Comparative transcriptome profiling uncovers a *Lilium regale* NAC transcription factor, *LrNAC35*, contributing to defence response against cucumber mosaic virus and tobacco mosaic virus

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**SUMMARY**

Cucumber mosaic virus (CMV) is a highly prevalent viral pathogen causing substantial damage to the bulb and cut-flower production of *Lilium* spp. Here, we performed an Illumina RNA sequencing (RNA-Seq) study on the leaf tissues of a virus-resistant species *Lilium regale* inoculated with mock control and CMV. A total of 1346 differentially expressed genes (DEGs) were identified in the leaves of *L. regale* upon CMV inoculation, which contained 34 up-regulated and 40 down-regulated DEGs that encode putative transcription factors (TFs). One up-regulated TF, *LrNAC35*, belonging to the NAM/ATAF/CUC (NAC) superfamily, was selected for further functional characterization. Aside from CMV, lily mottle virus and lily symptomless virus infections provoked a striking increase in *LrNAC35* transcripts in both resistant and susceptible *Lilium* species. The treatments with low temperature and several stress-related hormones activated *LrNAC35* expression, contrary to its reduced expression under salt stress. Ectopic overexpression of *LrNAC35* in petunia lines overexpressing *LrNAC35* resulted in reduced susceptibility to CMV and *Tobacco mosaic virus* infections, and enhanced accumulation of lignin in the cell walls. Four lignin biosynthetic genes, including *PhC4H*, *Ph4CL*, *PhHCT* and *PhCCR*, were found to be up-regulated in CMV-infected petunia lines overexpressing *LrNAC35*. *In vivo* promoter-binding tests showed that *LrNAC35* specifically regulated the expression of *Ph4CL*. Taken together, our results suggest a positive role of transcriptome-derived *LrNAC35* in transcriptional modulation of host defence against viral attack.

**Keywords:** Cucumber mosaic virus, lignin, *Lilium regale*, NAC transcription factor, petunia, *Tobacco mosaic virus*, transcriptome.

**INTRODUCTION**

Viral pathogens are the major constraining factors for crop growth and production worldwide. They occur ubiquitously in various environmental conditions. Disease symptoms resulting from virus invasion often include chlorotic or necrotic leaves, shortened internodes, stunted upright growth and weakened vitality (Galvez et al., 2014; Rojas et al., 2007). Plants have evolved multiple defensive mechanisms against the threats posed by massive virus proliferation. The recognition of foreign virions by plants firstly stimulates the establishment of a basal defence system, which involves the participation of reactive oxygen species, cell wall structural components, and antiviral proteins and compounds (He et al., 2007; van Loon et al., 2006). If viruses break through the basal guard, a second-line host defence called the innate immune response is activated (Liu et al., 2004).

Numerous virus-responsive genes have essential roles in the innate immunity system, such as the resistance (*R*) gene (Kang et al., 2005), tobacco *N* gene (Marathe et al., 2002; Whitham et al., 1996), and the genes associated with RNA silencing (Agius et al., 2012; Sun et al., 2016a) and translation suppression (Zorzatto et al., 2015). The transcription of these genes is potentially controlled by specific transcription factors (TFs), of which many members of various families, including MYB (Yang and Klessig, 1996), zinc finger (Guo et al., 2004), WRKY (Park et al., 2006), AP2/ERF (Huang et al., 2016), bZIP (Gagun cela et al., 2016), bHLH (Aparicio and Pallás, 2017) and NAC (Huang et al., 2017), have been revealed to be implicated in the response to viral stimuli. In particular, NAC domain-containing proteins comprise a large TF family in plants and play essential roles in plant growth, development and stress responses (Puranik et al., 2012). Approximately 105 and 75 NAC proteins are present in *Arabidopsis* and *Oryza sativa* genomes, respectively. Based on sequence similarity, NAC proteins from both species are classified into two large groups and 18 subgroups (Ooka et al., 2003). These proteins share five highly conserved subdomains (A to E) within...
the N-terminal region or a divergent C-terminal domain (Hegedus et al., 2003; Kikuchi et al., 2000).

Multiple studies have demonstrated the critical functions of NAC TFs in abiotic stress resistance, such as drought (Iran et al., 2004), salt (Balazadeh et al., 2010) and low temperature (Hao et al., 2011), as well as their disease resistance to fungal and bacterial pathogens, including Botrytis cinerea, Pseudomonas syringae and Alternaria brassicicola (Wang et al., 2009). In addition, a number of studies have also reported the involvement of NAC TFs in plant response to virus infection. For instance, GRAB1 and GRAB2 interact with wheat dwarf geminivirus (WDV) RepA protein, and their expression in wheat cells restrains DNA replication of WDV (Xie et al., 1999).

**Sinac1** overexpression causes an enhanced replication of tomato leaf curl virus by specifically binding to geminivirus replication enhancer (Ren) protein (Selth et al., 2005). The mutant of rim1-1, a novel NAC gene, shows reduced susceptibility to rice dwarf virus, whereas its overexpression enhances virus multiplication (Yoshii et al., 2009). Transgenic Arabidopsis plants overexpressing NAC TF gene ATAF2 exhibit decreased proliferation of tobacco mosaic virus (TMV) and up-regulation of some defence-associated marker genes (Wang et al., 2009). The interaction between NAC protein Pip and turnip crinkle virus coat protein interferes with the basal defence against virus invasion in Arabidopsis (Donze et al., 2014). Another NAC protein, NAC083, interacts with mungbean yellow mosaic India virus replication initiator protein in Arabidopsis, but its biological role in virus resistance has not yet been examined (Suyal et al., 2014). A recent study shows that six NAC genes differentially respond to tomato yellow leaf curl virus (TYLCV) infection in tomato plants, and among them virus-induced gene silencing (VIGS) of **Sinac61** leads to increased TYLCV accumulation (Huang et al., 2017). These findings indicate that NAC TFs have a conserved biological role in virus resistance mostly through the interaction with viral proteins. However, little is known about the transcriptional regulation of virus-responsive genes by NAC TFs in plants.

Lily belongs to the genus *Lilium* of the family Liliaceae. It is a summer-blooming perennial bulbous plant with various food, aesthetic, medicinal and economic values (Wang et al., 2016). It is a summer-blooming perennial bulbous plant with various food, aesthetic, medicinal and economic values (Wang et al., 2016). It is a summer-blooming perennial bulbous plant with various food, aesthetic, medicinal and economic values (Wang et al., 2016). It is a summer-blooming perennial bulbous plant with various food, aesthetic, medicinal and economic values (Wang et al., 2016). It is a summer-blooming perennial bulbous plant with various food, aesthetic, medicinal and economic values (Wang et al., 2016).

**RESULTS**

**Sampling, RNA sequencing and de novo assembly**

To investigate the host transcriptome response of *L. regale* to CMV infection, the second broad true leaves of seed-grown plantlets were used for virus inoculation (Fig. 1A). Viral gene expression tests showed a moderate increase in transcripts of CMV-1a, -2a and -coat protein (CP) during an initial 24 h of infection, followed by a rather sharp rise at 48 and 72 h post-inoculation (hpi) (Fig. 1B). The sampling time of *L. regale* leaves for RNA extraction and sequencing, representing a surge of virus replication, was thus determined to be 48 hpi.

