Global analysis of the AP2/ERF gene family in rose (Rosa chinensis) genome unveils the role of RcERF099 in Botrytis resistance

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Abstract

Background: The AP2/ERFs belong to a large family of transcription factors in plants. The AP2/ERF gene family has been identified as a key player involved in both biotic and abiotic stress responses in plants, however, no comprehensive study has yet been carried out on the AP2/ERF gene family in rose (Rosa sp.), the most important ornamental crop worldwide.

Results: The present study comprises a genome-wide analysis of the AP2/ERF family genes (RcERFs) in the rose, involving their identification, gene structure, phylogenetic relationship, chromosome localization, collinearity analysis, as well as their expression patterns. Throughout the phylogenetic analysis, a total of 131 AP2/ERF genes in the rose genome were divided into 5 subgroups. The RcERFs are distributed over all the seven chromosomes of the rose, and genome duplication may have played a key role in their duplication. Furthermore, Ka/Ks analysis indicated that the duplicated RcERF genes often undergo purification selection with limited functional differentiation. Gene expression analysis revealed that 23 RcERFs were induced by infection of the necrotrophic fungal pathogen Botrytis cinerea. Presumably, these RcERFs are candidate genes which can react to the rose’s resistance against Botrytis cinerea infection. By using virus-induced gene silencing, we confirmed that RcERF099 is an important regulator involved in the B.cinerea resistance in the rose petal.

Conclusion: Overall, our results conclude the necessity for further study of the AP2/ERF gene family in rose, and promote their potential application in improving the rose when subjected to biological stress.

Keywords: Rosa sp., AP2/ERF gene family, Botrytis cinerea, Virus-induced gene silencing

Background

Transcription factors are important regulators of the expression of various inducible genes in plants, and play an indispensable role in plant growth, development, stress response, as well as pathogen defence [1]. Transcription factors usually comprise a nuclear localization signal, a DNA binding domain, a transactivation domain, as well as an oligomerization site. These domains determine the subcellular localization, cis-regulatory elements binding, and the regulating function of transcription factors [2].

The AP2/ERF superfamily is one of the largest transcription factor gene family in plants, wherein a total of 147 AP2/ERF family members have been identified in Arabidopsis. The AP2/ERF gene family consists of the AP2/ERF domain comprising 60 to 70 amino acids, and recognizes the cis-regulatory element GCC box or DRE elements which regulate the reaction of target genes [3]. The AP2/ERF gene family can be further categorized...
into five subfamilies, to example ERF, AP2 (APET ALA2), DREB (dehydration-responsive element binding), RAV (related to ABI3/VP1) and Soloist [4–6]. The AP2/ERFs that regulate growth and development throughout the plant’s life cycle have been detected. The AP2/ERFs also play a very important role when the plant is exposed to abiotic stresses, such as dehydration, salinity, low temperature or heat stress. For example, transgenic Arabidopsis that overexpresses AtERF4 is more sensitive to drought stress and has a lower resistance to Sodium chloride [7]. In addition, overexpressing the RAP2.6 gene (RELATED TO AP2.6, encodes an ERF transcription factor) results in a sensitive phenotype to ABA (Abscisic Acid) and salt/osmotic stress during germination and the early growth stage of Arabidopsis [8].

More importantly, the AP2/ERF gene family is one of the transcription factors considered to be involved in plant defence responses against various phytopathogens [9–12]. For example, the transcript of ERF1 is induced significantly subsequent to the inoculation of necrotrophic fungi Botrytis cinerea, and overexpression of ERF1 in Arabidopsis enhanced its resistance to both B. cinerea and Plectosphaerella cucumerina [13]. Overexpressing ERF5 or ERF6 also increased resistance to B. cinerea in Arabidopsis, and the erf5 erf6 double mutant showed a significant increase in susceptibility [14].

Rose is the most popular ornamental crop and accounts for over 30% of total cut-flower sales worldwide [15]. However, the flower is a fragile organ and transportation over long distances causes rose flowers to be affected by post-harvest diseases such as gray mold caused by B. cinerea. The function of AP2/ERF transcription factors in disease resistance has been characterized in model plants Arabidopsis as well as many other plant species. However, no rose AP2/ERF family genes involved in disease resistance have yet been identified.

Recently, we performed a de novo RNA-Seq analysis of rose petals infected by B. cinerea. This transcriptome study revealed a large number of rose genes, including AP2/ERF family transcription factors, were significantly up-regulated and implied their involvement of resistance against B. cinerea [16]. In the present study, genome-wide identification and analysis of the AP2/ERF gene family in the rose were carried out. By using virus-induced gene silencing (VIGS), we further confirmed that RcERF099 plays a significant role in B. cinerea resistance in rose flowers.

**Results**

**Identifying RcERF genes in the rose genome**

In order to identify the potential AP2/ERFs of R. chinensis, we downloaded the AP2/ERF HMM profile (PF00847) from the Pfam database. Using this profile as a query, the HMM search of the rose genome finally lead to the identification of 137 candidate RcERF genes. Conserved Domains Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and ExPASy (http://web.expasy.org/protparam/) were employed to verify all candidate RcERFs contain a single AP2/ERF motif. We further removed any sequence having less than 150 amino acids, and finally obtained a total of 131 non-redundant RcERF genes. All these 131 ERF family genes can be mapped onto rose chromosomes and we designated the genes RcERF001 to RcERF131 in accordance with their chromosome order.

The length of proteins encoded by RcERF family genes varies from 150 to 832 amino acids, with an average length of 298 amino acids. The longest (RcERF052) contains 832 amino acids, whereas the shortest just has 150 amino acids (RcERF093 and RcERF095). Table 1 summarizes detailed information of all 131 RcERF genes, including their accession numbers, chromosome locations, exon and intron details, protein size and classification.

