



**ARTICLE**

## Transcriptome Profiling of Barley Cultivar Hua 30 MDEC in Response to *Agrobacterium* Infection

Yingbo Li<sup>1,2,#</sup>, Ting He<sup>1,2,#</sup>, Guimei Guo<sup>1,2</sup>, Hongwei Xu<sup>1,2</sup>, Yingjie Zong<sup>1,2</sup>, Shuwei Zhang<sup>1,2</sup>, Ruiju Lu<sup>1,2</sup>, Longhua Zhou<sup>1,2,\*</sup> and Chenghong Liu<sup>1,2,\*</sup>

<sup>1</sup>Biotech Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai, 201403, China

<sup>2</sup>Shanghai Key Laboratory of Agricultural Genetics and Breeding, Shanghai, 201106, China

\*Corresponding Authors: Chenghong Liu. Email: liuchenghong@saas.sh.cn; Longhua Zhou. Email: zhoulonghua@saas.sh.cn

#These authors contributed equally to this work

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### ABSTRACT

*Agrobacterium*-mediated transformation has been widely used in plants. However, the mechanism in plant cells' response to *Agrobacterium* infection was very complex. The mechanism of the determinants in host cell remains obscure, especially in barley, which is recalcitrant for *Agrobacterium*-mediated transformation. In the present study, microspore-derived embryogenic calli (MDEC) from barley elite cultivar were employed as unique subjects to characterize the mechanisms during the *Agrobacterium* infection process. Hua 30 MDEC can be successfully infected by *Agrobacterium*. RNA-sequencing at different infection points (0, 2, 6, 12, 24 hpi) was performed. The average expressional intensity of the whole genomics increased from 0 to 2 hpi, and then decreased subsequently. More upregulated than downregulated differentially expressed genes (DEGs) were counted at the same time. GO enrichment analysis showed that protein modification was significantly overrepresented in upregulated DEGs. Chromosome-related biological processes, gene expression and cellular metabolic processes were significantly overrepresented in downregulated DEGs. KEGG analysis showed that plant defense responses, phenylpropanoid biosynthesis and biosynthesis of amino acids were significantly enriched across the infection time course. Nine DEGs related to defense responses were identified. All DEGs were upregulated from 2 to 24 hpi. We speculate that these genes are possibly related to *Agrobacterium* infection. These findings will provide deep insights into the molecular events occurring during the process of *Agrobacterium*-mediated transformation.

### KEYWORDS

Barley; *Agrobacterium*-mediated transformation; microspore-derived embryogenic callus; transcriptome analysis

## 1 Introduction

*Agrobacterium tumefaciens* possesses the ability to integrate self T-DNA into plant genomic [1]. The *Agrobacterium tumefaciens*-mediated genetic transformation system has been widely used in both basic research and breeding in plants, based on the low copy number of large segments of DNA into host genome DNA and low cost. The *Agrobacterium*-mediated transformation system has been established in many crops, such as soybean, rice, cotton, maize, wheat and barley [2]. However, the transformation efficiency is still a restricting factor in the *Agrobacterium*-mediated transformation. Based on the previous



studies, the *Agrobacterium* infection process involves two steps: the attachment of the bacterium to host cells and then the transfer of the DNA-protein complex into the plant cell [3]. The plants have also evolved defense systems for the *Agrobacterium* infection, which may be an important factor influencing the infection efficiency of plant cells [4]. As a gram-negative bacterium, *Agrobacterium tumefaciens* possesses bacteria-derived compounds known as pathogen-associated molecular patterns (PAMP), which caused immune response termed as PAMP-triggered immunity (PTI) in host cells [5]. During infection, *Agrobacterium* transfer proteins (effectors) into the plant cell which activates a stronger defense response called effector-triggered immunity (ETI) [5]. The mechanisms in plant cells' response to *Agrobacterium* infection were very complex [6,7]; any molecular interactions at these stages can affect the infection efficiency. Thus, it is critically important to get more knowledge on the molecular mechanisms involved in the plant-*Agrobacterium* interaction [8].

