                | Contig1411_s_at         | 5.03                    |
| RNase S-like protein     | contig5059_s_at         | 2.212                   |
| Lectin                   | contig11641_s_at        | 2.099                   |
| subtilisin-like protease | Contig13847_s_at        | 3.188                   |
| α-L-arabinofuranosidase/β-D-xylosidase | Contig7032_s_at | 2.842                   |
| endo-1,3-β-glucanase     | HVSMEm0003C15r2_s_at    | 2.609                   |
| Glutathione synthetase   | Contig14516_at          | 2.379                   |
| CYP450                   | Contig15_s_at           | 6.113                   |
|                          | Contig 11818_at         | 5.409                   |
|                          | Contig 25477_at         | 3.52                    |
|                          | Contig 3160_at          | 3.235                   |
|                          | Contig 13847_at         | 3.191                   |
|                          | Contig 14804_at         | 2.308                   |
|                          | Contig 7194_at          | 2.292                   |
|                          | Contig 14663_at         | 2.111                   |
|                          | ContigCEb0006F_at       | 2.018                   |
| Fatty acid               | Contig23795_at          | 3.729                   |
|                          | Contig5664_at           | 2.535                   |
|                          | HVSMEn0002B08r2_at      | 2.164                   |
|                          | Contig2305_at           | 2.059                   |

Differential gene regulation in 2nd leaves of *eibi1* compared to WT based on microarray analysis. Genes up-regulated at >2-fold.

The Contig 6157_s_at, representing Horcolin (*Hordeum vulgare* coleoptile lectin), was up-regulated by 9.402-fold in *eibi1*. Database searches performed with the Horcolin protein sequence revealed its structure homology to the lectin family of jacin-related lectins (JRL). As a new member of the mannose-specific subgroup of jacin-related lectins in monocot species [13], overexpression of a wheat jasmonate-regulated lectin increases pathogen resistance, and the group of inducible lectins appears to function within the context of biotic/abiotic stress signaling in monocots and dicots [14,15]. Defense-related genes were up-regulated in the *eibi1* leaves including ribosome inactivating proteins, chitinases (Contig2990_at), protease inhibitors, amylases (Contig1411_s_at) and glucanases (HVSMEm0003C15r2_s_at). The function of the chitinases and b-glucanases may degrade the major structural component of the cell walls of many fungi [16].
The contigs for the fatty acid metabolism pathway were up-regulated, such as Contig5664 encoding very-long-chain fatty acid condensing enzyme, and HVSMEn0002B08r2_at encoding fatty acid elongase were up-regulated in eibi1 compared to the wild type. The enzymes called lipoxygenases (LOXs) (Contig 12574_at, Contig23795_at, Contig2305_at) can dioxygenate unsaturated fatty acids, which leads to lipoperoxidation of biological membranes. LOXs are known to be involved in the apoptotic (programmed cell death) pathway, and biotic and abiotic stress responses in plants [17]. GDSL-motif lipase/hydrolase family protein was reported to have protein localization and gene expression patterns that correlated with cutin biosynthesis [18] and the abundance of transcripts for GDSL-motif lipase/hydrolase, thought to contribute to cuticle reorganization and increased permeability [19]. The Contig15_s_at representing GDSL-motif lipase/hydrolase was significantly up-regulated 6-fold in eibi1 compared to the wild type.

Since the common activation of the stress-related genes of Thinion, Horcolin, and other lipid synthesis enzymes, their transcription was shown to be methyl jasmonate (MeJA)-inducible in leaves [20]. A total of eight out of 33 contigs involving methyl jasmonate-inducible protein were up-regulated; none of the contigs involved in Methyl jasmonate-inducible protein were down-regulated in the present differential analysis. The result suggested that eibi1 mutation can possibly activate the MeJA-inducible pathway in the response to stresses. Moreover, it is interesting to note that Horcolin was only expressed in barley coleoptiles [21]; however, it is highly expressed in leaves of eibi1. Considering the eibi1 candidate gene, ABC transporter was also confined to the coleoptiles, we hypothesized that the mutation of eibi1 may clearly affect the expression of horcolin, which may mediate related pathways for the response to stresses. We also found that by microarray gene expression profiling, eibi1 was similar to the reports of mutants lcr, fdh, and bdg, indicating that a number of up-regulated defense-related genes had accumulated [22]. The overall activation of a number of stress responsive genes in eibi1 may be associated with high resistance to barley fungi including rust and powdery mildew (data not shown), although eibi1 plant appeared thin leaves.