**Chromosomal localization and microsynteny analysis**

131 RcERF genes were located on all 7 rose chromosomes, as depicted in Fig. 1. Chromosome 2 contains the largest number of RcERF genes (31), followed by chromosome 7 (26). Chromosomes 3 and 5 contain the least number of chromosomes (11). The RcERF genes were unevenly distributed over 7 chromosomes. 8.40% of RcERFs were located in the long arm of chromosomes 3 and 5, 23.66% of RcERFs were located in chromosome 2, 15.27% of RcERFs were located in chromosome 1, 10.69% and 13.74% of RcERFs were distributed over chromosomes 4 and 6. Chromosome 7 contains 19.85% RcERFs, and they were distributed over both the long and short arms.

Furthermore, we studied RcERF duplication events, and discovered in total 21 gene pairs in the rose genome (Table 2). Only one gene pair was located on the same chromosome (RcERF021 and RcERF042), indicating that they are likely to be tandem repeats. The remaining 20 gene pairs were located on different chromosomes, and indicated that segmental duplication may occur in these regions (Fig. 2).

To explore the selective constraints among duplicated RcERF genes, we calculated the ratio of non-synonymous (Ka) to synonymous (Ks) nucleotide substitutions (Ka/Ks ratio) of 21 pairs of duplicated genes (Table 2). A Ka/Ks ratio < 1 indicates a negative or purifying selection of gene pairs, whereas Ka/Ks > 1 depicts a positive selection. Our study revealed that the Ka/Ks ratio for all RcERF gene pairs is < 0.4 (Table 2). These data indicate that RcERF gene pairs had undergone a purifying selection, and functional differentiation is limited.
Table 1 Members of the AP2/ERF gene family in rose genome

