Genomic analysis of NAC transcription factors in banana (Musa acuminata) and definition of NAC orthologous groups for monocots and dicots

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Received: 12 July 2013 / Accepted: 24 December 2013 / Published online: 26 February 2014
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Abstract Identifying the molecular mechanisms underlying tolerance to abiotic stresses is important in crop breeding. A comprehensive understanding of the gene families associated with drought tolerance is therefore highly relevant. NAC transcription factors form a large plant-specific gene family involved in the regulation of tissue development and responses to biotic and abiotic stresses. The main goal of this study was to set up a framework of orthologous groups determined by an expert sequence comparison of NAC genes from both monocots and dicots. In order to clarify the orthologous relationships among NAC genes of different species, we performed an in-depth comparative study of four divergent taxa, in dicots and monocots, whose genomes have already been completely sequenced: Arabidopsis thaliana, Vitis vinifera, Musa acuminata and Oryza sativa. Due to independent evolution, NAC copy number is highly variable in these plant genomes. Based on an expert NAC sequence comparison, we propose forty orthologous groups of NAC sequences that were probably derived from an ancestor gene present in the most recent common ancestor of dicots and monocots. These orthologous groups provide a curated resource for large-scale protein sequence annotation of NAC transcription factors. The established orthology relationships also provide a useful reference for NAC function studies in newly sequenced genomes such as M. acuminata and other plant species.

Keywords Comparative genomics · NAC transcription factors · Phylogenetic analysis · Gene family · Expert annotation · Gene duplication

Introduction Identifying the molecular mechanisms underlying tolerance to abiotic stresses is important in crop breeding. A comprehensive understanding of the gene families associated with drought tolerance is therefore highly relevant. The NAC gene family is one of the largest groups of plant transcription factors (TFs), which is known to regulate biotic and abiotic stress-responses such as osmotic stress and various plant developmental processes. NAC proteins are plant-specific TFs, and the NAC family has been recently reviewed by Puranik et al. (2012). NAC genes were originally characterized in a petunia NAM mutant (Sourer et al. 1996) and then in Arabidopsis CUC (Aida et al. 1997) and ATAF mutants (GenBank accession numbers X74755 and X74756). Two parts can be distinguished in the structure of NAC proteins: the NAC domain (InterPro IPR003441), in the N-terminal region, subdivided in five well-conserved subdomains (A-E); and the transcription regulatory regions (TRRs), in the C-terminal region, which is very variable in sequence and in length. The NAC domain is involved in dimerization and DNA binding, whereas the TRR region plays the role of transcription activator or repressor (Puranik et al. 2012). Evolutionary studies have been done on NAC genes for all major groups of land plants, and it has been shown that some NAC subfamilies were already present in early diverged land plants (Zhu et al. 2012).

Whole genome analyses of the NAC gene family have been performed in several species (Arabidopsis thaliana, Oryza sativa, Vitis vinifera, Populus trichocarpa, Glycine...
Whole genome duplications (WGDs) are an important evolutionary feature of plant genomes. Most plant taxa have experienced at least one WGD during their evolution (Van de Peer et al. 2009). A consequence of a WGD is the doubling of all genes. After a WGD event, genomes start to lose (by deletion or pseudogenization) the redundant copies of most of their genes, in a long evolutionary process (fractionation), but some duplicated copies are retained and fixed with modified functional properties. Moreover, differences in gene retention according with their function have been reported (Blanc and Wolfe 2004; Maere 2005). Consequently, in a multicentric family, the number of members in species that experienced independent WGD events can be highly variable. This variability is correlated with the number of WGDs that their genomes experienced in their evolution, the time elapsed from these events, the evolution rate and other evolutionary factors specific to each taxon.