Microspores can be induced into an embryogenic callus *in vitro*. Meanwhile, the micropores-derived embryogenic callus (MDEC) possesses high regeneration ability [9]. The easily accessible populations of callus make microspore embryogenesis excellent for transformation. Barley is one of the important cereal crops, and it is also recognized as an ideal model plant. In previous reports, stable *Agrobacterium*-mediated transformations of barley microspore cultures have been reported [10]. But the technique is still genotypic dependent. This suggested that some factors affect the efficiency of *Agrobacterium*-mediated transformation in barley MDEC [11]. The improvement of the infection efficiency depends on further modification of the plant itself. Thus, a comprehensive understanding of the mechanisms underlying the transformation process is indispensable.

RNA-seq is one of the most important approaches to understand the molecular events that are involved in the transformation. Meanwhile, the high-quality reference genome assembly for barley makes it an even more efficient way to investigate the mechanism of the Barley-*Agrobacterium* interaction [12]. Transcriptome analyses have been performed to determine the mechanism in response to *Agrobacterium* in several studies. However, there is little information in barley MDEC response to *Agrobacterium*.

In this study, we isolated and cultured the microspores from the barley cultivar Hua 30 according to Lu's protocol [13]. The Hua 30 MDEC can be successfully infected by the *Agrobacterium* strain LBA4404. With the aim to get a better understanding of the molecular mechanisms in barley MDEC response to *Agrobacterium* infection, RNA-seq was performed during *Agrobacterium* infection on the barley MDEC. Using time course transcriptome analysis to measure gene expressions throughout infection, different biological processes, pathways and genes were identified during the infection process. These findings will contribute to additional insights into the mechanisms during the *Agrobacterium* infection process in monocots plants.

## 2 Materials and Methods

### 2.1 Plant Materials and Microspore Culture

Barley (*Hordeum vulgare* L.) cv. Hua 30 was grown in the greenhouse under a 16 h/8 h light/dark cycle at  $20 \pm 2^\circ\text{C}$  with 60% relative humidity and approximately  $1000 \text{ mol m}^{-2} \text{ s}^{-1}$  light intensity. Microspores were isolated and cultured using the protocol described by Lu et al. [13]. After 21 days of culturing, the MDEC was used for *Agrobacterium*-mediated transformation.

### 2.2 *Agrobacterium* Infection on Barley MDEC

*Agrobacterium* strain LBA4404, harboring binary vector *pCAMBIA1305.1*, was used in the barley microspore induced callus transformation. When the  $\text{OD}_{600}$  of *Agrobacterium* was about 0.6–0.8,  $200 \mu\text{M}$

acetosyringone was added to the solution and treated for 2 h. The *Agrobacterium* was centrifugated at 3270 g for 15 min, and the suspension was resuspended in the MDEC induction solution (with 200  $\mu$ M acetosyringone) to an OD<sub>600</sub> of about 0.6–0.8.

More than 100 barley MDEC were incubated with 50 ml *Agrobacterium* suspensions with a slight agitation (100 rpm) for 30 min and then dried by wind from the clean bench during 30 min. The MDEC was then placed on filter paper soaked with 5 ml liquid induction medium containing 200  $\mu$ M acetosyringone in petri dishes, and co-cultivated at 23°C in a growth chamber. Samples were harvested for RNA seq (0, 2, 6, 12, 24 h) and GUS staining (3, 5 days). Three replicates were performed for each time point.

### 2.3 GUS Stain Assay

GUS staining was performed on the third and fifth day after co-cultivation with *Agrobacterium*. Callus pieces (more than 100) were randomly picked to stain them with X-Gluc solution at 37°C overnight. The number of blue staining calli was counted to calculate the efficiency. The frequency of transformation was calculated as follows: (GUS<sup>+</sup> calli/Total calli)  $\times$  100%. The significant difference was analyzed by *t*-test.

### 2.4 RNA Extraction, Sequencing and Reads Mapping

The total RNA of each sample was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality and integrity of RNA were determined by 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The cDNA libraries were constructed using Illumina RNA Seq library kit (Illumina, Inc., USA). RNA data were acquired on Illumina HiSeq™ 4000 platform (Illumina) in Beijing Novogene Biotech Co., Ltd. (China). Raw data were filtered to obtain clean reads by removing adapters and excluding low-quality reads (Q < 30 and length < 50 bp). The clean reads were mapped onto the reference genome of barley. Differential expression analysis was performed using the R package DESeq. Genes with a *P*-value < 0.05, and (Log<sub>2</sub> fold change)  $\geq$  1 were classed as significantly differentially-expressed genes (DEGs).