| Gene    | Accession number | Chr. | Position | Intro | Exon | CDS (bp) | AA \(^a\) | Subfamily |
|---------|------------------|------|----------|-------|------|----------|--------|-----------|
| RcERF001| RchiOBhm_Chr1g0331141 | 1    | 20.92    | 6     | 7    | 1203     | 401    | AP2       |
| RcERF002| RchiOBhm_Chr1g0346421 | 1    | 38.78    | 0     | 1    | 831      | 277    | DREB      |
| RcERF003| RchiOBhm_Chr1g0347621 | 1    | 40.31    | 0     | 1    | 819      | 273    | ERF       |
| RcERF004| RchiOBhm_Chr1g0347631 | 1    | 40.33    | 0     | 1    | 639      | 213    | ERF       |
| RcERF005| RchiOBhm_Chr1g0347641 | 1    | 40.38    | 0     | 1    | 717      | 239    | ERF       |
| RcERF006| RchiOBhm_Chr1g0347661 | 1    | 40.38    | 0     | 1    | 651      | 217    | ERF       |
| RcERF007| RchiOBhm_Chr1g0347671 | 1    | 40.38    | 0     | 1    | 612      | 204    | ERF       |
| RcERF008| RchiOBhm_Chr1g0349631 | 1    | 42.73    | 0     | 1    | 711      | 237    | ERF       |
| RcERF009| RchiOBhm_Chr1g0358681 | 1    | 50.76    | 0     | 1    | 903      | 301    | ERF       |
| RcERF010| RchiOBhm_Chr1g0360021 | 1    | 51.85    | 0     | 1    | 633      | 211    | DREB      |
| RcERF011| RchiOBhm_Chr1g0360081 | 1    | 51.90    | 2     | 3    | 1032     | 344    | DREB      |
| RcERF012| RchiOBhm_Chr1g0364341 | 1    | 55.52    | 8     | 9    | 1371     | 457    | AP2       |
| RcERF013| RchiOBhm_Chr1g0370631 | 1    | 60.12    | 0     | 1    | 987      | 329    | DREB      |
| RcERF014| RchiOBhm_Chr1g0371151 | 1    | 60.47    | 1     | 1    | 1152     | 384    | DREB      |
| RcERF015| RchiOBhm_Chr1g0373621 | 1    | 61.76    | 0     | 1    | 858      | 286    | ERF       |
| RcERF016| RchiOBhm_Chr1g0373631 | 1    | 61.77    | 0     | 1    | 879      | 293    | ERF       |
| RcERF017| RchiOBhm_Chr1g0373641 | 1    | 61.77    | 0     | 1    | 642      | 214    | ERF       |
| RcERF018| RchiOBhm_Chr1g0373641 | 1    | 63.85    | 0     | 1    | 693      | 231    | DREB      |
| RcERF019| RchiOBhm_Chr1g0376651 | 1    | 63.86    | 0     | 1    | 699      | 233    | DREB      |
| RcERF020| RchiOBhm_Chr1g0380021 | 1    | 65.82    | 0     | 1    | 1092     | 364    | ERF       |
| RcERF021| RchiOBhm_Chr2g0088321 | 2    | 2.93     | 1     | 2    | 615      | 205    | DREB      |
| RcERF022| RchiOBhm_Chr2g0091471 | 2    | 5.12     | 0     | 1    | 765      | 255    | DREB      |
| RcERF023| RchiOBhm_Chr2g0095581 | 2    | 8.53     | 0     | 1    | 630      | 210    | DREB      |
| RcERF024| RchiOBhm_Chr2g0105221 | 2    | 16.56    | 0     | 1    | 699      | 233    | ERF       |
| RcERF025| RchiOBhm_Chr2g0105401 | 2    | 16.68    | 0     | 1    | 726      | 242    | ERF       |
| RcERF026| RchiOBhm_Chr2g0105461 | 2    | 16.74    | 0     | 1    | 639      | 213    | ERF       |
| RcERF027| RchiOBhm_Chr2g0105481 | 2    | 16.76    | 0     | 1    | 579      | 193    | ERF       |
| RcERF028| RchiOBhm_Chr2g0105501 | 2    | 16.78    | 0     | 1    | 543      | 181    | ERF       |
| RcERF029| RchiOBhm_Chr2g0105521 | 2    | 16.81    | 0     | 1    | 624      | 208    | ERF       |
| RcERF030| RchiOBhm_Chr2g0106221 | 2    | 17.67    | 9     | 10   | 1605     | 535    | AP2       |
| RcERF031| RchiOBhm_Chr2g0106241 | 2    | 17.71    | 0     | 1    | 519      | 173    | DREB      |
| RcERF032| RchiOBhm_Chr2g0108831 | 2    | 20.29    | 8     | 9    | 1980     | 660    | AP2       |
| RcERF033| RchiOBhm_Chr2g0111031 | 2    | 22.67    | 8     | 8    | 1629     | 543    | AP2       |
| RcERF034| RchiOBhm_Chr2g0115041 | 2    | 27.01    | 1     | 1    | 1047     | 349    | ERF       |
| RcERF035| RchiOBhm_Chr2g0118211 | 2    | 30.54    | 1     | 2    | 966      | 322    | ERF       |
| RcERF036| RchiOBhm_Chr2g0118251 | 2    | 30.58    | 1     | 2    | 1164     | 388    | ERF       |
| RcERF037| RchiOBhm_Chr2g0126301 | 2    | 40.60    | 0     | 1    | 1398     | 466    | ERF       |
| RcERF038| RchiOBhm_Chr2g0130611 | 2    | 46.70    | 0     | 1    | 537      | 179    | ERF       |
| RcERF039| RchiOBhm_Chr2g0132251 | 2    | 48.70    | 6     | 7    | 1074     | 358    | AP2       |
| RcERF040| RchiOBhm_Chr2g0133451 | 2    | 50.24    | 1     | 2    | 603      | 201    | DREB      |
| RcERF041| RchiOBhm_Chr2g0133601 | 2    | 50.47    | 0     | 1    | 888      | 296    | ERF       |
| RcERF042| RchiOBhm_Chr2g0135921 | 2    | 53.15    | 1     | 2    | 582      | 194    | DREB      |
| RcERF043| RchiOBhm_Chr2g0139661 | 2    | 57.18    | 0     | 1    | 786      | 262    | DREB      |
| RcERF044| RchiOBhm_Chr2g0145271 | 2    | 62.91    | 8     | 9    | 1731     | 577    | AP2       |
| RcERF045| RchiOBhm_Chr2g0147651 | 2    | 65.22    | 2     | 2    | 1176     | 392    | ERF       |
| RcERF046| RchiOBhm_Chr2g0157901 | 2    | 74.24    | 0     | 1    | 693      | 231    | ERF       |