The main goal of this study was to set up a framework of Orthologous Groups (OGs) determined by an expert sequence comparison of NAC genes from both monocots (O. sativa and Musa acuminata) and dicots (V. vinifera and A. thaliana). A. thaliana and V. vinifera were chosen as dicot representatives because the former is a model plant species, while the genome of the latter contains a low number of NAC members in species that experienced independent WGD events can be slowly than genomes of other dicot taxa (Cenci et al. 2010; Yue et al. 2010). O. sativa, belonging to the Poales clade, was selected as a monocot model species. Instead of another gramineae, we chose M. acuminata, a member of the Zingiberales clade of monocots which diverged early from the Poales clade. Moreover, the sequence of the M. acuminata DH Pahang genome was recently published and comprehensively characterized (Shan et al. 2012). Among the species considered in the present study, O. sativa and M. acuminata experienced three independent WGDs in the evolution of their genome (D’Hont et al. 2012). V. vinifera and A. thaliana underwent a genome triplication (γ WGD, common to all the core eudicots) (Jaillon et al. 2007) and the A. thaliana genome experienced two additional and more recent WGDs (Van de Peer et al. 2009). WGDs and gene retention lead to multigenic gene families in which OG detection using automatic approaches is challenging. The members belonging to each OG that was defined in this process are supposed to derive from the same ancestor gene existing before the divergence of monocots and dicots, and possibly to share the same function. Our classification intends to be a reference for all monocot and dicot species, to facilitate the identification of the most interesting NAC genes in species for which functional information is lacking.

Methods

Sequence retrieval

The sequences of NAC protein-coding genes were extracted from gene family database GreenPhylDB (Rouard et al. 2011) based on the presence of the NAM (No apical meristem) InterPro domain (IPR003441). Excluding splice forms, 82, 111, 146 and 172 sequences were selected for V. vinifera, A. thaliana, O. sativa, and M. acuminata, respectively.

Datasets from V. vinifera, A. thaliana, and O. sativa were compared with sequences analysed in previous studies. No additional NAC members were found for V. vinifera (74 members (Wang et al. 2013), although only 69 sequences were considered in this study. Five sequences were excluded from the analyses: VvNAC35 and VvNAC38 because they are not NAC but VOZ transcription factors; VvNAC72 because it was identified as a cellulose synthase-like protein G3; and VvNAC50 and VvNAC66 because they are likely pseudogenes.

Among the 105 A. thaliana NAC members analysed by Ooka and Satoh (2003), splice forms of five genes were eliminated (ANAC021/022, 034/035, 038/039, 050/051, and 079/080). Among the GreenPhylDB-extracted sequences, after the eliminating the NAC pseudogenes, three sequences (At3g12910.1, At3g12977.1, At4g35580.2) which were not included in the study of Ooka and Satoh (2003) were added to the A. thaliana NAC members in this study. Comparisons between O. sativa NAC sequences extracted from GreenPhylDB with the 138 and the 151 members found by Fang et al. (2008) and Nuruzzaman et al. (2010), respectively, provided 165 independent sequences for our study. The previously reported M. acuminata NAC sequences had all been scanned for the presence of the conserved NAC domain using interproscan searches (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The sequences used in the present study are provided in Online Resources 1.
Expert annotation of NAC sequences

Since the *M. acuminata* NAC sequences were the result of an automatic annotation, an expert revision of the gene structure of this family was conducted. In order to detect additional InterPro protein domain IPR003441 not included in the annotated genes, a tBlastn search was performed on the *M. acuminata* pseudo-chromosome sequences with a sample of NAC genes. Banana sequences were curated using the Banana Genome Hub (Droc et al. 2013) and more specifically with the Artemis software (Carver et al. 2008) connected to the Community Annotation System (Guignon et al. 2012). Modifications of the gene structures were based on transcripts, when available, and protein similarity with published NAC genes of other plant species. The revised annotations are publically available via the Banana Genome Hub. Some modifications of published NAC annotations for *A. thaliana*, *V. vinifera* and *O. sativa* were also performed. The modified sequences are reported in Online Resources 1 and a concise description of modifications is provided in Online Resources 2.