Cytochrome P450 monooxygenases (CYP450) catalyze substrate-, region- and stereo-specific oxygenation steps in plant metabolism. Co-expression results for CYP450 related to plastidial functions/photosynthesis, and to phenylpropanoid, triterpenoid, and jasmonate metabolism [23]. In the present study, we found that eight contigs (Contig11818_at, Contig25477_at, Contig3160_at, Contig13847_at, Contig14804_at, Contig7194_at, Contig14663_at, and ContigCEb0006F_at) were up-regulated significantly in eibi1 compared to wild type. None of the contigs homologous to CYP450 were down-regulated in eibi1 compared to WT. Contig25477_at was up-regulated by 3.52-fold in eibi1, and it was homologous to At4g39490, a paralog of At1G57750 (AtMAH1) protein [24], which was involved in the cutin monomer synthesis [25]. It might be interpreted that cuticular mutant eibi1 would up-regulate the CYP450 related pathway to reduce levels of cutin monomers or wax and display conspicuous cuticle defects. Notably, the CYP450 family can be a candidate to investigate the mechanism of cutin synthetic pathway in eibi1.

2.3. The Metabolic Pathways Were Down-Regulated

We found that all the Contigs (Contig2670_x_at; Contig2670_s_at and Contig2672_at) encoding xyloglucan transglycosylases (XETs) were clearly down-regulated 3-fold in eibi1 (Table 2). The XETs
have been implicated in many aspects of cell wall biosynthesis [26]. The Contig5258_at encoding Endoxyloglucan transferase (EXT) was down-regulated 2.5-fold in eibi1. Xyloglucans are the principal components of the matrix polymers and bind tightly to the surface of cellulose microfibrils and, thereby, cross-link them to form an interwoven xyloglucan-cellulose network structure [27]. EXT is a newly identified class of transferase that catalyzes molecular grafting between xyloglucan molecules, thereby mediating molecular grafting between xyloglucan cross-links in plant cell walls [28]. The down-regulated of XETs and EXT genes in eibi1 may possibly affect the cell shapes and resulted in thin leaves.

### Table 2. Differentially down-regulated genes in eibi1.

| (Putative) gene function | Contig/gene designation | Regulation in eibi1/WT |
|---------------------------|--------------------------|------------------------|
| ribophorin I              | Contig4748_s_at          | 0.499                  |
| sucrose synthase 1        | Contig689_s_at           | 0.499                  |
| dTDP-glucose 4-6-dehydratase-like protein | Contig2918_s_at         | 0.48                   |
| UDP-glucuronic acid decarboxylase | Contig2915_at           | 0.469                  |
| UDP-glucuronic acid decarboxylase | Contig2031_s_at         | 0.206                  |
| ribosomal protein S8      | Contig1024_at            | 0.467                  |
| 60S                       | Contig2369_s_at          | 0.43                   |
| ribosomal protein L24     | HI02E24u_s_at            | 0.331                  |
| rpS28                     | Contig3403_s_at          | 0.321                  |
| ribosomal protein L17.1, cytosolic | rbagsi23_s_at         | 0.302                  |
| Ribosomal protein S7      | Contig1668_at            | 0.273                  |
| peroxidase (EC 1.11.1.7), pathogen-induced | Contig2118_at          | 0.325                  |
| HSP80-2                   | Contig1204_s_at          | 0.433                  |
| Endoxyloglucan transferase (EXT) | Contig5258_at          | 0.423                  |
| Serine carboxypeptidase III precursor (CP-MIII) | Contig682_s_at          | 0.413                  |
| glutathione peroxidase-like protein | Contig2453_at          | 0.406                  |
| Pyrophosphate phospho-hydrolase (PPase) | Contig2018_at          | 0.389                  |
| phenylalanine ammonia-lyase | Contig1805_s_at         | 0.496                  |
| phenylalanine ammonia-lyase (EC 4.3.1.5) | Contig1803_at          | 0.37                   |
| phenylalanine ammonia-lyase (EC 4.3.1.5) | HVSMEm0015M15r2_s_at    | 0.366                  |
| phenylalanine ammonia-lyase (EC 4.3.1.5) | Contig1800_at          | 0.353                  |
| Glyceraldehyde 3-phosphate dehydrogenase | Contig149_at          | 0.361                  |
| immunophilin               | HVSMEx0002K18r2_s_at     | 0.358                  |
| ascorbate peroxidase       | Contig1727_s_at          | 0.242                  |
| plasma membrane proton ATPase | Contig2_s_at          | 0.296                  |
| xyloglucan endotransglycosylase (XET) | Contig2670_x_at         | 0.3                    |
| xyloglucan endo-1,4-β-D-glucanase(XET) | Contig2672_at          | 0.335                  |
| xyloglucan endo-1,4-β-D-glucanase | Contig2670_s_at         | 0.358                  |
| promoter-binding factor-like protein | Contig10705_at         | 0.443                  |
| Actin                     | Contig1390_M_at          | 0.187                  |
| serine carboxypeptidase III | Contig682_s_at          | 0.413                  |
| glutathione peroxidase-like protein GPX54Hv | Contig2453_at         | 0.406                  |