| Gene      | Accession number | Chr. | Position | Intro | Exon | CDS (bp) | AA | Subfamily |
|-----------|------------------|------|----------|-------|------|----------|----|-----------|
| RcERF047  | RchiOBhm_Chr2g0160621 | 2    | 76.47    | 1     | 1    | 582      | 194| DREB      |
| RcERF048  | RchiOBhm_Chr2g0163201  | 2    | 78.78    | 0     | 1    | 909      | 303| RAV       |
| RcERF049  | RchiOBhm_Chr2g0166851  | 2    | 81.58    | 0     | 1    | 1071     | 357| ERF       |
| RcERF050  | RchiOBhm_Chr2g0167081  | 2    | 81.74    | 0     | 1    | 1257     | 419| ERF       |
| RcERF051  | RchiOBhm_Chr2g0169071  | 2    | 83.36    | 4     | 5    | 1377     | 459| AP2       |
| RcERF052  | RchiOBhm_Chr3g0447531  | 3    | 0.21     | 7     | 8    | 2496     | 832| AP2       |
| RcERF053  | RchiOBhm_Chr3g0449251  | 3    | 1.12     | 9     | 8    | 804      | 268| Soloist   |
| RcERF054  | RchiOBhm_Chr3g0450011  | 3    | 1.66     | 0     | 1    | 702      | 234| ERF       |
| RcERF055  | RchiOBhm_Chr3g0450351  | 3    | 1.92     | 0     | 1    | 900      | 300| ERF       |
| RcERF056  | RchiOBhm_Chr3g0461691  | 3    | 9.68     | 0     | 1    | 1791     | 597| DREB      |
| RcERF057  | RchiOBhm_Chr3g0468481  | 3    | 14.49    | 8     | 9    | 1026     | 342| AP2       |
| RcERF058  | RchiOBhm_Chr3g0472818  | 3    | 18.19    | 0     | 1    | 615      | 205| DREB      |
| RcERF059  | RchiOBhm_Chr3g0472361  | 3    | 18.24    | 0     | 1    | 600      | 200| DREB      |
| RcERF060  | RchiOBhm_Chr3g0480891  | 3    | 26.82    | 5     | 6    | 1212     | 404| AP2       |
| RcERF061  | RchiOBhm_Chr3g0481251  | 3    | 27.33    | 0     | 1    | 1047     | 349| DREB      |
| RcERF062  | RchiOBhm_Chr3g0482661  | 3    | 28.70    | 8     | 9    | 1275     | 425| AP2       |
| RcERF063  | RchiOBhm_Chr4g0392461  | 4    | 7.95     | 0     | 1    | 468      | 156| ERF       |
| RcERF064  | RchiOBhm_Chr4g0392501  | 4    | 7.98     | 0     | 1    | 804      | 268| ERF       |
| RcERF065  | RchiOBhm_Chr4g0401791  | 4    | 20.05    | 0     | 1    | 918      | 306| ERF       |
| RcERF066  | RchiOBhm_Chr4g0401801  | 4    | 20.08    | 8     | 9    | 1659     | 553| AP2       |
| RcERF067  | RchiOBhm_Chr4g0405371  | 4    | 25.78    | 6     | 7    | 1098     | 366| AP2       |
| RcERF068  | RchiOBhm_Chr4g0415231  | 4    | 39.84    | 0     | 1    | 1206     | 402| ERF       |
| RcERF069  | RchiOBhm_Chr4g0421551  | 4    | 47.20    | 1     | 2    | 1209     | 403| ERF       |
| RcERF070  | RchiOBhm_Chr4g0423581  | 4    | 49.24    | 1     | 2    | 765      | 255| ERF       |
| RcERF071  | RchiOBhm_Chr4g0428551  | 4    | 53.58    | 0     | 1    | 813      | 271| ERF       |
| RcERF072  | RchiOBhm_Chr4g0428891  | 4    | 53.79    | 1     | 2    | 708      | 236| ERF       |
| RcERF073  | RchiOBhm_Chr4g0330711  | 4    | 57.25    | 1     | 1    | 1284     | 428| ERF       |
| RcERF074  | RchiOBhm_Chr4g0352611  | 4    | 58.89    | 1     | 1    | 1041     | 347| DREB      |
| RcERF075  | RchiOBhm_Chr4g0357771  | 4    | 59.21    | 0     | 1    | 1098     | 366| RAV       |
| RcERF076  | RchiOBhm_Chr4g0405411  | 4    | 62.65    | 5     | 6    | 1299     | 433| AP2       |
| RcERF077  | RchiOBhm_Chr5g0089911  | 5    | 5.94     | 0     | 1    | 792      | 264| ERF       |
| RcERF078  | RchiOBhm_Chr5g0097711  | 5    | 6.43     | 0     | 1    | 510      | 170| ERF       |
| RcERF079  | RchiOBhm_Chr5g0097411  | 5    | 6.45     | 0     | 1    | 804      | 268| ERF       |
| RcERF080  | RchiOBhm_Chr5g0032721  | 5    | 26.47    | 0     | 1    | 750      | 250| ERF       |
| RcERF081  | RchiOBhm_Chr5g0041261  | 5    | 36.01    | 0     | 1    | 678      | 226| ERF       |
| RcERF082  | RchiOBhm_Chr5g0046591  | 5    | 42.67    | 0     | 1    | 1098     | 366| RAV       |
| RcERF083  | RchiOBhm_Chr5g0061501  | 5    | 67.00    | 5     | 6    | 855      | 285| AP2       |
| RcERF084  | RchiOBhm_Chr5g0073531  | 5    | 79.54    | 0     | 1    | 798      | 266| ERF       |
| RcERF085  | RchiOBhm_Chr5g0077201  | 5    | 83.01    | 7     | 8    | 1659     | 553| AP2       |
| RcERF086  | RchiOBhm_Chr5g0080541  | 5    | 86.52    | 0     | 1    | 1095     | 365| RAV       |
| RcERF087  | RchiOBhm_Chr5g0083271  | 5    | 88.95    | 0     | 1    | 846      | 282| ERF       |
| RcERF088  | RchiOBhm_Chr5g00257181 | 6    | 12.45    | 0     | 1    | 804      | 268| ERF       |
| RcERF089  | RchiOBhm_Chr5g00274591 | 6    | 36.05    | 1     | 2    | 1353     | 451| ERF       |
| RcERF090  | RchiOBhm_Chr5g00276671 | 6    | 38.87    | 0     | 1    | 969      | 323| ERF       |
| RcERF091  | RchiOBhm_Chr5g00284081 | 6    | 47.38    | 6     | 6    | 669      | 223| Soloist   |
| RcERF092  | RchiOBhm_Chr5g00288231 | 6    | 51.49    | 0     | 1    | 789      | 263| ERF       |
Phylogenetic and exon-intron structural analysis of RcERF genes