Analysis of NAC duplication in *Musa* genome

NAC genes separated by not more than 10 other genes were considered as tandem duplications. NACs generated by segmental duplication of the *Musa* genome were determined by the analysis of the output file (Online Resources 3) of the CoGe SynMap program (http://genomevolution.org/CoGe/SynMap.pl) obtained using a default parameter (Lyons et al. 2008).

Expert inference of the OGs

Orthologous group (OG) reconstruction was based on sequence similarity inferred from protein–protein BLAST analyses (analyses performed with default Blastp parameters) that were all carefully examined by human expertise (Fig. 1). The method used is based on two assumptions. Firstly, the best Blastp hit indicates the lowest protein distance from the query sequence. Secondly, for each member of the query species, the smallest protein distance will be found with an orthologous gene of the subject species, although, this second assumption cannot be true in all circumstances, particularly for rapid sequence evolution of the orthologous gene(s). However, since all the NAC genes have a similar function (regulation of gene transcription), dramatic differences in their evolution rates are expected to be infrequent.

The orthologous inference was performed in two steps. First, all the NAC amino acid sequences of *A. thaliana*, *M. acuminata* and *O. sativa* were grouped with the *V. vinifera* NAC member having the higher Blastp score. Then, Blastp analyses were performed for all the other “species by species” combinations to verify the highest sequence-similarity among the sequences of each group. When inconsistencies were found (i.e. the best NAC protein found for the three species belonged to two different groups) the majority
rule was applied and the query sequence included inside the group containing the two best hits. The Blastp analyses of V. vinifera NAC sequences also allowed grouping those genes never found having best Blastp hits with the other species. Sequences having low Blastp scores with other species (see results) were considered species-specific and not considered in subsequent comparative analyses.

**OrthoMCL clustering**

The whole set of NAC sequences included in the OGs were clustered using OrthoMCL (Li et al. 2003) after Blastp all vs all (e-value 1e–10). The inflation parameter was gradually increased from 1.5 (default) to 14, to vary levels of granularity and until we reached the number of OGs identified by the expert annotation. Then, the resulting groups were compared to check if the manually obtained results were reproducible by a common strategy for orthology group detection (i.e. OrthoMCL).

**Phylogenetic analyses**

Phylogenetic analyses were performed using an in-house phylogenetic workflow powered by Galaxy (available at http://gohelle.cirad.fr/galaxy/u/reviewer/w/greenphyl-phylogenetic-analysis-workflow) that reproduces the main steps of the GreenPhyl pipeline. These steps included: multiple alignment (MAFFT v.6 (Katoh and Toh 2008)); masking (GBLOCKS (Talavera and Castresana 2007)); NNI and SPR methods with aLRT support (Anisimova and Gascuel 2003) with tree improvement with best of the group containing the two best hits. The Blastp analyses of V. vinifera NAC sequences also allowed grouping those genes never found having best Blastp hits with the other species. Sequences having low Blastp scores with other species (see results) were considered species-specific and not considered in subsequent comparative analyses.

**Results**

**Number of NAC genes in M. acuminata genome**

Among the 36,542 automatically predicted genes in the M. acuminata genome, 172 contained the InterPro protein domain IPR003441. To improve their structural annotation, all these sequences were manually curated, as necessary. Additional genomic regions containing the domain IPR003441 were also detected and analyzed. Globally, our analysis resulted in 167 potentially functional NAC genes (Online Resources 1) that we retained for this study. Five pseudogenes and a number of remnants were also found in the M. acuminata genome. We then refined the selection of NAC sequences for other species, taking into account previous publications dedicated to NAC transcription factors (see “Methods”).

**NAC member duplication in M. acuminata**

The banana genome contains the largest number of NAC genes among the already sequenced genomes of angiosperms. Despite the large number of NAC genes observed, only twelve tandem duplication regions (involving 27 genes) were detected in M. acuminata. By contrast, at least 18 segmental duplications (involving 43 NAC genes) were detected by CoGe SynMap (Online Resources 3), that originated from one of the last two WGDs that occurred for the Musa genome (D’Hont et al. 2012).