Differential gene regulation in second leaves of eibi1 compared to WT based on microarray analysis. Genes were down-regulated at >2-fold.
The UDP-xylose is an important sugar donor in the synthesis of hemicellulose and glycoproteins [29]. Arabinoxylans in crop plants such as rice, maize, wheat, and barley are major components of the cell wall of the starchy endosperm, as well as the aleurone layer [30]. UDP-D-glucuronic acid decarboxylase (UXS) (Contig2915_at 0.409, Contig2031_at 0.206) catalyzed the conversion of UDP-D-glucuronic acid to UDP-D-xylose. UDP-xylose is not only used as a substrate for xyloglucan biosynthesis, but also as a substrate of β-(1,4)-xylosyltransferase that catalyses the synthesis of the xylan backbone [31]. The down-regulated of these genes may be associated with the low plant height and grain traits of *eibi1* compared with the wild type.

Actin, which is vital for pectin synthesis and for cytoskeleton [32], is significantly down-regulated (Contig1390_M_at) 7-fold in the *eibi1* mutant. Phenylalanine ammonia-lyase (PAL) (Contig1805_s_at, Contig1803_at, Contig1800_at, HVSMEm0015M15r2_s_at) was down-regulated (Table 2). It catalyzes the first reaction in the general phenylpropanoid pathway leading to the production of phenolic compounds with a significant range of biological functions. The heat shock protein members HSP80 and HSP90 were down-regulated 2.1–2.3-fold in the *eibi1* mutant. In the case of inactivation of heat-shock protein synthesis, it is well recognized the damaged/denatured proteins signals in *eibi1*.

The transcriptome profiling of molecular pathways in *eibi1* were down-regulated, in particular, the genes involved in cell wall modification. The expression of the related gene network underlying cell wall biosynthesis will deteriorate the cuticle development, particularly as is evident in the thinner leaves and shorter plants of *eibi1* than that of wild type.

### 2.4. The Epigenetic Related Pathways

Epigenetic phenomena have been associated with the regulation of active and silent chromatin states achieved by modifications of chromatin structure through DNA methylation and histone post-translational modifications [32].

As shown in Table 3, the micro-molecular proteins involved in methionine synthase (MSY) (Contig1424_at) were down-regulated 4-fold in *eibi1*. All three enzymes, MSY, SAM1 (*S*-adenosyl-L-methionine synthetase), and SAH (*S*-adenosyl-L-homocysteine hydrolase) were involved in the methyl cycle [33]. The co-expressed enzyme genes are likely responsible for DNA methylation involved in determining plastid division and amyloplast differentiation as included in seeds development of barley [34].

| (Putative) gene function | Contig/gene designation | Regulation in *eibi1*/WT |
|--------------------------|-------------------------|-------------------------|
| Methionine adenosyltransferase 1 | Contig1269_at | 0.456 |
| Methionine adenosyltransferase 1 | Contig1271_x_at | 0.427 |
| Methionine adenosyltransferase 1 | Contig1269_s_at | 0.327 |
| S-adenosyl-L-homocysteine hydrolase | Contig1791_x_at | 0.477 |
| S-adenosyl-L-homocysteine hydrolase | Contig1794_s_at | 0.271 |
| Methionine synthase protein | Contig1424_at | 0.244 |
| putative histone deacetylase HD2 | Contig1625_at | 0.348 |