The GO and KEGG enrichment analysis were performed to assign possible functional categorization and metabolic pathways using AgriGO tool (<http://systemsbiology.cau.edu.cn/agriGOv2/>). Venn diagrams software (<https://magic.novogene.com/>) was used to sort the DEGs.

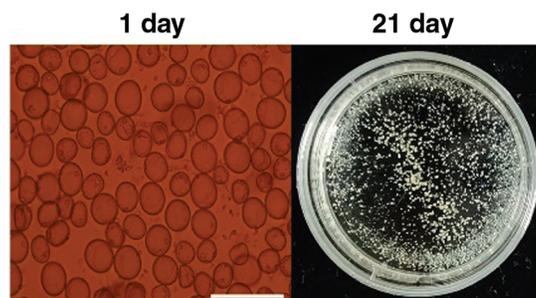
### 2.5 Quantitative RT-PCR Analysis

To validate the data of RNA-seq, 1  $\mu$ g total RNA of each sample was used for qRT-PCR assay. First strand cDNA was synthesized according to the manufacturer's recommended procedures (Toyobo). Primer pairs were designed using Primer 3 (<http://www.ncbi.nlm.nih.gov/tools/primerblast/>), and the selected gene name and primer information are listed in Supplementary Table 1. qRT-PCR was performed in the ABI 7500 fast system (Applied Biosystems, USA) using SYBR qPCR Mix (Toyobo). *Hv-Actin* was used as internal control [14].  $\Delta\Delta$ CT method was used to quantify the relative expression of identified genes [15].

## 3 Results

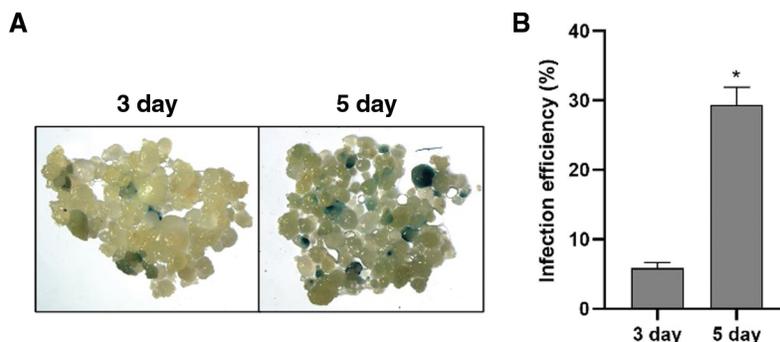
### 3.1 Infection of Hua 30 MDEC by *Agrobacterium*

Microspores isolated from the anthers of Hua 30 were cultured under controlled aseptic conditions. After 21-day of culturing, the microspores derived embryogenic callus (MDEC) were obtained and subsequently used for *Agrobacterium*-mediated transformation (Fig. 1).



**Figure 1:** Microspores-derived embryogenic callus culturing process of Hua 30 at 1 day (single cells) and 21 days (calli). Bars = 125  $\mu$ m

Infection efficiency was measured by counting blue spots after three- and five-day co-cultivation (Fig. 2A). As shown in Fig. 2B and Supplementary Table 2, the infection efficiency was 6.1% at 3 days post inoculation (dpi). At 5 dpi, infection efficiency increased to 32.3%. The results indicated that Hua 30 MDEC can be successfully infected by *Agrobacterium* strain LBA4404, but the infection efficiency was not high.



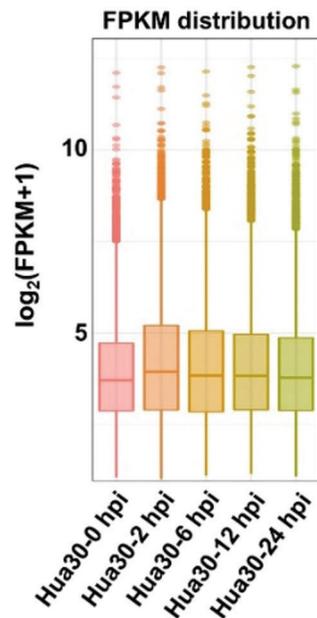
**Figure 2:** Infection efficiency of Hua 30 MDEC. A: GUS staining of Hua 30 MDEC at 3 and 5 dpi. B: Infection efficiency of Hua 30 MDEC at 3 and 5 dpi. The data are presented as average  $\pm$  s.e. of three independent samples. \* indicates significant differences at the level of  $P < 0.05$