Illumina paired-end sequencing in total generated 154,921,638 and 154,400,476 raw reads for the mock control and CMV-infected *L. regale* libraries. Correspondingly, 151,810,642 and 150,595,506 clean data were obtained after trimming off the adaptor sequences, and ambiguous and low-quality reads. *De novo* assembly resulted in a total of 115,826 unigenes. The minimum, maximum and mean lengths of the assembled unigenes are 11,418, 224 and 615.8 bp, respectively (Table 1).

The sequences and functional annotation of all assembled unigenes are shown in Fig. 51 and Table 51 (see Supporting
information). Their size distribution revealed that 75,601 (65.3%) unigenes ranged from 200 to 499 bp, 22,496 (19.4%) from 500 to 999 bp and 17,729 (15.3%) were more than 1000 bp (Fig. S2, see Supporting information). To corroborate the accuracy of RNA-Seq data, nine unigenes, comprising one unknown and eight annotated transcripts, were randomly selected and analysed via quantitative real-time PCR (qRT-PCR). The qRT-PCR results for the unigenes tested were consistent with the transcriptome data (Fig. S3, see Supporting information). The reliability of differential gene expression identified by RNA-Seq was accordingly validated. Principal component analysis (PCA) of transcriptome data showed that principal components 1 and 2 explained 60.8% and 21% of the variance, respectively, and CMV-infected samples were significantly different from the mock control (Fig. S4, see Supporting information).

Detection of differentially expressed genes during CMV infection

Comparative transcriptome analysis of mock control and CMV-infected L. regale leaves generated 1346 differentially expressed genes (DEGs). Of all DEGs, 500 unigenes were significantly up-regulated in CMV-infected leaves, while 846 unigenes were down-regulated (Fig. 1C and Table S2, see Supporting information). Gene Ontology (GO) enrichment analysis showed that 663, 418 and 158 DEGs were assigned to three functional categories: biological process, cellular component and molecular function, respectively. The largest number of enriched genes fell into the metabolic process, response to stimulus and single-organism process (Fig. S5, see Supporting information). We further implemented a pathway-based categorization of DEGs by searching against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and found that 219 DEGs were mapped to 58 biochemical pathways. Among these mapped pathways, the metabolic pathway contained the largest number of DEGs (36), followed by secondary metabolites biosynthesis (28) and plant–pathogen interaction (22) pathways (Table S3, see Supporting information).

Candidate transcriptional regulators involved in response to CMV

To understand the transcriptional modulation mechanism of CMV defence in L. regale, 74 DEGs encoding putative TFs, of which 34 were up-regulated and 40 were down-regulated, were obtained.
Identification of LrNAC35

We selected LrNAC35 (GenBank accession no. MK805884) from the expression-quantified TF genes above for further functional characterization. The full length of LrNAC35 cDNA includes a complete coding region encoding 358 amino acids (Fig. S6, see Supporting information), possessing five subdomains A to E. Alignment and the phylogenetic tree showed that LrNAC35 has high homology to NAC49 and NAC75 from O. sativa, and NAC35 proteins from Petunia hybrida, Arabidopsis thaliana, Triticum aestivum, Solanum lycopersicum and Zea mays (Fig. 3A,B).

To investigate the protein structural features of LrNAC35, particularly its DNA-binding potential, homology-based protein structural modelling was performed for the NAC DNA-binding domain. A combination of multiple homologous protein structures (chain A of Protein Data Banks (PDBs) 1UT4, 1UT7, 4DUL, 3SWM, SWP and 3ULX) was identified and used as a template for protein modelling. As shown in Fig. 3C, LrNAC35 harbours a conserved NAC domain (N-terminal) and a divergent transcription regulatory domain (C-terminal). The NAC domain of LrNAC35 adopts the typical NAC protein fold, which mainly consists of beta-sheets. Based on the report on the structure of Arabidopsis AtNAC19 bound with DNA (PDB 3SWM) (Welner et al., 2012), six residue groups (G1–G6) have been suggested to have DNA-binding potential (Fig. 3D). The spatial positions of the corresponding G1, G2 and G6 in LrNAC35 were displayed in superimposition with the chain A of AtNAC19 (PDB 3SWM). The protein model of LrNAC35 resembles the spatial coordination of AtNAC19. The side chains of the residues in G1, G2 and G6 are in close proximity to the bound DNA molecule. Each of these three residue groups inserts into the adjacent grooves of the DNA, similar as that observed for AtNAC19 (Fig. 3E).

Biotic, abiotic stresses and hormone treatments alter LrNAC35 expression

To assess the expression patterns of LrNAC35 during multiple plant–virus interactions, additional virus infection tests using CMV, LMoV and LSV were carried out in five wild Lilium species, including virus-resistant species L. regale, L. pumilum and L. duchartrei, and susceptible species L. brownii and L. tigrinum (Sun et al., 2016b). The overall expression levels of LrNAC35 increased dramatically in all species tested when challenged with three viruses, and in particular L. regale displayed the maximum abundances at 48 or 72 hpi (Fig. 4A–C). CMV infection resulted in significantly higher transcript levels of LrNAC35 in L. regale, L. pumilum not L. duchartrei than in two susceptible species at 48 or 72 hpi (Fig. 4A), while only L. regale exhibited higher LrNAC35 expression than the susceptible ones at 24 or 48 hpi with LMoV (Fig. 4B). A different expression profile was observed after inoculation with LSV, showing that LrNAC35 transcripts in the susceptible species L. brownii surpassed those in the resistant species L. pumilum or L. duchartrei at 24 or 48 hpi (Fig. 4C).

For treatment with abiotic stresses and plant growth regulators, LrNAC35 transcripts increased markedly at low temperature but reduced under high salinity (Fig. 4D). A pronounced elevation in transcript levels of LrNAC35 occurred following treatments with ethylene (ET) and abscisic acid (ABA), while a moderate and delayed up-regulation was observed upon exposure to salicylic acid (SA) (Fig. 4E). No significant change in the expression of LrNAC35 was found under dehydration and Jasmonic acid (JA) treatments (Fig. 4D,E). Furthermore, LrNAC35 was constitutively expressed in stems and leaves with higher abundances than in roots, scales, seeds and floral tissues (Fig. 4F).
Table 2  Putative transcription factors associated with the defence response of *Lilium regale* to CMV infection