Differential gene regulation in second leaves of *eibi1* compared to WT based on microarray analysis. Genes were down-regulated at >2-fold.
Expression of \( S \)-Adenosylmethionine Synthetase gene (SAM1) appeared to be 2–3-fold down-regulated (Contig1269_at, Contig1269_s_at and Contig1271) in \textit{eibi1} compared to WT (Table 3). SAM catalyzes the biosynthesis of \( S \)-adenosyl-L-methionine (AdoMet), a universal methyl-group donor. In \textit{Arabidopsis} and rice, the SAM gene is expressed primarily in the vascular tissue and is also preferentially accumulated in lignified tissues [35]. Moreover, we found that SAH (Contig1791_x_at and Contig1794_at) were clearly down-regulated by 2–5-fold in \textit{eibi1} compared to WT (Table 3). The \( S \)-adenosyl-L-homocysteine metabolic pools were highly regulated on cytosine methylation [36], and down-regulation of SAH reveals the role of cytokinin in promoting transmethylation reactions [37].

Histone deacetylases (HDAC) are important in plant gene expression (Contig1625_at). HDAC function has been best studied in \textit{Arabidopsis}. Inactivation of \textit{Arabidopsis} HD1 (AtHD1/HDA19), mutant (athd1-t1) is induced by pleiotropic developmental abnormalities [38,39]. Down-regulation of HDAC reduced rice peduncle elongation and fertility, altered plant height and flag leaf morphology, leading to the production of narrowed leaves and stems [40].

The co-expression of the contigs involved in the methylation and histon modification in \textit{eibi1} and WT infer that the \textit{eibi1} may affect the epigenetic pathways. The relationship of phenotypical changes between \textit{eibi1} and the WT with respect to the epigenetic modification need to be further investigated.

2.5. Validation of Microarray Data by qRT-PCR

To assess the accuracy of microarray data, we selected 10 differential expressed contigs including stress responsive genes, secondary metabolism biosynthesis genes and epigenetic modifying genes as shown in Table 4. The expression profiles of the genes that show up or down-regulated up-regulation between \textit{eibi1} and WT identified by microarray. We tested the similarity between gene expression identified by microarray and those by qRT-PCR, and observed that microarray and qRT-PCR data, which were calculated based on the median of three repeats, showed good correlation at different water stress treatments and overall water stress conditions (\( r = 0.902–0.960 \)). Hence, the results of the differential expressed genes identified through microarrays confirm actual differences between \textit{eibi1} and WT genotypes.

| Contig       | Predict functional gene | EST accession | Primers                                      |
|--------------|-------------------------|---------------|----------------------------------------------|
| Contig1579_s_at | Thionin                | AK250720      | TGAATCTTCTCCCTGAATCCGCAAATAGATTCATCGTGCGGGA |
| Contig6157_s_at | horcolin                | AY033628      | CTACGTGACCGAAATCTCCGGCCATGTAAGGGCCTTCACAAA  |
| Contig2990_at  | chitinase               | X78671        | CAACACCTTCCCGGGCTTGCGTCATCCAGAAACCACATC     |
| Contig 25477_at | CYP450                  | AK252707      | CTCTCCACAACGGACCTGACTAGCACCTGACATCAAGGTTC    |
| Contig23795_at | lipoxygenase            | AJ507213      | TGATCCATCTGAAGCAGCAGCTTTGACGGTGAAAGAGGCAG   |
| Contig23796_at | Fatty acid              | AJ507214      | CGCAGGCAGCAATGGTGCGACCGGCAGGTTGACTT         |
Table 4. Cont.

| Contig         | Predict functional gene                  | EST accession | Primers                                      |
|----------------|------------------------------------------|---------------|----------------------------------------------|
| Contig2031_s_at| UDP-glucuronic acid decarboxylase         | AY677177      | CCATACGTGGTGAAGCCCTGGACCAACAGTCCGCAA         |
|                |                                          |               | CATCTTCACTGAAACCCTGAAACGAAAGTGACAGACCGA     |
| Contig1269_s_at| Methionine adenosyltransferase 1          | AK249878      | ACATGACCATCCAGACCGCTGGCCCTCATGAGACCAT        |
| Contig1794_s_at| S-adenosyl-L-homocysteine hydrolase      | AM039898      | ATGAATTGGCCGGCTTCTATCCCTCAAGCAGCGG          |
| Contig1424_at  | methionine synthase protein              | AM039905      | ATGAATTGGCCGGCTTCTATCCCTCAAGCAGCGG          |