### 3.2 RNA Sequencing of Hua30 MDEC and Data Analysis

To investigate the mechanism of Hua 30 MDEC in response to *Agrobacterium*, RNA was extracted from Hua 30 MDEC inoculated with *Agrobacterium* at five time points (0, 2, 6, 12, 24 h). Fifteen digital gene expression libraries were constructed (Supplementary Table 3). On average, the raw reads generated from each library were 47.27 million. The clean-reads numbers generated from each library ranged from 44.28 to 51.55 million. The number of reads, ranging from 83.74% to 92.96% were mapped to the barley reference genome. 'Phred value'  $> 30$  (Q30) of each library ranged from 93.39% to 94.64%. The transcriptome data were further used for bioinformatics analysis.

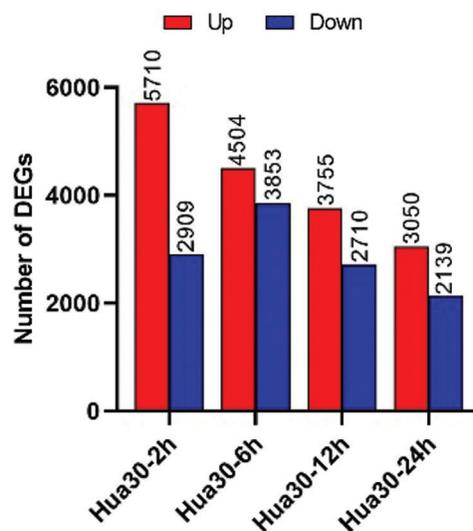
### 3.3 Identification of Differentially Expressed Genes (DEGs)

Whole genomic gene expression profiling of Hua 30 MDEC was analyzed during the *Agrobacterium* infection process. The results showed that the average expression level was highest at 2 hpi, and then decreased in the subsequently time points (Fig. 3).

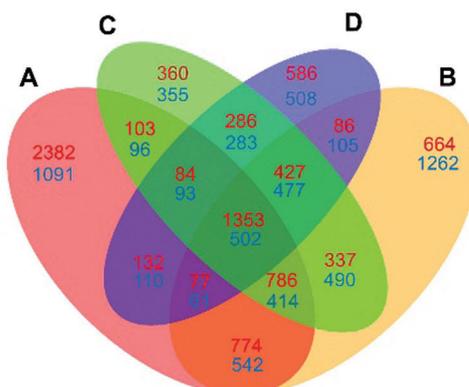


**Figure 3:** Expression profiling of the whole genomic gene expression under *Agrobacterium* infection at five time points (0, 2, 6, 12, and 24 hpi)

The number of DEGs at four infection time points was counted [0 hpi was used as control,  $P < 0.05$ , and  $\log_2(\text{fold change}) \geq 1$ ]. The DEGs were separated as upregulated- and downregulated- groups at each time point (Fig. 4). The number of the upregulated DEGs decreased from 2 to 24 hpi. The changing trend of downregulated DEGs was different from that of the upregulated DEGs. The number of downregulated DEGs increased from 2 to 6 hpi and then decreased from 6 to 24 hpi. More upregulated than downregulated DEGs were detected at any time. These results indicated that complex regulatory mechanisms were involved in Hua 30 MDEC response to *Agrobacterium* infection.



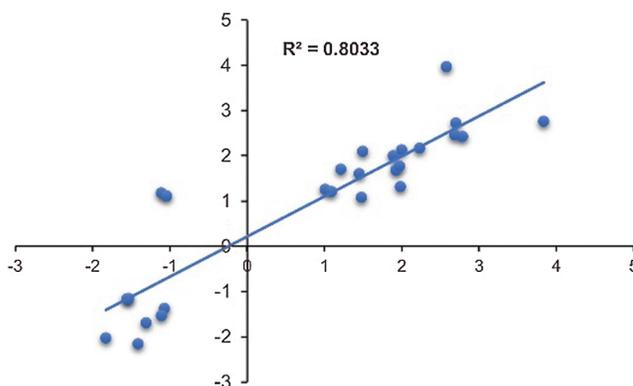
**Figure 4:** Number of DEGs at four time points (2, 6, 12, and 24 hpi). The y axis represents DEGs number. Red columns show the number of up-regulated DEGs, blue columns show the number of down-regulated DEGs