**Expert orthologous grouping of NAC sequences**

The V. vinifera NAC sequences were chosen as a reference for two main reasons: (a) V. vinifera proteins appear to have a slower evolution rate than other dicots (Cenci et al. 2010; 2013; Yue et al. 2010), and (b) its genome experienced the lowest number of WGDs after the monocot/dicot lineage divergence (Bowers et al. 2003; Jaillon et al. 2007; Tang et al. 2010; D’Hont et al. 2012; Paterson et al. 2009) which is the probable main reason for its low number of NAC members.

Among the 103 A. thaliana NAC members, 19 displayed very low Blastp scores (lower than 170) with the best hit of the V. vinifera protein database and with the other two analysed species. Consequently, only the 84 NAC members (showing Blastp score higher than 250) were considered for the grouping. Similarly, 73 Oryza sativa NAC sequences showed very low similarity with NACs of other analysed species and were not considered in the orthologous grouping.

Finally, among the 167 M. acuminata NAC sequences, those five having a very low score with the best V. vinifera hit (less than 130) were discarded, and the remaining 162 with BLAST scores higher than 200 were retained for the orthologous grouping.

Thus, 40 OGs containing NAC members were obtained (Table 1). The nomenclature we used for OGs is based on the eight V. vinifera clusters (Wang et al. 2013) with a letter to distinguish different groups inside the clusters (Table 1). For example, among the nine V. vinifera NAC sequences included in cluster 1, eight OGs were named from 1a to 1h (being two sequences, VvNAC05 and VvNAC11, included
Table 1 List of the 40 OGs of the NAC gene family based on sequence analysis in four angiosperm species