2.6. Transcriptomic Comparison of eibi1 with Other Barleys under Stresses

Since the whole genome transcript analysis revealed that eibi1 activated some signaling pathways in response to stress factors, we performed a comparative analysis of differentially expressed genes from eibi1 to other barleys’ transcriptome change under various stress treatments on PlexDB database [41–43]. The available Affymetrix Barley1 GeneChip data included barley transcriptome change in response to four abiotic factors including chilling and freezing temperature (BB81, BB95), drought (BB84, BB89), and three biotic factors from powdery mildew resistance genes mlo-5 (BB7), mla-13 (BB4) and rar1 (BB5). The differentially expressed genes from eibi1 down-regulated more than 2-fold as compared to WT were selected for the cluster analysis (Figure 2). The cluster analysis showed that the set of differentially expressed genes from eibi1 was different from the data from all analyzed datasets, and was clustered as an out group.

Figure 2. Hierarchical cluster analysis of differentially expressed genes from eibi1 and barley cultivars transcriptome change under various stress treatments (from data available at PlexDB database).

Based on the cluster analysis, we supposed that the eibi1 mutant might have different reaction mechanisms for the osmotic stress of high water loss rate, which is not similar to the stress-induced expressional changes and other reported mutations for barley biotic and abiotic stresses.
3. Experimental

3.1. Plant Materials and Experimental Design

Germplasm used for this study was derived from *eibi1* mapping population [2]. The F$_3$ populations of 23–19/eibi1 for BSA analysis were chosen to evaluate the water loss rate [3]. We isolated the second leaves of the seedling after 10 days after germination at 22 °C in growth chamber. The detached leaves of *eibi1* plants lost about 50% of their initial weight during 1 h of dehydration while the wild type detached leaves lost only 5% (Figure 3). Half leaves were immediately frozen by liquid nitrogen, and the other half was used to test the water loss rate. Each of the 20 leaves with significant water loss rate representing the genotype of *eibi1* and normal water loss rate for wild type was mixed, respectively, and three replicates of samples were used for RNA extraction and microarray analyses.

**Figure 3.** Phenotypes of leaves of *eibi1* and wild type before or after dehydration.

3.2. Leaf Transcriptomic Microarray Analysis

Total RNAs were extracted from each replicate using the RNeasy Mini Kit (Qiagen). Each sample was hybridized to a barley chip (22-k Barley1 DNA microarray, Affymetrix, Santa Clara, CA, USA). Chip preparation, hybridization and analysis were done at the CaptitalBio Corporation (Beijing, China). cDNA synthesis (first and second strand), biotin-labeled cRNA synthesis, fragmentation, hybridization, washing, staining, and scanning were performed according to the standard manufacturer’s (Affymetrix) recommendations. The microarray data were further analyzed using the GOEAST program [44].

3.3. Quantitative RT-PCR Validation of Eibi1 and Wild Type Gene Expression

Microarray data were further validated using qRT-PCR for a selected number of genes using gene-specific primer sets. Primer pairs (Table 4) were designed using OligoPerfect Designer software [45].
Specificity of the primer sets and their product length was verified by agarose gel electrophoresis. The qRT-PCR reaction mixture was consist of the SYBR Premix EX-Taq™ II Kit (TakaRa, Japan) on iCycler iQ (BIO-RAD) with three biological repeats of cDNA of eibi1 and wild type.

3.4. Comparative Transcriptomic Analysis

The differentially expressed (at least two-fold up or down-regulated) probe sets from our experiment were compared with the expression of the same probe sets in public barley GeneChip experiments from the PlexDB database [43]. Seven transcriptome changes representing barley in response to abiotic and biotic factors were chosen. Bootstrap confidence levels of transcriptome analysis of the barley eibi1 mutant were calculated from 100 iterations using the seqboot programme from the PHYLIP package. A graphical tree representing the comparison was visualized using TreeView [46].

4. Conclusions

The phenotypic observation of leaf profiles suggested that eibi1 appeared significant effects on the cuticle formation compared to the wild type. We hypothesize that the differences in eibi1 leaf cells might not be under the direct control of the HvABCG31 gene, but are probably the result of pleiotropic effects of the mutation. Based on the present whole genome expression analyses on eibi1 leaves compared with wild-type leaves, we found that the pleiotropic effects of eibi1 mutation were primarily activated by CYP450 regulated genes, fatty acid metabolic pathway, and the jasmonate signal transduction pathway, as well as, possibly, epigenetic related pathways to deal with the osmotic stress of high water loss rate in leaves. We conclude that the single gene mutation of eibi1 showed that the unique in vivo dehydration stress leading to the morphological and physiological changes, and the genetic and epigenetic mechanisms responsible for the eibi1 plant defects were different from the reported barley abiotic mutation or treatments.