| Gene ID     | Annotation                                                      | RPKM       |        | Log₂ (CMV/mock) |
|-------------|-----------------------------------------------------------------|------------|--------|-----------------|
|             |                                                                  | Mock       | CMV    |                 |
| **AP2/ERF family** |                                                                 |            |        |                 |
| Unigene000389 | Ethylene-responsive transcription factor ERF01-like             | 0.362      | 2.026  | 2.486           |
| Unigene000390 | Ethylene-responsive transcription factor ERF01-like             | 0.371      | 2.501  | 2.752           |
| Unigene0014641 | Ethylene-responsive transcription factor ERF01-like            | 0.169      | 0.957  | 2.503           |
| Unigene0052014 | Ethylene-responsive transcription factor ERF01-like            | 0.140      | 0.580  | 2.047           |
| Unigene0052016 | Ethylene-responsive transcription factor ERF01-like            | 0.401      | 1.947  | 2.281           |
| Unigene0072729 | Ethylene-responsive transcription factor ERF01-like             | 0.001      | 0.832  | 9.701           |
| Unigene0073936 | Ethylene-responsive transcription factor TINY-like              | 0.001      | 0.731  | 9.515           |
| Unigene0089226 | Ethylene-responsive transcription factor ERF061-like            | 0.038      | 1.575  | 5.377           |
| Unigene0020351 | Ethylene-responsive transcription factor PLT2-like              | 6.224      | 1.947  | 2.281           |
| Unigene0037049 | Ethylene-responsive transcription factor ERF012-like            | 0.644      | 0.001  | -9.331          |
| **AUX/IAA family** |                                                                 |            |        |                 |
| Unigene0090221 | AUX/IAA transcriptional regulator family protein                | 0.979      | 0.203  | -2.272          |
| Unigene0108320 | Auxin-responsive protein IAA17-like                            | 129.788    | 48.931 | -1.407          |
| **bHLH family** |                                                                 |            |        |                 |
| Unigene0015592 | Transcription factor ABA-inducible bHLH-type-like              | 0.001      | 0.495  | 8.951           |
| Unigene0038912 | Transcription factor bHLH100-like                              | 1.341      | 6.279  | 2.227           |
| Unigene0038913 | Transcription factor bHLH100-like                              | 0.727      | 4.607  | 2.600           |
| Unigene00111737 | Transcription factor bHLH18-like                              | 4.817      | 25.620 | 2.411           |
| Unigene0009308 | Transcription factor bHLH75-like                               | 1.880      | 0.045  | -5.395          |
| Unigene0029409 | Transcription factor PAR1-like                                 | 0.414      | 0.050  | -3.064          |
| Unigene0031740 | Transcription factor bHLH137-like                              | 1.508      | 0.020  | -6.255          |
| Unigene0038757 | Transcription factor bHLH35-like                               | 0.832      | 0.094  | -3.141          |
| Unigene0042758 | BHLH family transcriptional factor                             | 6.980      | 1.631  | -2.098          |
| Unigene0051700 | Transcription factor bHLH35-like                              | 0.223      | 0.001  | -7.802          |
| Unigene0059321 | Transcription factor bHLH30-like                              | 1.232      | 0.431  | -1.514          |
| Unigene0011609 | BHLH family protein                                            | 1.397      | 0.134  | -3.379          |
| **bZIP family** |                                                                 |            |        |                 |
| Unigene0010955 | Transcription factor RF2a-like                                 | 5.961      | 0.724  | -3.041          |
| Unigene0027495 | BZIP-like transcription factor-like                            | 1.748      | 0.459  | -1.928          |
| **MYB family** |                                                                 |            |        |                 |
| Unigene0003524 | Transcription factor MYB98-like                                | 0.139      | 2.429  | 4.123           |
| Unigene0006408 | Transcription factor RL9                                       | 0.122      | 0.408  | 1.736           |
| Unigene0007577 | MYB-related protein Zm1-like                                   | 0.226      | 1.360  | 2.592           |
| Unigene0054070 | Transcription factor CPC-like                                  | 0.604      | 6.966  | 3.527           |
| Unigene0003522 | Transcription factor MYB98-like                                | 0.335      | 0.001  | -8.386          |
| Unigene0097039 | Transcription factor R2R3-MYB                                 | 1.876      | 0.135  | -3.800          |
| **MYC family** |                                                                 |            |        |                 |
| Unigene0053370 | Transcription factor MYC4-like                                 | 0.001      | 3.317  | 11.696          |
| Unigene0076682 | Transcription factor ICE1-like                                 | 0.956      | 0.051  | -4.237          |

(Continues)
Ectopic overexpression of *LrNAC35* alters flowering time in petunia

To elucidate the possible function of *LrNAC35*, we performed a genetic transformation assay in petunia, which is a common model platform for molecular research of floral crops. *LrNAC35* was ectopically overexpressed under the control of the CaMV 35S promoter in petunia. Three transgenic lines (3-1, 5-6 and 12-3) with *LrNAC35* overexpression showed delayed flowering phenotypes compared with wild-type (WT) plants (Fig. 5A,C). A dual expression analysis using semiquantitative and quantitative methods confirmed the substantial transcription of *LrNAC35* in transgenic lines (Fig. 5B).

**LrNAC35** affects susceptibility to CMV

In view of *LrNAC35*’s prominent induction when viruses invade, the function of *LrNAC35* in the defence response against CMV was investigated. Both WT and transgenic lines were inoculated with CMV. At 18 days post-inoculation (dpi), *LrNAC35*-overexpressing transgenic petunia lines showed milder leaf distortion and necrosis than WT plants (Fig. 6A). Accumulation of CMV-CP at both transcript and protein levels was reduced in systemically infected leaves of overexpression lines compared with the control at 8 or 12 dpi (Fig. 6B,C). In the inoculated leaves, CMV infection elicited a higher level of HR-like cell death in WT lines than that in transgenic lines overexpressing *LrNAC35*, where relatively smaller lesion areas were observed (Fig. 6D,E). Transgenic lines generated significantly lower levels of electrolyte leakage than the WT control after CMV inoculation (Fig. 6F). There was a notable increase in Klason lignin content in the CMV-inoculated leaves of three overexpression lines compared to WT lines (Fig. 6G).

For functional validation of *LrNAC35*, we selected a petunia orthologue of *LrNAC35*, designated *PhNAC35*, to perform TRV-based virus-induced gene silencing (VIGS) assay. Petunia leaves infiltrated with TRV empty vector or TRV-*PhNAC35* were inoculated with CMV. Genes with increased expression in the TRV-*PhNAC35*-treated leaves are annotated in Table 2.