**Figure 5:** Venn diagram comparison of DEGs at four infection stages (2, 6, 12 and 24 hpi) compared each with 0 hpi. A: DEGs at 2 hpi; B: DEGs at 6 hpi; C: DEGs at 12 hpi; D: DEGs at 24 hpi. The numbers of up-regulated DEGs are shown in red, and the numbers of down-regulated DEGs are shown in blue

Venn diagrams were further constructed using DEGs at four time points (Fig. 5). 2382 upregulated DEGs were specially detected at 2 hpi, which were higher than those detected at any other time point (ranging from 360 to 664). At 6 hpi, more downregulated DEGs (1262) were specially detected as compared to those at any other time points. Meanwhile, 1353 up- and 502 down-regulated DEGs were detected at all four time points.

Nine DEGs were randomly selected to validate the RNA-seq data (Supplementary Table 4). There was a good coefficient ( $R^2 = 0.80$ ) between RNA-seq data and qRT-PCR analysis (Fig. 6), indicating that the RNA-seq data were reliable.

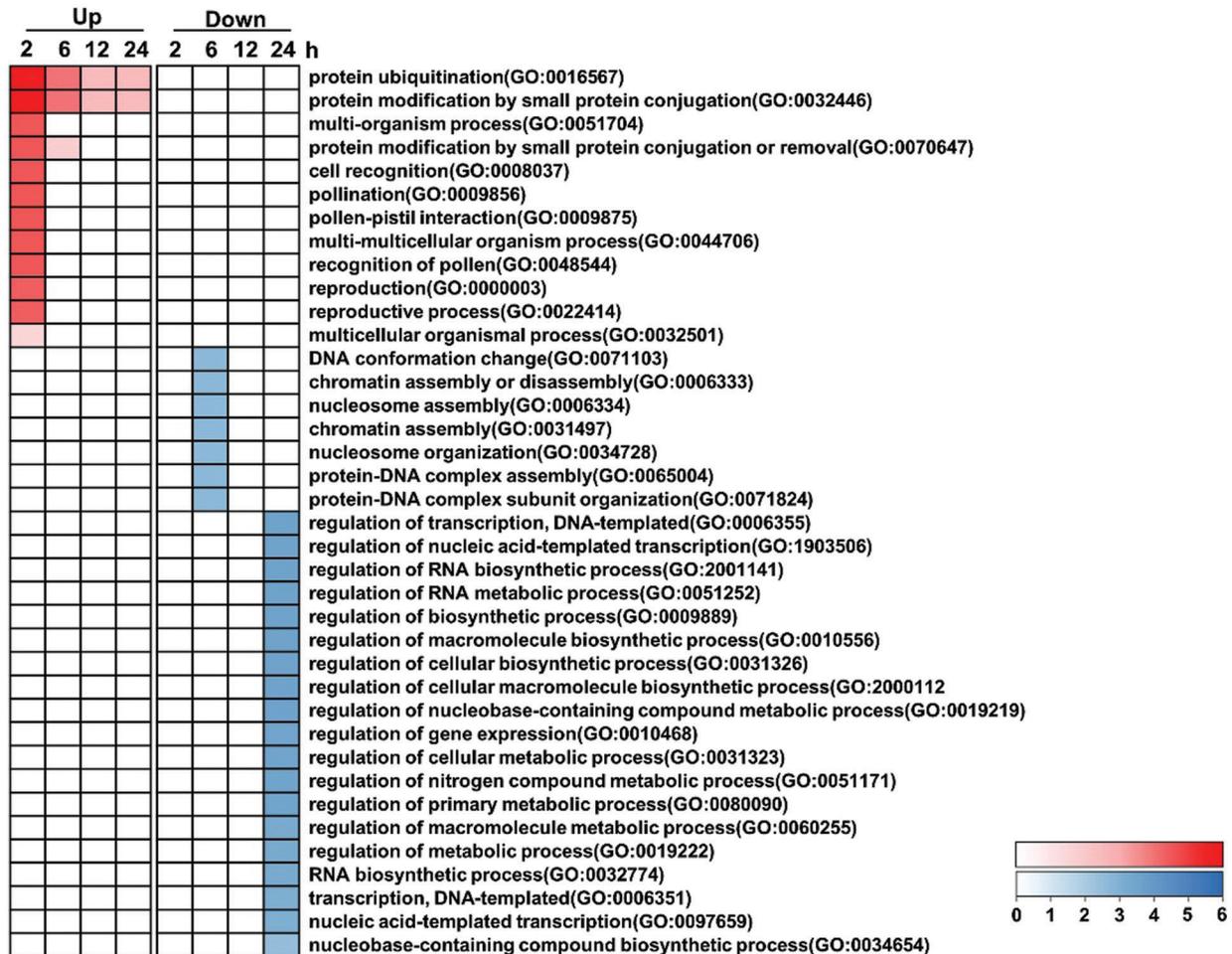


**Figure 6:** Validation of transcriptome data by qRT-PCR. Scatter plots indicate the transcriptional changes of qRT-PCR analysis and RNA-seq for 26 data points from 9 genes. The Pearson correlation coefficient ( $R^2$ ) was 0.80

### 3.4 Gene Ontology (GO) Enrichment Analysis of the DEGs in *Hua 30*

GO functional analysis was performed by assigning the DEGs across the infection time course. Biological processes were categorized into separate sets of upregulated and downregulated groups (Fig. 7, Supplementary Data s1). Most of the biological processes were enriched at 2 hpi among the upregulated groups. Protein ubiquitination and protein modification by small protein conjugation were significantly over-represented among the upregulated genes from 2 hpi to 24 hpi. In the downregulated group, function