Acknowledgments

Zujun Yang and Guangrong Li were supported by grant 31171542 and 31101143 from the National Natural Science Foundation of China (NSFC), and Fundamental Research Funds for the Central Universities of China (ZYGX2010J099) for the financial support. Eviatar Nevo thanks the Ancell-Teicher Research Foundation for Genetic and Molecular Evolution for continued financial support.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Chen, G.; Sagi, M.; Song, W.N.; Krugman, T.; Fahima, T.; Korol, A.B.; Nevo, E. Wild barley eibi1 mutation identifies a gene essential for leaf water conservation. *Planta* **2004**, *219*, 684–693.

2. Chen, G.; Komatsuda, T.; Pourkheirandish, M.; Sameri, M.; Sato, K.; Krugman, T.; Fahima, T.; Korol, A.B.; Nevo, E. Mapping of the eibi1 gene responsible for the drought hypersensitive cuticle in wild barley (*Hordeum spontaneum*). *Breed. Sci.* **2009**, *59*, 21–26.
3. Chen, G.; Komatsuda, T.; Pourkheirandish, M.; Sameri, M.; Sato, K.; Krugman, T.; Fahima, T.; Korol, A.B.; Nevo, E. Genetic targeting of candidate genes for drought sensitive gene eibi1 of wild barley (Hordeum spontaneum). Breed. Sci. 2009, 59, 637–644.
4. Chen, G.; Komatsuda, T.; Ma, J.F.; Nawrath, C.; Pourkheirandish, M.; Tagiri, A.; Hu, Y.G.; Sameri, M.; Li, X.; Zhao, X.; et al. An ATP-binding cassette subfamily G full transporter is essential for the retention of leaf water in both wild barley and rice. Proc. Natl. Acad. Sci. USA 2011, 108, 12354–12359.
5. Peters, J.L.; Cnudde, F.; Gerats, T. Forward genetics and map-based cloning approaches. Trends Plant Sci. 2003, 8, 484–491.
6. Alonso, J.M.; Ecker, J.R. Moving forward in reverse, genetic technologies to enable genome-wide phenomic screens in Arabidopsis. Nat. Rev. Genet. 2006, 7, 524–536.
7. Close, T.J.; Wanamaker, S.I.; Caldo, R.A.; Turner, S.M.; Ashlock, D.A.; Dickerson, J.A.; Wing R.A.; Muehlbauer, G.J.; Kleinhofs, A.; Wise, R.P. A new resource for cereal genomics, 22K Barley GeneChip comes of age. Plant Physiol. 2004, 134, 960–968.
8. Mitra, R.M.; Gleason, C.A.; Edwards, A.; Hadfield, J.; Downie, J.A.; Oldroyd, G.E.D.; Long, S.R. A Ca$^{2+}$/calmodulin-dependent protein kinase required for symbiotic nodule development, Gene identification by transcript-based cloning. Proc. Natl. Acad. Sci. USA 2004, 101, 4701–4705.
9. Zhang, L.; Fetch, T.; Nirmala, J.; Schmierer, D.; Brueggeman, R.; Steffenson, B.; Kleinhofs, A. Rpr1, a gene required for Rpg1-dependent resistance to stem rust in barley. Theor. Appl. Genet. 2006, 113, 847–855.
10. Xi, L.; Moscou, M.J.; Meng, Y.; Xu, W.; Caldo, R.A.; Shaver, M.; Nettleton, D.; Wise, R.P. Transcript-based cloning of RRP46, a regulator of rRNA processing and R gene-independent cell death in barley-powdery mildew interactions. Plant Cell 2009, 21, 3280–3295.
11. Zhang, L.; Lavery, L.; Gill, U.; Gill, K.; Steffenson, B.; Yan, G.; Chen, X.; Kleinhofs, A. A cation/proton-exchanging protein is a candidate for the barley NecS1 gene controlling necrosis and enhanced defense response to stem rust. Theor. Appl. Genet. 2009, 118, 385–397.
12. Stec, B. Plant thionins—The structural perspective. Cell. Mol. Life Sci. 2006, 63, 1370–1385.
13. Grunwald, I.; Heinig, I.; Thole, H.H.; Neumann, D.; Kahmann, U.; Kloppstech, K.; Gau, A.E. Purification and characterisation of a jacalin-related, coleoptile specific lectin from Hordeum vulgare. Planta 2007, 226, 225–234.
14. Van Damm, E.J.M.; Barre, A.; Rouge, P.; Peumans, W.J. Potato lectin, an updated model of a unique chimeric plant protein. Plant J. 2004, 37, 34–45.
15. Ma, Q.H.; Tian, B.; Lia, Y.L. Overexpression of a wheat jasmonate-regulated lectin increases pathogen resistance. Biochimie 2010, 92, 187–193.
16. Rushton, P.J.; Somssich, I.E. Transcriptional control of plant genes responsive to pathogens. Curr. Opin. Plant Biol. 1998, 1, 311–315.
17. Umate, P.; Tuteja, N. Genome-wide analysis of lipoxygenase gene family in Arabidopsis and rice. Plant Signal. Behav. 2011, 6, 335–338.
18. Yeats, T.H.; Howe, K.J.; Matas, A.J.; Buda, G.J.; Thannhauser, T.W.; Rose, J.K.C. Mining the surface proteome of tomato (Solanum lycopersicum) fruit for proteins associated with cuticle biogenesis. J. Exp. Bot. 2010, 61, 3759–3771.
19. Williams, C.E.; Nemacheck, J.A.; Shukle, J.T.; Subramanyam, S.; Saltzmann, K.D.; Shukle, R.H. Induced epidermal permeability modulates resistance and susceptibility of wheat seedlings to herbivory by Hessian fly larvae. *J. Exp. Bot.* **2011**, *62*, 4521–4531.