| Gene ID          | Annotation                          | RPKM | Log2 (CMV/mock) |
|------------------|-------------------------------------|------|-----------------|
| Unigene0105852   | Transcription factor MYC2-like       | 5.815| −5.917          |
| Unigene0111554   | Transcription factor MYC4-like       | 5.449| −2.776          |
| NAC family       |                                      |      |                 |
| Unigene0012070   | NAC domain-containing protein 35-like| 5.611| 2.760           |
| Unigene0025824   | NAC domain-containing protein 100-like| 0.088| 2.656           |
| Unigene00050073  | NAC domain-containing protein 90-like| 1.611| −3.718          |
| Unigene0093619   | NAC domain-containing protein 2122-like| 3.284| −2.881          |
| Unigene0112474   | NAC domain-containing protein 48-like| 8.865| −1.571          |
| WRKY family      |                                      |      |                 |
| Unigene0001022   | WRKY transcription factor 28         | 0.385| 2.296           |
| Unigene0039394   | WRKY transcription factor 28         | 1.759| 2.838           |
| Unigene0104357   | WRKY transcription factor 48         | 14.561| 1.264          |
| Unigene0042962   | WRKY family protein                  | 5.930| −1.512          |
| Unigene0051891   | WRKY transcription factor 53         | 4.355| −3.404          |
| Unigene0078584   | WRKY transcription factor 51         | 0.665| −9.377          |
| Zinc finger family|                                    |      |                 |
| Unigene0071735   | Zinc finger protein WIP3-like        | 0.429| 1.932           |
| Unigene0076256   | Zinc finger protein RICESLEEPER 2-like| 0.723| 1.668           |
| Unigene0109816   | Zinc finger protein ZAT11            | 0.001| 10.453          |
| Unigene0111263   | Dof zinc finger protein DOF5.6-like  | 0.240| 3.530           |
| Unigene0018640   | Zinc finger protein 7                | 1.513| −3.645          |
| Unigene0092120   | Zinc finger CCCH domain-containing protein 28-like| 1.094| −2.792          |
| Unigene0093967   | Zinc finger protein ZNFhex133       | 0.713| −2.239          |
| Unigene0027578   | RING-H2 zinc finger protein ATL79-like| 32.849| −1.359         |
| Unigene0060238   | RING-H2 zinc finger protein ATL3-like| 1.615| −7.357          |
subsequently inoculated with CMV, and the mock control was used to differentiate the symptoms triggered by TRV vector and CMV. PhNAC35 transcripts were markedly down-regulated in TRV-PhNAC35-infected petunia leaves. We observed more severe symptoms in CMV-infected petunia plants with PhNAC35 silencing. CMV-CP transcript and protein levels were significantly higher in PhNAC35-silenced petunia leaves than those in non-silenced ones (Fig. S7A–D, see Supporting information), suggesting that PhNAC35 silencing caused a compromised resistance to CMV.

**LrNAC35 modulates expression of lignin biosynthesis-related genes**

Lignification creates a non-degradable mechanical barrier to hinder pathogen spread within the host tissues (Moura et al., 2010). To verify if LrNAC35 can regulate lignin biosynthesis, the expression of the core genes in the lignin biosynthetic pathway were examined. Transgenic petunia lines with LrNAC35 overexpression showed higher transcript abundances of cinnamate 4-hydroxylase (PhC4H), 4-coumarate: CoA ligase (Ph4CL), hydroxycinnamoyl CoA: quinate /shikimate hydroxycinnamoyl transferase (PhHCT) and cinnamoyl-CoA reductase (PhCCR) than WT plants at 0 and 48 hpi with CMV. The expression levels of the other structural genes, such as phenylalanine ammonia-lyase (PhPAL), coumaroyl-quinate:shikimate 3-hydroxylase (PhC3H), caffeoyl CoA O-methyltransferase (PhCCoAOMT), ferulate 5-hydroxylase (PhF5H), caffeic acid O-methyltransferase (PhCOMT) and cinnamyl alcohol dehydrogenase (PhCAD), remained unchanged between WT and overexpression lines during CMV infection (Fig. 7A).

**Fig. 2** Expression of candidate transcription factors (TFs) associated with Lilium regale defence against CMV infection. Sixteen unigenes, classified into nine families, were chosen from up- or down-regulated TFs and evaluated by qRT-PCR at given time points. Transcript abundances were normalized to LrActin. SE of the mean from three biological replicates is shown as error bars.
To investigate the possible transactivation of \textit{PhC4H}, \textit{Ph4CL}, \textit{PhHCT} and \textit{PhCCR} promoters by LrNAC35, the promoter binding sites of these genes were predicted. Two types of NAC protein binding sites with the core CACG and CATGTG (or CGTG and CACATG in the opposite strand, respectively) elements were found in the 2.0 kb promoter regions upstream...
of their coding sequences (Fig. 7B). Based on the effector and reporter constructs (Fig. 7C), a dual luciferase assay was performed to examine the potential interaction of LrNAC35 with PhC4H, Ph4CL, PhHCT and PhCCR promoters in petunia leaves. The co-expression of 35S::LrNAC35 with pPh4CL::LUC led to a 5.9-fold rise in firefly luciferase (LUC) activity, whereas no significant change in LUC activity was detected for the pPhC4H/pPhHCT/pPhCCR::LUC constructs (Fig. 7D).

**LrNAC35 affects susceptibility to TMV**

To determine whether LrNAC35 participates in the response to other viruses, a TMV vector expressing green fluorescent protein (TMV-GFP) was used to inoculate the transgenic and WT petunia lines. Under UV irradiation, the LrNAC35-overexpressing lines had visibly smaller sizes of fluorescent foci than the WT lines at 6 dpi (Fig. 8A,B). This fluorescence variation was consistent with the measured transcript levels of GFP, which were decreased significantly in transgenic petunia lines compared to WT lines (Fig. 8C). At 4 and 6 dpi, the petunia plants overexpressing LrNAC35 accumulated lower abundances of TMV-CP transcripts than the WT plants (Fig. 8D).

To further determine the impact of PhNAC35 silencing on the defence response to TMV, TRV empty vector- and TRV-PhNAC35-infected petunia leaves were challenged with TMV-GFP. A clearly enlarged fluorescent area and increased accumulation of TMV-CP were found in PhNAC35-silenced petunia leaves compared to empty vector control (Fig. S7E,F, see Supporting information).

**DISCUSSION**

The wild lily species *L. regale* displays exceptional resistance to various biotic stress factors and has drawn extensive attention from lily breeders and plant biologists. Interspecific hybridization between *L. regale* and *L. rubellum* (Niimi et al., 1996) or *L. nobilissimum* (Obata et al., 2000) has been performed with attempts to introduce resistance traits of *L. regale* to other species sensitive to fungi or viruses. The *L. regale* × *L. nobilissimum* hybrids show some resistance to fungal disease, including *Fusarium* and *Botrytis*. Analogously, Lim et al. (2003) found that *L. regale* is highly resistant to *Fusarium oxysporum*. In recent reports, differentially expressed transcripts in *L. regale* response to *F. oxysporum* have been identified using the SSH method.
Transcriptional regulation of antiviral defence

(Rao et al., 2013). Transcriptome-wide identification has also been performed to characterize the microRNAs (Gao et al., 2017) and genes (Cui et al., 2018a) with significantly changed expression in Botrytis elliptica-infected L. regale, and two TFs, LrWRKY4 and LrWRKY12, have been characterized as important regulators of resistance to B. cinerea (Cui et al., 2018b). In this study, we employed a comparative transcriptome approach to dissect the antiviral molecular mechanism in L. regale.

We screened the DEGs encoding putative TFs in CMV-infected L. regale leaves. One NAC TF gene, LrNAC35, was identified to be consistently and significantly up-regulated upon virus infection. Protein modelling analysis revealed that three residue groups (G1, G2 and G6) of LrNAC35 within the NAC domain seem to be associated with potential DNA binding (fig. 3E). As reported by Welner et al. (2012), G2 has been suggested to be related to DNA binding specificity recognition, whilst G1 and G6 may affect the binding affinity. The results also showed a great resemblance with AtNAC19 in DNA binding, suggesting that the NAC domain is highly conserved in LrNAC35 in comparison with other NAC proteins. LrNAC35 may adopt a similar DNA-binding mechanism with AtNAC19.