We performed a phylogenetic analysis on all RcERF genes using the neighbor-joining method and established a phylogenetic tree. According to their evolutionary relationships, RcERF genes are further categorized into five subfamilies with supported bootstrap values, including ERF, DREB, AP2, RAV and...
Chromosome localization of rose AP2/ERF family members. The physical distribution of each RcERF gene is listed on the seven chromosomes of *Rose chinensis*.

### Table 2: Duplication analysis of the AP2/ERF gene family

| Sequence1 | Sequence2 | Ka     | Ks     | Ka_Ks  | Effective Len | Average S-sites | Average N-sites |
|-----------|-----------|--------|--------|--------|---------------|----------------|----------------|
| RcERF021  | RcERF042  | 0.29553678 | 1.72567726 | 0.1712584 | 582           | 132           | 450            |
| RcERF012  | RcERF050  | 0.40300562 | 1.380835301 | 0.2918527 | 924           | 212.75        | 711.25         |
| RcERF048  | RcERF075  | 0.4114621 | NaN    | NaN    | 900           | 197.4166667   | 702.5833333    |
| RcERF051  | RcERF076  | 0.3331089 | 2.56556843 | 0.129917 | 1209          | 275.3333333   | 933.66666667   |
| RcERF046  | RcERF081  | 0.3163392 | 1.85921206 | 0.1701469 | 609           | 153.4166667   | 455.5833333    |
| RcERF025  | RcERF088  | 0.57783254 | 1.78941311 | 0.329174 | 708           | 160.9166667   | 547.0833333    |
| RcERF064  | RcERF092  | 0.35723109 | NaN    | NaN    | 699           | 158           | 541            |
| RcERF063  | RcERF093  | 0.36996467 | 1.47353077 | 0.2510736 | 432           | 104.4166667   | 327.5833333    |
| RcERF070  | RcERF098  | 0.6685266 | 1.81097809 | 0.369152 | 753           | 174           | 579            |
| RcERF021  | RcERF100  | 0.38250295 | 1.50870683 | 0.253303  | 612           | 138.916667    | 473.0833333    |
| RcERF040  | RcERF101  | 0.27568714 | NaN    | NaN    | 561           | 126.0833333   | 434.9166667    |
| RcERF022  | RcERF103  | 0.41399228 | 1.28764002 | 0.3215124 | 735           | 178.916667    | 556.0833333    |
| RcERF031  | RcERF104  | 0.2707983 | 1.29440563 | 0.2091327 | 429           | 104.0833333   | 324.9166667    |
| RcERF032  | RcERF105  | 0.27018563 | 1.27442854 | 0.2120053 | 1797          | 397.166667    | 1399.5833333   |
| RcERF074  | RcERF107  | 0.76307193 | NaN    | NaN    | 969           | 216.666667    | 752.8333333    |
| RcERF072  | RcERF109  | 0.57052476 | 1.55144847 | 0.3677368 | 684           | 155.416667    | 528.5833333    |
| RcERF009  | RcERF112  | 0.56506363 | 2.56420719 | 0.2203658 | 852           | 194.25        | 657.75         |
| RcERF020  | RcERF112  | 0.48408323 | NaN    | NaN    | 972           | 229.5         | 742.5          |
| RcERF019  | RcERF119  | 0.62960209 | 2.53219954 | 0.2486384 | 666           | 161.75        | 504.25        |
| RcERF003  | RcERF123  | 0.5452034 | 2.76643897 | 0.1970777 | 759           | 188.8333333   | 570.166667    |
| RcERF034  | RcERF131  | 0.34870274 | 1.24794199 | 0.2870468 | 1011          | 238.8333333   | 772.166667    |
Soloist, comprising 64, 42, 18, 4 and 3 members, respectively.

Subsequent analysis of the exon-intron structure proved to be consistent with the phylogenetic analysis results. Most of the genes clustered in the same subfamily exhibit a similar exon-intron structure. Members of the RAV subfamily do not comprise intron, however, in contrast, AP2 and Soloist subfamily genes comprise four to twelve introns. Most of the ERF and DREB subfamily members have either no intron or only one, however, some exceptions were also observed; for example, \textit{RcERF011} and \textit{RcERF045} have two introns and \textit{RcERF107} has three (Fig. 3; Table 1). These results demonstrate the presence of highly conserved structures within the subfamilies and diversity among the different subfamilies.

There is increasing evidence that AP2/ERF transcription factors play a key role in disease resistance in various plant species (Table 3). In order to evaluate \textit{RcERFs}’ involvement in rose disease resistance, we generated a composite phylogenetic tree that included defence-related ERFs in other plant species and all RcERFs (Fig. 4). In this composite phylogenetic tree, each subfamily is marked with a different colour, and all plant ERFs that are known to be involved in disease resistance are in bold. ERFs involved in regulating defence responses are distributed in ERF and DREB subfamilies, but not in AP2, RAV, or Soloist.

The expression of \textit{RcERF} genes in response to \textit{Botrytis cinerea} infection

There has been an increasing rise in evidence gained from studying various plant species which indicates that plant AP2/ERF transcription factors play a significant role in pathogen response. In order to study the role of \textit{RcERFs} in \textit{B. cinerea} resistance, we analyzed transcriptome data in rose petals at 30 hpi and 48 hpi of this pathogen. The 30 hpi timepoint represents the early response to infection, whereas the 48 hpi timepoint corresponds to the late response [16]. A total of 23 \textit{RcERF} genes (\textit{RhERF004}, \textit{RhERF005}, \textit{RhERF015}, \textit{RhERF019}, \textit{RhERF023}, \textit{RhERF024}, \textit{RhERF054}, \textit{RhERF063}, \textit{RhERF064}, \textit{RhERF066}, \textit{RhERF068}, \textit{RhERF070}, \textit{RhERF072}, \textit{RhERF080},...
Fig. 3 Phylogenetic and gene structural analysis of rose AP2/ERF transcription factors. The phylogenetic tree is constructed by MEGA6.0 using a Neighbor-joining method. Numbers on the nodes of the branches represent bootstrap values. The gene structure diagram represents UTRs, exons and introns with green boxes, yellow boxes and gray lines, respectively. The scale at the bottom estimated the size of UTRs, exons and introns.
Table 3: Plant AP2/ERF family genes involved in disease resistance

| Gene name | Gene ID     | Species                  | Pathogens                          | References |
|-----------|-------------|--------------------------|------------------------------------|------------|
| OSERF922  | Os01t54890.1| Oryza sativa L.          | Magnaporthe oryzae                 | [17]       |
| GmERF3    | ACD47129.1  | Glycine max              | disease resistance                 | [18]       |
| GmERF113  | XP_003548854.1| Glycine max                | Phytophthora sojae                  | [19]       |
| GmERF5    | AEX25891.1  | Glycine max              | Phytophthora sojae                  | [20]       |
| ATERF15   | At4g31060   | Arabidopsis thaliana      | B. cinerea and DC3000               | [21]       |
| ATERF14   | At1g04370   | Arabidopsis thaliana      | Fusarium oxysporum                 | [22]       |
| ATERF1    | At3g2340    | Arabidopsis thaliana      | B. cinerea                         | [23]       |
| ATERF5    | At5g47230   | Arabidopsis thaliana      | B. cinerea                         | [14]       |
| ATERF4    | At3g15210   | Arabidopsis thaliana      | Plant defense systems               | [7]        |
| ATERF6    | At4g17490   | Arabidopsis thaliana      | B. cinerea                         | [14]       |
| ATERF094(CRA59) | At1g06160 | Arabidopsis thaliana      | plant defense                      | [24]       |
| SIERF.A1  | Solyc08g078180.1 | Solanum lycopersicum     | B. cinerea                         | [12]       |
| SIERF.B4  | Solyc03g093540 | Solanum lycopersicum     | B. cinerea                         | [12]       |
| SIERF.C3  | Solyc09g066360 | Solanum lycopersicum     | B. cinerea                         | [12]       |
| SIERF.A3  | Solyc05g052050 | Solanum lycopersicum     | B. cinerea                         | [12]       |
| SIERF.C6  | Solyc02g077370 | Solanum lycopersicum     | Pseudomonas syringae to pv.        | [25]       |
| SIERF.C4  | Solyc09g089930 | Solanum lycopersicum     | Ralstonia Solanecarum Strain BJ1057 | [26]       |