20. Akiyama, T.; Jin, S.; Yoshida, M.; Hoshino, T.; Opassiri, R.; Cairns, J.R.K. Expression of an endo-(1,3;1,4)-β-glucanase in response to wounding, methyl jasmonate, abscisic acid and ethephon in rice seedlings. *J. Plant Physiol.* **2009**, *166*, 1814–1825.

21. Van Damme, E.J.; Zhang, W.; Peumans, W.J. Induction of cytoplasmic mannose-binding jacalin-related lectins is a common phenomenon in cereals treated with jasmonate methyl ester. *Commun. Agric. Appl. Biol. Sci.* **2004**, *69*, 23–31.

22. Voisin, D.; Nawrath, C.; Kurdyukov, S.; Franke, R.B.; Reina-Pinto, J.J.; Efremova, N.; Will, I.; Schreiber, L.; Yephremov, A. Dissection of the complex phenotype in cuticular mutants of *Arabidopsis* reveals a role of SERRATE as a mediator. *PLoS Genet.* **2009**, *5*, e1000703.

23. Eckermann, C.; Eichel, J.; Schroder, J. Plant methionine synthase, new insights into properties and expression. *Biol. Chem.* **2000**, *381*, 695–703.

24. Li, C.; Wang, A.; Ma, X.; Nevo, E.; Chen, G. Consensus maps of cloned plant cuticle genes. *Sci. Cold Arid Reg.* **2010**, *2*, 465–476.

25. Greer, S.; Wen, M.; Bird, D.; Wu, X.; Samuels, L.; Kunst, L.; Jetter, R. The cytochrome P450 enzyme CYP96A15 is the midchain alkane hydroxylase responsible for formation of secondary alcohols and ketones in stem cuticular wax of *Arabidopsis*. *Plant Physiol.* **2007**, *145*, 653–667.

26. Bourquin, V.; Nishikubo, N.; Abem, H.; Brumer, H.; Denman, S.; Eklund, M.; Christiernin, M.; Teeri, T.T.; Sundberg, B.; Mellerowicz, E.J. Xyloglucan Endotransglycosylases have a function during the formation of secondary cell walls of vascular tissues. *Plant Cell* **2002**, *14*, 3073–3088.

27. Nishitani, K. Endo-xyloglucan transferase, a new class of transferase involved in cell wall construction. *J. Plant Res.* **1992**, *108*, 137–148.

28. Tabuchi, A.; Mori, H.; Kamisaka, S.; Hoson, T. A new type of endo-xyloglucan transferase devoted to xyloglucan hydrolysis in the cell wall of azuki bean epicotyls. *Plant Cell Physiol.* **2001**, *42*, 154–161.