LrNAC35 belongs to the ONAC022 subgroup of the NAC family in plants (fig. 3B). Several members of the ONAC022 subgroup have been linked with responses to fungal or viral stimuli.

LrNAC35 transcripts also increased significantly in all resistant or susceptible lily species infected with two other common lily viruses, LMoV and LSV. It is particularly interesting that the expression of LrNAC35 appears not to be in complete accord with virus-resistant levels of five species when challenged with LSV (fig. 4C). This may be explained by the potential genetic variations in LrNAC35 across different lily species. This hypothesis is supported by several previous studies in which plant sensitivity to viral diseases has been associated with a single-nucleotide or amino acid polymorphism in the defensive gene or protein (Lee et al., 2010; Ling et al., 2009; Wang et al., 2011). Thus, we performed sequence analysis of LrNAC35 by amplifying its complete coding sequences and translating the nucleotides to amino acids. Sequence alignment verified that L. brownii and L. tigrinum had four and one amino acid variations in the amino acid sequences for example, AtNAC94 transcripts accumulate more at fungus-infected sites with hypersensitive cell death in Arabidopsis (Michel et al., 2006). Arabidopsis rosette leaves infected with cabbage leaf curl virus display elevated abundances of AtNAC36 and AtNAC42 (Ascencio-Ibánez et al., 2008). Our RNA-Seq and qRT-PCR analyses based on CMV infection tests showed that the up-regulation of LrNAC35 is probably associated with CMV resistance in L. regale (Table 2 and fig. 4A).

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Fig. 6  Increased resistance to CMV infection in transgenic petunia plants overexpressing LrNAC35. (A) Disease symptoms of wild-type (WT) and LrNAC35-overexpressing lines (3-1, 5-6 and 12-3) at 18 days post-inoculation (dpi) with CMV. The magnified views of symptoms in systemically infected leaves with CMV are indicated as the insets. Four-leaf-stage seedlings were used for inoculation. qRT-PCR (B) and western blot (C) analyses of CMV coat protein (CMV-CP) transcript and its protein levels in the uppermost leaves of WT and overexpression lines at 8 or 12 dpi with CMV and the leaf samples at 12 dpi were used for western blots. 26S rRNA and actin were used as a reference gene and protein, respectively. Disease symptoms (D) and relative cell death area (E) of inoculated leaves of WT and LrNAC35-overexpressing lines (3-1, 5-6 and 12-3) at 6 dpi with CMV. (F) Electrolyte leakage (conductivity) in the inoculated leaves of WT and transgenic lines challenged with CMV at intervals. (G) Klason lignin content in CMV-inoculated leaves of WT and transgenic lines at different time points. Error bars represent SE of the mean from three biological replicates. Asterisks indicate significant difference as evaluated by Student’s t-test at P < 0.05.
of LrNAC35, respectively, compared to three resistant species (Fig. S8A, see Supporting information). *L. brownii also exhibited the most polymorphic nucleotide sites (Fig. S8B, see Supporting information). This could perhaps explain the discrepancy between higher LrNAC35 transcript levels and lower resistance levels in *L. brownii during LSV infection.

Apart from virus-caused induction, increased expression of LrNAC35 under low temperature treatment may suggest the involvement of LrNAC35 in *L. regale cold tolerance (Fig. 4D). This notion is supported by the report that a mutant of AtLOV1, an Arabidopsis homologue of LrNAC35, exhibits enhanced hypersensitivity to cold, while the AtLOV1 gain-of-function allele displays reduced hypersensitivity (Yoo et al., 2007). An opposite expression of LrNAC35 was observed in *L. regale leaves during salt stress (Fig. 4D). The biological role of LrNAC35 in response to high salinity awaits further investigation.

Moreover, we also measured the effects of plant hormone treatments, including ET, ABA, SA and JA, on LrNAC35 transcripts. These plant hormones have all been shown to affect virus resistance in different plants (Alazem and Lin, 2015). In the present study, expression analysis demonstrated that LrNAC35 was significantly up-regulated in ET-, ABA- and SA-treated *L. regale leaves (Fig. 4E), providing further support that these hormones may be involved in virus resistance in plants. The crucial involvement of LrNAC35 in *L. regale response to viruses may be attributed to the interplay between LrNAC35 and these hormones. Future studies
will examine the hormone variation in WT and transgenic plants overexpressing LrNAC35.

We verified the enhanced resistance to CMV conferred by LrNAC35 overexpression in transgenic petunia plants (Fig. 6A–F). We also detected reduced fluorescence signals and TMV-CP expression in transgenic lines infiltrated with an artificially modified vector, TMV-GFP (Fig. 8), but no significant difference in disease symptoms was observed between TMV-GFP-infected transgenic and WT lines (data not shown). It was speculated that the GFP tag may have repressed the full manifestation of visual symptoms caused by TMV infection. In addition, LrNAC35's function in antiviral defence was validated through the TRV-VIGS assay of a petunia orthologous gene PhNAC35 (Fig. S7, see Supporting information). We observed lessened symptoms of HR-like necrotic lesions and reduced electrolyte leakage in systemically infected or inoculated leaves of overexpression plants with CMV compared to WT lines. Earlier studies have indicated that virus-induced HR is correlated with increased lignin, which is a major constituent of the plant secondary cell wall (Kimmins and Wuddah, 1977). Lignin deposition perhaps acts as a physical barrier in preventing the spread of viruses (Candela et al., 1994; Nicholson and Hammerschmidt, 1992).

There have been a large number of publications on the role of lignification or lignin biosynthetic genes in plant responses to fungal penetration (Moura et al., 2010; Wang et al., 2018; Zeyen et al., 1995). However, the reports on lignin’s function in response to viruses are fewer and sometimes controversial. Tobacco necrosis virus and tomato spotted wilt virus infections increase lignin content in dwarf bean (Kimmins and Wuddah, 1977) and petunia leaves (Quecini et al., 2007), respectively, but Kofalvi and Nassuth (1995) found no significant change in wheat streak mosaic virus-infected wheat leaves. Application of aminooxyacetate, a competitive inhibitor of phenylalanine ammonia-lyase upstream of the lignin biosynthesis pathway, to tobacco leaves enlarges the size of TMV-induced necrotic lesions (Massala et al., 1980). A significant discovery is that synthetic or natural lignin could inhibit the replication and cytopathogenicity of human immunodeficiency virus infection (Nagashima et al., 1992).