**RhERF088, RhERF089, RhERF092, RhERF093, RhERF095, RhERF099, RhERF114, RhERF123 and RhERF125** were significantly up-regulated, indicating they could be key regulators in resisting *B. cinerea* infection in rose. Amongst these *B. cinerea*-induced *ReERFs*, the expression of 10 *ReERF* genes was increased significantly at 30 hpi, suggesting that these *ReERFs* may well be involved in an early response to *B. cinerea* (Table 4).

In order to further verify the expression profile from RNA-seq, the expression of six *ReERFs* was analyzed by qPCR. The results of the qPCR analysis proved to be consistent with the expression profile obtained from the transcriptome analysis (Fig. 5).

**RcRF099 is required for rose resistance to *B. cinerea***

In order to further illustrate the potential role of *B. cinerea*-induced *ReERF* genes in resistance of this pathogen, we used VIGS to knock down the expression of *ReERF099* in rose petals. *ReERF099* was selected to conduct this VIGS study because: 1) *ReERF099* is up-regulated upon *B. cinerea* infection (Fig. 5; Table 4); and 2) based on phylogenetic analysis, *ReERF099* belongs to the DREB subfamily which comprises many disease-resistant ERFs originating from other plant species, such as *AtERF001, AtERF004, AtERF005, AtERF006, AtERF014*, and *AtERF015* (Fig. 4; Table 3).

In order to silence *ReERF099* in rose petals, we cloned a 230 bp fragment of *ReERF099* into a pTRV2 vector [27] to generate TRV- *ReERF099*. *Agrobacterium tumefaciens* carrying TRV- *ReERF099* and TRV1 [27] were co-infiltrated into rose petal discs to generate TRV- *ReERF099*-silenced rose petals. The infiltrated rose petal discs were then inoculated with *B. cinerea*. Comparing the control petal (TRV-00) inoculated with an empty TRV, the plant inoculated with TRV-TRV- *ReERF099* showed more serious disease symptoms displaying a significant increase in the size of the disease lesion (Fig. 6a and b). Furthermore, we confirmed the silencing efficiency of VIGS with qPCR (Fig. 6c). These results indicated that *ReERF099* is required for rose resistance to *B. cinerea*.

**Discussion**

Plant disease resistance-related genes are often induced by the invasion of pathogens, and are regulated at the transcriptional level by specific transcription factors. The AP2/ERFs is a major transcription factor family in plants, and has proved to have important functions in disease resistance in various plant species [28–32]. A genome-wide analysis of the AP2/ERF gene family has been performed in arabidopsis and rice [4]. So far, no comprehensive analysis of the rose AP2/ERF gene family has yet been reported, and the function of most *ReERFs* is largely generally unknown. In the current study, using the recently available rose genome, we performed a comprehensive analysis of the AP2/ERF gene family, including their gene structure, phylogeny, chromosomal location, gene duplication, as well as expression profiles during infection of gray mold caused by necrotrophic fungal pathogen *B. cinerea*. 
The number of AP2/ERF genes in rose (131) has proved to be lower than those in arabidopsis (147) and rice (164) [4], which indicates that the AP2/ERF gene family in different plants has expanded in various degrees during its evolution. Furthermore, we indicated that gene duplication is involved in the expansion of the RcERF gene family, in which a total of 21 duplication events were identified. Most of the duplicated genes (20) were involved in segmental duplication, whereas only one was involved in tandem duplication. Interestingly, the Ka/Ks ratio of all these 21 RcERF duplicates was < 1, indicating that the RcERF gene family undergoes a purification rather than a positive selection, suggesting a highly conservative evolution of this important transcription factor in the gene family. Previously, it has been demonstrated that the plant immune receptor genes involved in race-specific recognition of an invading pathogen undergo positive selection pressure [15]. It further indicates that the RcERFs generally involved in the basal defense against pathogens, are not race-specific resistance.

Although the role of RcERFs in disease resistance remains unclear, increasing evidence has proved that plant AP2/ERF genes are important players involved in regulating plant disease resistance. It prompts us to search for candidate RcERFs that are involved in the resistance to B. cinerea in roses. Based on their expression in response to gray mold infestation, we identified 23 RcERFs that could well be involved in gray mold resistance in rose petals.

We subsequently added plant ERFs that are known to be involved in disease resistance in the RcERFs phylogenetic tree. We discovered that these disease-related ERFs are mainly distributed within ERF and DREB subfamilies. The RcERF099 belongs to the DREB subfamily, which includes certain members of known disease-related plant ERF genes (Fig. 4). Especially, RcERF099 has a close homolog with Arabidopsis AtERF014, which has proved to play an important role in resistance against both bacterial pathogen Pseudomonas syringae pv. tomato, as well as fungal pathogen Fusarium oxysporum and B. cinerea [22].
More importantly, \textit{RcERF099} was induced significantly with \textit{B. cinerea}. We therefore consider that \textit{RcERF099} should be regarded as an important candidate gene involved in the regulation of rose disease resistance. The silencing of \textit{RcERF099} in rose petals by VIGS increased its susceptibility to \textit{B. cinerea}, indicating that it has a positive regulatory function in gray mold resistance.