categories were enriched at 6 and 24 hpi. Chromosome-related biological processes (chromatin assembly, protein-DNA complex assembly and nucleosome assembly) were significantly enriched at 6 hpi. Categories related to gene expression (regulation of transcription, regulation of RNA biosynthetic process, regulation of gene expression and RNA biosynthetic-related process) and cellular metabolic processes (regulation of biosynthetic process, regulation of cellular metabolic process, regulation of nucleobase-containing compound and nitrogen compound, regulation of primary metabolic process) were significantly enriched at 24 hpi.



**Figure 7:** Selected overrepresented biological processes of GO categories across the time course. The categories are significantly enriched ( $P < 0.05$ ) among either upregulated or downregulated genes.  $P$ -values from each category enrichment test have been negatively  $\log_{10}$ -transformed and plotted for each time point. Please increase the size of the text at the right of the figure

### 3.5 KEGG Enrichment Analysis of the DEGs in *Hua 30*

KEGG is a useful tool for the analysis of the roles of genes in various biological functions. We further performed KEGG enrichment using the DEGs from 4 infection time points. As shown in Table 1, the upregulated pathways were more than those downregulated. Five pathways were significantly enriched through the time course. One of them was plant-pathogen interaction, which was upregulated from 2 to 24 hpi, except at 12 hpi. Plant hormone signal transduction was downregulated at 2 hpi and upregulated

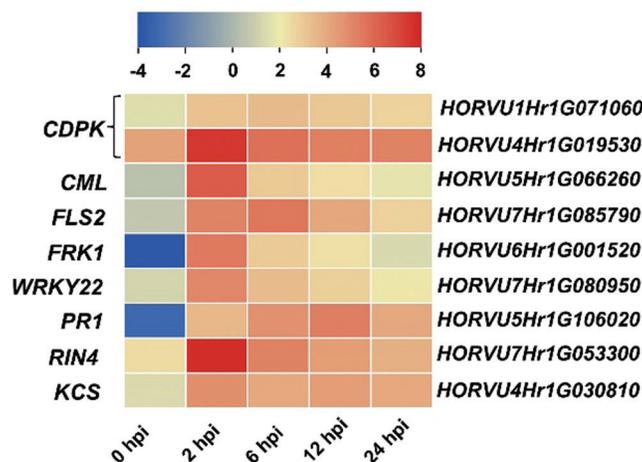
from 12 to 24 hpi. Phenylpropanoid biosynthesis and phenylalanine, tyrosine and tryptophan biosyntheses were upregulated from 2 to 24 hpi. Biosynthesis of amino acids was upregulated from 6 to 24 hpi. Glutathione metabolism was upregulated from 6 to 12 hpi. Seven pathways, including DNA replication, carbon metabolism, arginine biosynthesis, citrate cycle (TCA cycle), cysteine and methionine metabolism, pyruvate metabolism and glycine, serine and threonine metabolisms were upregulated at 24 hpi. Protein processing at the endoplasmic reticulum was downregulated at 12 hpi. The results suggested that more upregulated than downregulated pathways were induced during *Agrobacterium* infection in barley MDEC.