Indeed, we detected higher lignin content in LrNAC35-overexpressing plants than WT plants inoculated with CMV (Fig. 6G). It appears likely that lignin is an essential intermediate in transcriptional modulation of antiviral defence by LrNAC35. This hypothesis is further confirmed by the altered abundances of several lignin biosynthesis-related genes in LrNAC35-overexpressing lines, and a particularly direct activation of Ph4CL promoter by LrNAC35 (Fig. 7A,D). However, the specific DNA binding sites recognized by LrNAC35 remain enigmatic. To date, multiple cis-acting elements have been clearly identified in the target gene promoters

Fig. 8 Enhanced resistance to TMV infection in LrNAC35-overexpressing transgenic petunia plants. GFP fluorescent foci (A) and relative fluorescent area (B) in the inoculated leaves of wild-type (WT) and transgenic petunia lines (3-1, 5-6 and 12-3) at 6 days post-inoculation (dpi) with Agrobacterium bearing no TMV vector (mock control) or TMV-GFP. Photographs were taken under UV light. Scale bars = 2.0 mm. qRT-PCR analysis of transcripts of GFP (C) and TMV-CP (D) encoding TMV coat protein in the leaves of WT and transgenic plants at 4 or 6 dpi with TMV-GFP, and the samples at 6 dpi were used for assessment of GFP expression. Error bars represent SE of the mean from three biological replicates. Asterisks denote significant difference as calculated by Student's t-test at P < 0.05.
of NAC members. For example, a complete NAC recognition sequence containing the core elements CATGT or CACG was determined in Arabidopsis (Tran et al., 2004). AtNAC19, AtNAC55 and AtNAC72 physically bind to the CATGTG motif of the ERD1 promoter (He et al., 2005). Two NAC proteins, SND1 and VND7, specifically bind to a secondary wall NAC binding element (SNBE) (Zhong et al., 2010). The binding sequence of ATAF2, an NAC TF, harbours an imperfect palindrome CAAATNNNATTTG (Huh et al., 2012). In a recent study, ThNAC13 was reported to bind to NACRS and CBNAC elements with the core sequence CGT(A/G) and GCTT, respectively (Wang et al., 2017). In the present study, we only identified the CACG and CATGTG motifs in the promoter regions of Ph4CL (Fig. 7B). The unanswered question is whether LrNAC35 could bind to these two motifs or to other unrevealed ones. Thus, a deletion analysis of the Ph4CL promoter is required to identify the exact DNA binding sites of LrNAC35 in the subsequent studies.

Given the impact of LrNAC35 overexpression on several lignin biosynthesis-related genes, we searched the corresponding orthologous unigenes against our RNA-Seq data in CMV-infected L. regale, and two 4CL and three CCR homologues were identified. Although all these homologues displayed a false discovery rate (FDR) > 0.05, their transcript abundances were increased by 2.0- to 4.8-fold upon CMV infection (Table S5, see Supporting information). We found no orthologous genes of PhC4H and PhHCT in our transcriptome data, probably due to a very large genome size in Lilium (Siljak-Yakovlev et al., 2003) and an incomplete coverage in our Illumina RNA-Seq approach.

In summary, our results suggest that LrNAC35 plays an important role in antiviral defence by mediating the biosynthesis of lignin. The identification of LrNAC35, arising from a comprehensive transcriptome sequencing effort, provides a valuable genetic solution to reduce the susceptibility of lilies to viral disease. To find more promising candidate genes, functional screening of massive transcriptome data is required. For instance, high-throughput VIGS is an attractive tool for permitting the quick knockdown of gene expression in plants, and a CMV-based VIGS system has been established in L. leichtlinii (Tasaki et al., 2016). Using the VIGS method to unravel the functions of candidate genes in response to CMV or other viruses in Lilium, not in heterologous model plants, should be pursued in future work.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Wild lily species L. regale seeds were planted in small pots filled with sterile soil mix (peat:perlite:vermiculite = 3:1:1 v/v/v) and germinated in a growth chamber at 22/20 °C day/night temperature with a 16/8 h light/dark photoperiod. The second newly sprouted leaves with relatively large sizes were inoculated with CMV for subsequent viral detection, transcriptome assay and expression analysis of candidate genes. Uppermost young leaves of 6-week-old plantlets of bulb-propagated L. regale, L. pumilum, L. duchartrei, L. brownii and L. tigrinum were used for assessment of LrNAC35 expression upon CMV, LMoV and LSV inoculation, abiotic stress and hormone treatments. These lily plants were cultivated in the germplasm resource nursery under 25/22 °C day/night temperature and natural photoperiods. Tissue-specific expression analysis of LrNAC35 was carried out using rootlets, outer bulb scales, top stems, young leaves, floral tissues at anthesis (petals, pistils and stamens) and mature seeds collected from L. regale plants at 12 or 16 weeks after bulb germination. To study the LrNAC35's function in resistance response to CMV, we selected petunia (P. hybrida 'Mitchell Diploid') as material for genetic transformation experiments. Petunia seeds were sown in a 36-well tray, and four-leaf-stage seedlings were then transferred to pots under identical chamber conditions to those described above. The leaves three to eight from terminal were collected as explant source for genetic transformation.

Inoculation assay

Viral inoculum of CMV strain LS (CMV-LS, subgroup II) was obtained from the infected leaves of Lilium Oriental hybrids cultivar Sorbonne, while both strains of LMoV and LSV were isolated from the wild species L. davidii. Leaf tissues infected with three types of viruses were separately homogenized with kieselguhr in 100 mM phosphate buffer adjusted at pH 7.0 (1:6 w/v) to prepare infectious sap. The sap from leaves of virus-free plants was used as mock control. The virus preparations were rub-inoculated onto healthy young leaves of wild lily species, WT and transgenic petunia plants through the mechanical method (Hull, 2009). An agroinjection method with TMV-GFP plasmids was used to inoculate petunia (Dorokhov et al., 2006). For functional analysis of LrNAC35 in petunia, virus symptom development was evaluated during the post-inoculation growth periods. Accumulation levels of CMV coat protein (CMV-CP), GFP and TMV-CP in the infected leaves were tested via qRT-PCR (Sun et al., 2017) or western blot (Jeon et al., 2017). GFP fluorescence was monitored using a Blak-Ray long-wave ultraviolet lamp (UV products, Upland, CA, USA; Model B 100AP) and photographed using a Canon EOS 40D digital camera. The cell death and GFP fluorescence at the infection sites were quantified by measuring pixel sizes of the necrotic and fluorescent spots using Photoshop CS6. The calculation of relative cell death and fluorescence area was based on the quantitative data, and the lowest relative data was set to 1.0.

RNA extraction, library construction and sequencing

Lilium regale leaves from 20 seedlings at 48 hpi with CMV or mock were randomly divided into two groups serving as two independent biological replicates. Ten seedlings for each replicate were pooled to prepare RNA samples. Total RNA was isolated using
the modified cetyltrimethylammonium bromide (CTAB) method (Li et al., 2011) and purified with RNase-free DNase I (Promega, Madison, WI, USA) to avoid genomic DNA contamination. The quality and quantity of RNA was assessed using a NanoDrop ND-2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Generation of cDNA libraries and sequencing projects was carried out using Illumina protocols at Gene Denovo Bio-Technology Co., Ltd (Guangzhou, China). Briefly, mRNA harbouring poly (A) (A) was enriched through magnetic beads with oligo(dT) and randomly chopped into small fragments. These pieces were used as templates to synthesize cDNAs. The resulting cDNAs were subjected to purification and the 3'-end repair process and then ligated with sequencing adaptors. The ligation products were size-selected, PCR-amplified and sequenced on an Illumina HiSeq2000 platform. The raw transcriptome reads were submitted to the NCBI Sequence Read Archive (SRA) database under accession no. SRP193127.