29. Suzuk, K.; Suzuki, Y.; Kitamura, S. Cloning and expression of a UDP-glucuronic acid decarboxylase gene in rice. *J. Exp. Bot.* **2004**, *54*, 1979–1979.

30. Izydorczyk, M.S.; Biliaderis, C.G. Cereal arabinoxylans, advances in structure and physicochemical properties. *Carbohydr. Polym.* **1995**, *28*, 33–48.

31. Faik, A.; Price, N.J.; Raikhel, N.V.; Keegstra, K. An Arabidopsis gene encoding an α-xyllosyltransferase involved in xyloglucan biosynthesis. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 7797–7802.

32. Mirouze, M.; Paszkowski, J. Epigenetic contribution to stress adaptation in plants. *Curr. Opin. Plant Biol.* **2011**, *14*, 267–274.

33. Astolfi, S.; Zuchi, S.; Hubberten, H.M.; Pinton, R.; Hoefgen, R. Supply of sulphur to S-deficient young barley seedlings restores their capability to cope with iron shortage. *J. Exp. Bot.* **2010**, *61*, 799–806.

34. Radchuk, V.V.; Sreenivasulu, N.; Radchuk, R.I.; Wobus, U.; Weschke, W. The methylation cycle and its possible functions in barley endosperm development. *Plant Mol. Biol.* **2005**, *59*, 289–307.
35. Sanchez-Aguayo, I.; Rodriguez-Galan, J.M.; Garcia, R.; Torreblanca, J.; Pardo, J.M. Salt stress enhances xylem development and expression of S-adenosyl-L-methionine synthase in lignifying tissues of tomato plants. *Planta* **2004**, *220*, 278–285.

36. Fojtova, M.; Kovar, A.; Votruba, I.; Holy, H. Evaluation of the impact of S-adenosylhomocysteine metabolic pools on cytosine methylation of the tobacco genome. *Eur. J. Biochem.* **1998**, *252*, 347–352.

37. Li, C.H.; Yu, N.; Jiang, S.M.; Shangguan, X.X.; Wang, L.J.; Chen, X.Y. Down-regulation of S-adenosyl-L-homocysteine hydrolase reveals a role of cytokinin in promoting transmethylation reactions. *Planta* **2008**, *228*, 125–136.

38. Tian, L.; Fong, M.P.; Wang, J.J.; Wei, N.E.; Jiang, H.; Doerge, R.W.; Chen, Z.J. Reversible histone acetylation and deacetylation mediate genome-wide, promoter-dependent and locus-specific changes in gene expression during plant development. *Genetics* **2005**, *169*, 337–345.

39. Tian, L.; Chen, Z.J. Blocking histone deacetylation in Arabidopsis induces pleiotropic effects on plant gene regulation and development. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 200–205.

40. Hu, Y.; Qin, F.; Huang, L.; Sun, Q.; Li, C.; Zhao, Y.; Zhou, D.X. Rice histone deacetylase genes display specific expression patterns and developmental functions. *Biochem. Biophys. Res. Commun.* **2009**, *388*, 266–271.

41. Caldo, R.A.; Nettleton, D.; Wise, R.P. Interaction-dependent gene expression in Mla-specified response to barley powdery mildew. *Plant Cell* **2004**, *16*, 2514–2528.

42. Guo, P.; Baum, M.; Grando, S.; Ceccarelli, S.; Bai, G.; Li, R.; von Korff, M.; Varshney, R.K.; Graner, A.; Valkoun, J. Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. *J. Exp. Bot.* **2009**, *60*, 3531–3544.

43. Wise, R.P.; Caldo, R.A.; Hong, L.; Shen, L.; Cannon, E.; Dickerson, J.A. BarleyBase/PLEXdb: A Unified Expression Profiling Database for Plants and Plant Pathogens. In *Plant Bioinformatics: Methods and Protocols*; Edwards, D., Ed.; Humana Press: Totowa, NJ, USA, 2008; pp. 347–363.

44. Zheng, Q.; Wang, X.J. GOEAST: A web-based software toolkit for Gene Ontology enrichment analysis. *Nucleic Acids Res.* **2008**, *36*, W358–W363.

45. *Custom Primers—OligoPerfect Designer*; Invitrogen: Carlsbad, CA, USA, 2006.

46. Page, R.D. *TreeView: An application to display phylogenetic trees on personal computers. Comp. Appl. Biosci.* **1996**, *12*, 357–358.

© 2013 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).