**Table 1:** DEGs enriched on the pathways at 2, 6, 12 and 24 hpi.  $\geq 1$  means the number of upregulated genes,  $\leq -1$  means the number of downregulated genes

Pathway	2 hpi		6 hpi		12 hpi		24 hpi	
	$\geq 1$	$\leq -1$						
Plant-pathogen interaction	48		32				21	
Plant hormone signal transduction		20			28		25	
Phenylpropanoid biosynthesis	27		32		31		25	
Phenylalanine, tyrosine and tryptophan biosynthesis	12		15		11		9	
Biosynthesis of amino acids			39		42		43	
Glutathione metabolism			16		17			
DNA replication							15	
Carbon metabolism							35	
Arginine biosynthesis							11	
Citrate cycle (TCA cycle)							13	
Cysteine and methionine metabolism							17	
Pyruvate metabolism							14	
Glycine, serine and threonine metabolism							11	
Protein processing in endoplasmic reticulum						20		

### 3.6 DEGs Involved in Plant Defense Response

Plant immune responses play important roles during *Agrobacterium*- mediated transformation [16,17]. Plant defense was also significantly enriched in Hua 30 MDEC response to *Agrobacterium*. We further screened defense response genes that upregulated at all four infection time points (2, 6, 12, 24 hpi). Nine DEGs were identified in the plant-pathogen interaction pathway. The identified genes include two CDPK (*HORVU1Hr1G071060* and *HORVU4Hr1G019530*), CML (*HORVU5Hr1G066260*), FLS2 (*HORVU7Hr1G085790*), FRK1 (*HORVU6Hr1G001520*), WRKY22 (*HORVU7Hr1G080950*), PR1 (*HORVU5Hr1G106020*), RIN4 (*HORVU7Hr1G053300*) and KCS (*HORVU4Hr1G030810*). Heat maps of these genes were constructed, and the transcript accumulation of all genes was more from 2 to 24 hpi than that at 0 hpi (Fig. 8).



**Figure 8:** Heat maps of the nine identified genes across the infection process. The bottom color bars represent the  $\log_2$  of FPKM for each gene, ranging from blue ( $-4$ ) to red ( $8$ ). A deeper color indicates more transcript accumulation. Please increase the size of the text at the right of the figure

#### 4 Discussion

Genotypic dependency is still the main limiting factor that influences the *Agrobacterium* infection efficiency. In the present study, the infection efficiency of Hua 30 MDEC increased from 3 to 5 dpi. This suggested that the genotype was compatible with the *Agrobacterium* strain LBA4404. Thus, RNA-seq was performed on Hua 30 MDEC during the infection process to detect more information on the molecular mechanism.

Our results showed that the *Agrobacterium* infection brought rapid changes in gene expression at the early stages of infection (2 hpi). This was similar to previous reports. The defense response genes were activated as early as 3–6 h in tobacco BY2 cell suspensions upon *Agrobacterium* infection [4]. Host defense response was stimulated by *Agrobacterium* as early as 3 hpi in *Arabidopsis* [16]. One study in rice embryogenic calli showed gene expression changes as early as 1 hpi upon *Agrobacterium* infection [18]. In addition, plant defense response was also stimulated quickly in the Hua 30 MDEC under *Agrobacterium* infection. These studies suggested that the MDEC cell senses the *Agrobacterium* immediately and subsequently influences the transcripts.