RNA-Seq data processing

Raw data/reads from the sequencing machine were yielded through base calling. Before assembly, raw reads were handled by removing reads harbouring adaptor sequences, more than 10% of unknown nucleotides (N) and more than 40% of low-quality (Q value ≤ 10) bases using a Perl script tool. The generated high-quality clean reads were assembled de novo using Trinity software (v. 2.1.1) with k-mer size parameter set to 25 by default (Grabherr et al., 2011). Clean data were mapped back onto the assembled transcriptome sequences, and the reads for each unigene were counted based on the mapping results. Trinity combined reads with certain length of overlap to create longer fragments not containing N or contigs. Next, these contigs were processed using sequence clustering software TGICL (v. 2.1) (Pertea et al., 2003) to form sequences longer than 200 bp, which were defined as unigenes.

A homology search of all unigenes against the public Non-Redundant (NR) (released on July 24, 2015, http://blast.ncbi.nlm.nih.gov/), Swiss-Prot (released on July 24, 2015, http://www.expasy.ch/sprot), Clusters of Orthologous Groups (COG) (released on July 24, 2015, http://www.ncbi.nlm.nih.gov/COG) and KEGG (released on July 27, 2015, https://www.genome.jp/kegg) databases with a cut-off e value of 1 × 10−5 was performed for functional annotation, sequence orientation and coding region prediction, using the standoff BLAST (v. 2.2.29). Transcript levels of unigenes were calculated and normalized to reads per kilobase of unigenes was carried out using Blast2GO (v. 2.3.5) (Conesa et al., 2005) and then the WEGO tool (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) (Ye et al., 2006) was used to perform GO functional categorization of unigenes. To identify the active metabolic pathways in CMV-infected L. regale leaves, a BLAST search against the KEGG database was employed to perform the mapping of DEGs to reference canonical pathways (Wixon and Kell, 2000). A formula was used for calculation of P values (Lu et al., 2012), which was gone through FDR correction. KEGG pathways with FDR ≤ 0.05 were defined as significantly enriched.

**Semiquantitative RT-PCR and qRT-PCR**

Total RNA from lily and petunia tissues was extracted using the modified CTAB method and TRIzol reagent (Invitrogen, Carlsbad, CA, USA), respectively. After genomic DNA elimination, 2–5 μg of RNA samples was reversely transcribed to first-strand cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan). PCR amplification was performed using Premix Taq DNA polymerase (TaKaRa, Kyoto, Japan), and the abundances of products were analysed by electrophoresis on 1% agarose gel stained with GelRed (Biotium, Hayward, CA, USA). q-RT-PCR was conducted using the SYBR Green PCR Master Mix (2×) (Applied Biosystems, Foster City, CA, USA) in a LightCycler480 Real-Time PCR System (Roche Diagnostic, Basel, Switzerland). *Glyceraldehyde-3-phosphate dehydrogenase* (*LrGAPDH*) and *LrActin* served as internal controls in Lilium species, and the reference gene in petunia was 26S ribosomal RNA. Data were calculated using the 2−△△CT method (Livak and Schmittgen, 2001). A set of oligonucleotide primers used for analyses of virus accumulation, RNA-Seq validation and gene expression are listed in Table S6 (see Supporting information). The unigene sequences used for expression or functional analysis in this study are provided in Fig. S9 (see Supporting information).

**Isolation and sequence analysis of LrNAC35**

A 1366-bp cDNA sequence of *LrNAC35* containing a complete coding region was selected from up-regulated TFs in CMV-infected *L. regale* leaves. To amplify the coding sequences of *LrNAC35* from various *Lilium* species, the forward primer 5′-ATGGCAATTACCGCAGCCATGAG-3′ and reverse primer 5′-TCACTCCCATGCTTCTGGGAT-3′ were used. Its deduced amino acids were obtained by the ExPaSy translated tool (http://web.expasy.org/translate/). Proteins homologous to LrNAC35 were found by a BLAST search. The conserved motifs were identified based on comparative NAC domains analysis between *O. sativa* and *Arabidopsis* (Ooka et al., 2003). Multiple protein alignments were performed using DNAMAN (v. 5.2.2) (Wang, 2015). A phylogenetic tree was constructed using MEGA (v. 4.0.2) (Tamura et al., 2007). Homologous templates for protein modelling were identified from the RCSB Protein Data Bank (https://www.rcsb.org/). The modelling process was carried out using the Modeller server (v. 9.20).
(Webb and Sali, 2014) based on sequence alignment performed in Chimera (v. 1.12) (Pettersen et al., 2004). A combination of multiple protein structures (chain A of PDBs: 1UT4, 1UT7, 4DUL, 3SWM and 3SWP) was used for the homologous modelling of LrNAC35. Only the NAC domains were used and modelled. The best model was chosen based on the lowest Discrete Optimized Protein Energy (DOPE) values and GA 341 score of 1, which suggest that these models are reliable. The final model was validated by Ramachandran plot analysis using PROCHECK (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK). Molecular visualizations were performed using PyMOL (v. 1.3r1. Schrodinger, LLC, Cambridge, MA, USA).

Abiotic stress and hormone treatments

To determine the impacts of abiotic stresses and stress-related hormones on transcript levels of LrNAC35, c. 20-cm long stems from the top were cut from 6-week-old L. regale plantlets and used for the follow-up experiments. For the cold treatment, the plants were placed into a large glass container with fresh deionized water at 4 °C in cold storage. For the salinity and dehydration treatments, the seedlings were exposed to a NaCl solution with a concentration of 150 mM and no water. For treatments with hormones, the stems were immersed in water without addition and treated with continuous 15 μM MA, 100 μM ET in a sealed chamber, or in water supplemented with 50 μM ABA, 100 μM SA and 100 μM JA. Terminal upper leaves were excised from three individual plants at five time points (0, 3, 6, 12 and 24 h) after the treatments.

Plasmid construct and petunia plant transformation

The full-length of the LrNAC35 open reading frame (ORF) sequence was PCR-amplified using the forward primer 5′-ATGGTA CCATGGCAATTACCGCAGCATT-3′ bearing a KpnI restriction site, and reverse primer 5′-ATGTGACTCTCCATAGCTTGTCTG-3′ bearing a SaI restriction site. To generate the overexpression construct, the amplified DNA fragment was inserted into the corresponding position of a modified pCAMBIA1300 vector downstream of the CaMV 35S promoter in the sense orientation. Agrobacterium tumefaciens LBA4404 was transformed with the recombinant plasmid by electroporation. Agrobacterium-mediated genetic transformation and regeneration of 'Mitchell Diploid' leaf discs were carried out according to a previously described protocol (Sun et al., 2016a). After a continuous hygromycin selection on MS plates and cultivation process in soil mixtures, the T2 lines 3-1, 5-6 and 12-3, verified as homozygotes, were finally chosen for phenotype and further antiviral analyses of LrNAC35.