Conclusion

In this study, a genome-wide analysis of \textit{RcERFs} was carried out. A total of 131 non-redundant AP2/ERF family members were identified in the rose genome, and these \textit{RcERFs} were divided into 5 subfamilies on the basis of phylogeny and conserved domains. Expression analysis indicated that the transcriptional regulation of certain \textit{RcERF} family genes was induced by \textit{B. cinerea} infection in rose petals. In addition, plant ERFs involved in disease resistance are usually clustered on the same branch of the phylogenetic tree. Based on these analyses, using VIGS, we further proved that \textit{RcERF099} is involved in regulating resistance to \textit{B. cinerea} in rose petals. The information ensuing from these results may facilitate further research into \textit{RcERFs} functions and crop improvement.

Methods

Identification of the rose AP2/ERF family gene

The genome sequences and CDS sequences of rose were downloaded from the website (https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/) to construct a local genome database. Based on AP2/ERF HMM (Hidden Markov model) from Pfam (PF00847, http://pfam.xfam.org), we initially identified AP2/ERF candidate genes in the rose genome with E-value $<1 \times 10^{-3}$. Finally, all candidate AP2/ERF sequences were verified that they contain at least one AP2/ERF domain through the CDD (Conserved Domains Database; https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and ExPASy (http://web.expasy.org/protparam/). Sequences without relevant domains or conserved motifs were removed. Chromosomal distribution

| Gene\textsuperscript{b} | Accession number | Subfamily | log2Ratio 30hpi | log2Ratio 48hpi |
|--------------------------|-----------------|-----------|-----------------|----------------|
| RcERF004 \textsuperscript{c} | RchiOBHm_Chr1g0347631 | ERF | – | 14.996 |
| RcERF005 | RchiOBHm_Chr1g0347641 | ERF | – | 5.460 |
| RcERF015 | RchiOBHm_Chr1g0373621 | ERF | 1.582 | 2.148 |
| RcERF019 | RchiOBHm_Chr1g03766651 | DREB | – | 2.259 |
| RcERF023 | RchiOBHm_Chr2g0095581 | DREB | 2.100 | 5.019 |
| RcERF024 | RchiOBHm_Chr2g0105221 | ERF | – | 16.346 |
| RcERF054 | RchiOBHm_Chr3g0450001 | ERF | – | 8.381 |
| RcERF063 | RchiOBHm_Chr4g0392461 | ERF | – | 8.895 |
| RcERF064 | RchiOBHm_Chr4g0392501 | ERF | 4.876 | 6.106 |
| RcERF066 | RchiOBHm_Chr4g0401801 | AP2 | – | 14.732 |
| RcERF068 | RchiOBHm_Chr4g0415231 | ERF | – | 5.509 |
| RcERF070 | RchiOBHm_Chr4g0423581 | ERF | 2.100 | 3.775 |
| RcERF072 | RchiOBHm_Chr4g0428891 | ERF | 1.087 | 1.803 |
| RcERF080 | RchiOBHm_Chr5g0032721 | ERF | 2.367 | 2.197 |
| RcERF088 | RchiOBHm_Chr6g0257181 | ERF | – | 3.241 |
| RcERF089 | RchiOBHm_Chr6g0274591 | ERF | 1.206 | 2.469 |
| RcERF092 | RchiOBHm_Chr6g0288231 | ERF | 6.085 | 6.755 |
| RcERF093 | RchiOBHm_Chr6g0288241 | ERF | 3.650 | 6.087 |
| RcERF095 | RchiOBHm_Chr6g0288271 | ERF | – | 7.574 |
| RcERF099 | RchiOBHm_Chr6g0295481 | DREB | – | 4.523 |
| RcERF114 | RchiOBHm_Chr7g0199231 | DREB | – | 3.194 |
| RcERF123 | RchiOBHm_Chr7g0204641 | ERF | 1.837 | 2.980 |
| RcERF125 | RchiOBHm_Chr7g0231481 | DREB | – | 5.621 |

\textsuperscript{a}The log2 transformed expression profiles were obtained from the RNA-seq dataset [16]

\textsuperscript{b}The \textit{RcERFs} undergo duplicate events are marked in bold

\textsuperscript{c}In this study, a genome-wide analysis of \textit{RcERFs} was carried out. A total of 131 non-redundant AP2/ERF family members were identified in the rose genome, and these \textit{RcERFs} were divided into 5 subfamilies on the basis of phylogeny and conserved domains. Expression analysis indicated that the transcriptional regulation of certain \textit{RcERF} family genes was induced by \textit{B. cinerea} infection in rose petals. In addition, plant ERFs involved in disease resistance are usually clustered on the same branch of the phylogenetic tree. Based on these analyses, using VIGS, we further proved that \textit{RcERF099} is involved in regulating resistance to \textit{B. cinerea} in rose petals. The information ensuing from these results may facilitate further research into \textit{RcERFs} functions and crop improvement.
of each AP2/ERF gene was mapped using Mapchart 2.2 software [33].

**Gene structure and phylogenetic analysis of RcERFs**

The map of exon-intron structures of the RcERF genes was carried out using TBtools software [34] by comparing the coding sequences (CDS) with their corresponding protein sequences. Furthermore, the phylogenetic analysis of RcERFs in the rose was conducted using the NJ method in MEGA 6.0 software and the bootstrap test was carried out with 1000 replicates.