GO enrichment analysis showed that DEGs involved in protein ubiquitination and protein modification processes by small protein conjugation were upregulated across the infection process. This was consistent with a previous report in wheat, which showed that ubiquitin protein ligase was upregulated during the *Agrobacterium* infection process [19]. Protein ubiquitination was necessary for the *Agrobacterium*-mediated transformation [20]. Chromosome-related biological processes were overrepresented among downregulated DEGs at 6 hpi. Chromosome-related biological processes were important for *Agrobacterium* infection. In *Arabidopsis*, the overexpression of one histone gene (H2A-1) results in increased *Agrobacterium*-mediated transformation [21]. In rice, Tie et al. [18] showed that chromatin modification was repressed at the early time of infection in a recalcitrant genotype. There was a successful infection but low infection efficiency in Hua 30 MDEC. Our results suggested that the chromosome-related biological processes may be one influencing factor on the infection efficiency in barley MDEC.

Most plant secondary metabolites might play important roles during the *Agrobacterium* infection process. Phenylpropanoid biosynthesis was upregulated across the infection process. Zhou et al. showed

that genes involved in this metabolic process are significantly upregulated upon *Agrobacterium* infection in wheat calli [19]. In soybean, genes involved in this metabolic process are also upregulated. But these genes' expression was reduced when a-aminooxyacetic acid (AOA, transformation promotion factor) was added in tissue culture following transformation [22]. This suggested that the upregulation of phenylpropanoid biosynthesis pathway may negatively affect the *Agrobacterium* infection.

KEGG enrichment analysis showed that the plant-pathogen interaction was significantly upregulated during the infection process. As a soilborne pathogenic bacterium, *Agrobacterium tumefaciens* can activate plant defense and immune responses [23]. Defense genes were rapidly induced in tobacco BY2 cells upon *Agrobacterium* infection. But these defense genes were repressed at later stages of transformation by the transfer-competent instead of transfer-deficient *Agrobacterium* strains [4]. Similar results were reported in rice. Some defense genes were downregulated in the compatible genotype and upregulated in the recalcitrant genotype at the later stages of the *Agrobacterium* infection process [18]. In our results, the defense response ('Plant-pathogen interaction') was upregulated at the later stage (24 hpi) in Hua 30 MDEC. This suggested that the pathway may negatively affect the *Agrobacterium* infection.

Nine DEGs related defense responses were identified. All the genes exhibited high transcript accumulation after *Agrobacterium* infection. *CDPK* and *CML* (calcium-binding protein) were functional in the  $Ca^{2+}$  signal transduction [24].  $Ca^{2+}$  was recognized as a conserved second messenger and principal mediator in the plant immune response. Six genes (*Fls2*, *FRK1*, *WRKY22*, *PR1*, *RIN4* and *KCS*) were reported to function in the flg22-dependent defense response. The defense responses were always considered as PTI [25], which includes reactive oxygen species (ROS) burst, calcium flux and transcriptional reprogramming [5]. One PTI loss mutant showed increased susceptibility to *Agrobacterium* in *Arabidopsis*. This indicated that PTI plays an important role during the *Agrobacterium* transformation process. Thus, we speculate that the identified genes are related to *Agrobacterium* infection. The function of these genes needs further investigation.

In conclusion, this study showed the response of barley MDEC under *Agrobacterium* infection. Upon *Agrobacterium* infection, more upregulated than downregulated DEGs were counted at the same time point. Genes involved in protein modification, defense response, phenylpropanoid biosynthesis and biosynthesis of amino acids were upregulated. Meanwhile, genes involved in chromosome-related biological processes, gene expression and cellular metabolic processes were downregulated. Nine genes related to defense response were identified, which may affect the *Agrobacterium* infection. The finding will provide additional insights of the molecular events occurring during the process of *Agrobacterium*-mediated transformation.

**Availability of Data and Materials:** The datasets generated and analyzed during the current study are available in the National Center for Biotechnology Information: Submission ID: SUB10402909; BioProject ID: PRJNA764780.

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**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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## Supplementary Materials

**Supplementary Table 1:** Primer information for qRT-PCR

**Supplementary Table 2:** Infection efficiency of Hua 30 MDEC

**Supplementary Table 3:** An overview of sequencing and assembly of Hua 30 MDEC

**Supplementary Table 4:** Validation of the transcriptome data by qRT-PCR

**Supplementary Data s1:** Data of significant enriched Biological Process of GO categories across time-course comparisons in Hua 30 MDEC. The categories are separated as upregulate and downregulate groups

**Supplementary Data s2:** Data of pathways by KEGG enrichment in Hua 30 MDEC at 2, 6, 12 and 24 hpi. The pathways are separated as upregulate and downregulate groups