Western blot assay

The CMV-infected leaves were homogenized in liquid nitrogen, and proteins were prepared using a Plant Total Protein Extraction Kit (Sigma-Aldrich, St Louis, MO, USA). Equal amounts of samples were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The fractionated proteins were transferred to a polyvinylidene fluoride membrane (0.45 μm) on a Mini Trans-Blot Cell (Bio-Rad, Richmond, CA, USA). The primary antibody anti-CMV-CP was applied to probe the blots, which were then incubated with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase. The antigen–antibody complexes were visualized using an ECL western blot detection kit (Pierce, Waltham, MA, USA). The actin antibody was used for examination of reference protein.

VIGS assay

A 310-bp fragment of PhNAC35, the petunia homologue of LrNAC35, was PCR-amplified and cloned into TRV plasmid. The generated TRV-PhNAC35 was electrotansformed into A. tumefaciens GV3101. The transformed bacteria were cultured, centrifuged and resuspended in the infiltration buffer as previously described (Sun et al., 2017). The bacterial mixture containing TRV1 and TRV2 plasmids in a 1:1 ratio was used to infiltrate four-leaf-stage petunia seedlings. For virus inoculation, mock control and CMV or TMV-GFP were inoculated onto the leaves of petunia seedlings at 5 dpi with TRV empty vector and TRV-PhNAC35. PCR primers to sequence beyond the inserted fragment for silencing were used to examine expression levels of PhNAC35 in VIGS assay (Table S6, see Supporting information).

Electrolyte leakage assay

Leaf discs, 8 mm in diameter, were prepared from the leaves of WT and LrNAC35-overexpressing transgenic petunia lines at intervals after CMV inoculation using a hole punch, and thoroughly immersed in 20 mL of deionized water with gentle shaking at room temperature. Conductivity was measured using an Orion conductivity meter (Thermo Scientific, Waltham, MA, USA).

Measurement of Klason lignin content

The CMV-inoculated leaves of WT and LrNAC35-overexpressing transgenic petunia plants were harvested, ground into a fine powder in liquid nitrogen and sequentially extracted using a method previously described (Wang et al., 2012). Sulphuric acid (72%) was added to the methanol extract upon air-dry treatment for hydrolysis reaction. The hydrolysate was diluted with distilled water to adjust the acid concentration to 4% and boiled under reflux for 1 h. Lignin obtained as an insoluble solid residue was filtered, washed with hot water and freeze dried. Determination of Klason lignin content, expressed as a weight percentage of dried cell wall residues, was conducted using the Klason technique (Dence, 1992).

Dual luciferase assay

The dual luciferase assay was performed using a previously reported method (Chen et al., 2017). The full-length ORF of LrNAC35 was PCR-amplified and introduced into pGreenII62-SK vector,
with the recombinant construct being used as effector. The complete promoter regions of PhC4H, Ph4CL, PhHCT and PhCCR were amplified using the primers listed in Table S6 (see Supporting information). Next, these promoter sequences were ligated into pGreenII0800-LUC vector to generate reporter plasmids, where the promoters drive a firefly luciferase (LUC) gene and the CaMV 35 promoter drives a Renilla luciferase (REN) gene. The A. tumefaciens GV3101 cells transformed with the effector and reporter plasmids were co-infiltrated into the petunia plants at the four-leaf stage. The LUC and REN enzyme activities were tested by a Tecan Infinite M200 luminometer (Männedorf, Switzerland) and represented as the LUC/REN ratio.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Sequences of all assembled unigenes in *Lilium regale*.

Fig. S2 Length distribution of unigenes in *Lilium regale*.

Fig. S3 Validation of RNA-Seq data by qRT-PCR. Nine unigenes were randomly selected for expression analysis in mock- and CMV-inoculated *Lilium regale* leaves at 48 h post-inoculation (hpi). *LrActin* was used as a reference gene. Error bars indicate standard error (SE) of the mean from three biological replicates. Significance of difference was calculated using Student’s *t* test (*P* < 0.05) and is shown as asterisks. Unigene0000074, cytochrome *P450 B61B1*; Unigene0004763, lecithine-cholesterol acyltransferase 4; Unigene0007097, endonuclease exonuclease phosphatase family protein; Unigene0011570, auxin-induced 15A; Unigene0013897, unknown protein; Unigene0017759, mannose-specific lectin 3; Unigene0063829, argonaute 1; Unigene0073681, ABC transporter G family member 11; Unigene0079669, geranylgeranyl dipshosphate synthetase.

Fig. S4 Principal component analysis of *Lilium regale* transcriptome data.

Fig. S5 GO functional classification of differentially expressed genes in *Lilium regale*.

Fig. S6 Full-length cDNA sequence of *LrNAC35* and its deduced amino acids. *LrNAC35* cDNA sequence harbours a 1077-bp open reading frame region encoding a polypeptide of 358 amino acids. *LrNAC35* cDNA sequence harbours a 1077-bp open reading frame region encoding a polypeptide of 358 amino acids.
plants with PhNAC35-VIGS silencing. (A) Disease symptoms of TRV empty vector- and TRV-PhNAC35-infected petunia plants at 14 days post-inoculation (dpi) with mock control and CMV. (B) qRT-PCR analysis of PhNAC35 expression levels in uppermost leaves of TRV empty vector- and TRV-PhNAC35-infected petunia plants at 14 dpi with mock control and CMV. qRT-PCR (C) and western blot (D) analyses of CMV coat protein (CMV-CP) transcript and its protein levels in the uppermost leaves of TRV constructs-infected petunia plants at 14 dpi with CMV. 26S rRNA and actin were used as a reference gene and protein, respectively. GFP fluorescent foci (E) and qRT-PCR analysis of transcripts of TMV-CP (F) encoding TMV coat protein in the leaves of TRV constructs-inoculated petunia plants at 6 dpi with TMV-GFP. Four-leaf-stage petunia seedlings were used for VIGS assay, and the seedlings at 5 dpi with TRV constructs were thereafter inoculated with mock control, CMV and TMV-GFP. Error bars represent SE of the mean from three biological replicates. Asterisks indicate significant difference as determined by Student’s t-test at \( P < 0.05 \).

**Fig. S8** Sequence analysis of LrNAC35 from five *Lilium* species. Alignment of LrNAC35 amino acid sequences (A) and nucleotide sequences (B) from *L. regale*, *L. pumilum*, *L. duchartrei*, *L. brownii* and *L. tigrinum*. Identical amino acids and nucleotides are shaded in black, while similar ones are shaded in grey.

**Fig. S9** Sequences of unigenes used for qRT-PCR or functional analysis.

**Table S1** Functional annotation of all assembled unigenes in *Lilium regale*.

**Table S2** Differentially expressed genes in *Lilium regale*.

**Table S3** KEGG pathway annotation of differentially expressed genes in *Lilium regale*.

**Table S4** Differentially expressed transcription factors in *Lilium regale*.

**Table S5** Unigenes associated with lignin synthesis exhibiting increased expression in *Lilium regale*.

**Table S6** Primers used for qRT-PCR, gene promoter and fragment amplification.