In addition, 17 ERFs were previously reported that involved in disease resistance. These ERFs originate from various plant species, including tomato (*Solanum lycopersicum*), rice (*Oryza sativa*), soybean (*Glycine max*), and *Arabidopsis thaliana*. Amino acid sequences of these disease resistance-related ERFs and rose AP2/ERFs were then aligned using ClustalW. The alignment of protein sequences which resulted was subsequently used for phylogenetic analysis. A phylogenetic analysis was conducted using the NJ method in MEGA 6.0 software [35] and the bootstrap test was carried out with 1000 replicates. On the phylogenetic dendrograms, the percentage of replicated trees in which the associated taxa clustered together in the bootstrap test is indicated alongside the branches.

**Collinearity analyses**

For the purpose of identifying the collinearity of RcERFs, we downloaded the genome sequence of rose on a local server, and a Multiple Collinearity Scan toolkit [36] was
used to determine microsyntenic relationships between 
RcERF genes. The resultant microsynteny relationships 
were further evaluated by CollinearScan set at an E-
value of <1e−10.

Calculation of non-synonymous (Ka) to synonymous (Ks) 
substitution rates
TBtools was used to calculate the synonymous (Ks) and 
non-synonymous (Ka) nucleotide substitution rates. The 
Ka/Ks ratios of duplicated gene pairs were calculated to 
determine the selection mode driving the evolution of 
RcERFs.

Expression of RcERFs in response to B. cinerea
RNA-Seq data (accession number PRJNA414570) of rose 
petals undergoing B. cinerea infection was downloaded 
from the National Center for Biotechnology Information 
(NCBI) database. The clean sequencing reads were 
mapped to the Rosa chinensis ‘Old Blush’ reference gen-
ome. Gene expression levels of RcERFs were calculated by 
Reads per kb per million reads (RPKM). And differentially 
expressed gene based on Log2 fold change was performed 
by DEseq2. In order to verify the RNA-Seq results, the ex-
pression of 6 RcERF genes was analyzed using quantitative 
PCR (qPCR). To this end, total RNA was extracted from 
rose petals at 30 h and 48 h post-inoculation (hpi) 
respectively with B. cinerea using the hot borate method 
as previously described [37]. One microgram of DNase-
treated RNA was used to synthesize the first-strand cDNA 
by using HiScript II Q Select RT SuperMix (Vazyme) in a 
20-μL reaction volume. An qPCR reaction was performed 
using the SYBR Green Master Mix (Takara), and detection 
was achieved in StepOnePlus Real-Time PCR System 
(Thermo Fisher Scientific). RcUBI2 was used as an in-
ternal control. A delta-delta-Ct method calculation 
method was used for expression analysis. All primers that 
were used as qPCR are listed in Supplementary Table S1.

VIGS and B. cinerea inoculation assays
The rose plants (Rosa hybrida) used in this study were 
grown in soil in a greenhouse in Yunnan, China. In order 
to obtain the constructs for silencing, a 230 bp sequence of 
RcERF099 was amplified using primers TRV-RcERF099-F
(5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTCATTTGGGTCCTATACT
3′) and TRV-RcERF099-R (5′-GGGGACCACTTTGTACAAAGAAAGCTGGGTA
GTAATATCTTCAAGCAATT3′). The fragment gener-
ated was subsequently cloned into TRV2 vectors [27]. The 
VIGS of detached rose petal discs has been described previ-
ously [38]. In brief, detached petals are obtained from the 
outermost whorls of the rose, and 15-mm petal discs were 
punched. Agrobacterium consisting of TRV1 [27] and 
TRV2 constructs were mixed at a ratio of 1: 1 and vacuum

Fig. 6 Functional analysis of rose AP2/ERF transcription factor gene RcERF099. a Compromised B. cinerea resistance upon silencing of RcERF099
(TRV- RcERF099) was observed at 60 hpi post-inoculation. b Quantification of B. cinerea disease lesions on TRV-RcERF099- and TRV-00-inoculated 
rose petal discs. The graph indicates the lesion size of three biological replicates (n = 48) with the standard deviation. c Expression of RcERF099 
relative to that during the control at 6 days of post-silencing. All statistical analyses were performed using Student’s t-test; ** p < 0.01
infiltrated into petal discs. Petal discs were then inoculated with *B. cinerea* at 6 days after TRV infection. At least three biological repeats were performed, using at least 16 discs for each repeat. The disease lesion was estimated at 60 h post-inoculation, and a Student’s t-test conducted to determine the significance. All primers used for this study are listed in Supplementary Table S1.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12870-020-02740-6.

Additional file 1: Table S1. List of primers used in this study.

Additional file 2: Figure S1. Melting curves for qPCR.

**Abbreviations**

hpi: Hours post-inoculation; NJ: Neighbor-joining; HMM: Hidden Markov Model; CDD: Conserved Domains Database; VIGS: Virus-induced gene silencing

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Not Applicable.

**Authors’ contributions**

ZZ, Y.S., DL, and XL conceived and designed the experiments. DL, X.L, LS, and Y.S. carried out the experiments and analyzed the data. ZZ, Y.S., H.Z. and DL have written the paper. All the authors have read and approved the final version of the manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study have been included within supplemental data. The raw data of RNA-Seq of rose petals undergoing *B. cinerea* infection can be found in the BioProject database (accession nr. PRJNA414570). The plant materials are available from the corresponding author on request.

**Ethics approval and consent to participate**

Not applicable. Our research did not involve any human or animal subjects, material, or data. The plant materials used in this study were provided by the China Agricultural University and are freely available for research purposes following institutional, national and international guidelines.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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