| Orthologous group | V. vinifera | A. thaliana | O. sativa | M. acuminata | Function |
|-------------------|-------------|-------------|-----------|--------------|----------|
| 1a VvNAC56        | ANAC074     | Os02g41450.1 | Achr1T10860 | MaNAC6 (Achr11T00880), banana fruit ripening (Shan et al. 2012) |
|                   |             | Os02g35660.1 | Achr1T20530 |              |
|                   |             | Os03g01870.1 | Achr5T18670 |              |
|                   |             | Os04g43560.1 | Achr6T36550 |              |
|                   |             | Os10g33760.1 | Achr8T18980 |              |
|                   |             |              | Achr11T00880|              |
|                   |             |              | Achr11T22760|              |
|                   |             |              | AchrUn_randomT17260| |
|                   |             |              | AchrUn_randomT02760| |
| 1b VvNAC33        | ANAC021/022 | Os02g06950.1 | Achr3T23360 | NAC1 (ANAC21/022) |
|                   |             | Os04g52810.1 | Achr5T00500 | Root development (Guo et al. 2004); Os12g41680, abiotic stresses (Nuruzzaman et al. 2012); MaNAC5 (Achr9T26140), banana fruit ripening (Shan et al. 2012) |
|                   |             | Os06g46270.1 | Achr5T26640 |              |
|                   |             | Os08g10080.1 | Achr6T30050 |              |
|                   |             | Os12g41680.1 | Achr6T31350 |              |
|                   |             |              | Achr8T07120 |              |
|                   |             |              | Achr8T33310 |              |
|                   |             |              | Achr9T10210 |              |
|                   |             |              | Achr9T26140 |              |
|                   |             |              | Achr11T25720|              |
| 1c VvNAC65        | ANAC038/039 | Os09g32260.1 | Achr6T01770 | CUC1 and CUC2 (ANAC054 and 098), shoot apical meristem development (Takada et al. 2001) |
|                   |             |              | Achr7T19680 |              |
|                   |             |              | Achr8T24680 |              |
| 1d VvNAC16        | ANAC054     | Os06g23650.1 | Achr10T22350| Shoot apical meristem development (Hibara et al. 2006) |
|                   | ANAC098     |              | Achr10T26180|              |
| 1e VvNAC14        | ANAC031     | Os08g40030.1 | Achr9T20090 | Shoot apical meristem development (Hibara et al. 2006) |
| 1f VvNAC06        | ANAC058     | Os03g42630.1 | Achr8T18420 | AtNAC2 (ANAC059), salt stress response and lateral root development (He et al. 2005); ANAC092, salt stress (Balazadeh et al. 2010); OsNAC2 (Os04g38720) Shoot branching (Mao et al. 2007); MaNAC3 (Achr9T27530), banana fruit ripening (Shan et al. 2012) |
| 1g VvNAC61        | ANAC046     | Os01g01470.1 | Achr3T09520 | OsNAC45 (Os11g03370), drought and salt tolerance (Zheng et al. 2009); Os11g03370, Os12g03050, virus infection (Nuruzzaman et al. 2010) |
|                   | ANAC087     | Os01g29840.1 | Achr3T18020 |              |
|                   |             | Os03g21030.1 | Achr5T07600 |              |
|                   |             | Os07g48550.1 | Achr6T08600 |              |
|                   |             | Os11g03310.1 | Achr6T32290 |              |
|                   |             | Os11g03370.1 | Achr7T18330 |              |
|                   |             | Os12g03050.1 | Achr8T21470 |              |
|                   |             |              | Achr9T16920 |              |
|                   |             |              | Achr10T05070|              |
| 1h VvNAC05        | ANAC059     | Os02g36880.1 | Achr6T30570 |              |
| VvNAC11           | ANAC079/080 | Os04g38720.1 | Achr7T11500 |              |
|                   | ANAC092     |              | Achr9T27530 |              |
|                   | ANAC100     |              |              |              |
| 2a VvNAC02        | ANAC007     | Os02g42970.1 | Achr6T36840 | VND4-6 (ANAC007, 026, 101), vascular development (Kubo 2005) |
| VvNAC22           | ANAC026     | Os04g45340.1 | Achr7T06640 |              |
|                   | ANAC101     | Os06g01480.1 | Achr8T11590 |              |
|                   |             |              | Achr11T03780|              |
|                   |             |              | Achr11T17510|              |
| Orthologous group | V. vinifera | A. thaliana | O. sativa | M. acuminata | Function |
|-------------------|-------------|-------------|----------|-------------|----------|
| 2b                | VvNAC23     | ANAC037     | Os03g03540.1 | Achr8T12100 | VND1-3 (ANAC037, 076,105), vascular development (Kubo 2005); Os10g38834, drought stress (Nuruzzaman et al. 2012) |
|                   | ANAC076     |             | Os10g38834.1 | Achr11T03040 |          |
|                   | ANAC105     |             |           |             |          |
| 2c                | VvNAC63     | ANAC030     | Os04g59470.1 | Achr3T22360 | VND7 (ANAC030), vascular development (Kubo 2005) |
|                   |             |             | Os08g01330.1 | Achr7T23170 |          |
| 2d                | VvNAC70     | ANAC015     | Os04 g (unannotated, OsI_35493) | Achr2T05640 | Bearskin 1-2 (ANAC015, 070), Root cap maturation (Bennett et al. 2010) |
|                   | ANAC070     |             |           |             |          |
| 2e                | VvNAC68     | ANAC033     | Os02g15340.1 | Achr2T20020 | Sombrero (ANAC033), Root cap maturation (Willemens et al. 2008) |
|                   |             |             | Os06g33940.1 | Achr10T14400 |          |
|                   |             |             |           | Achr10T21750 |          |
| 2f                | VvNAC24     | ANAC043     | Os06g04090.1 | Achr3T12230 | NST1-2 (ANAC043-066) and SND1 (ANAC012), Secondary wall thickening (Mitsuda et al. 2005; 2007) |
|                   | VvNAC49     | ANAC066     | Os08g02300.1 | Achr7T05980 |          |
|                   | ANAC012     |             |           | Achr9T24450 |          |
|                   |             |             |           | AchrUn_randomT21980 |          |
| 3a                | VvNAC08     | ANAC002     | Os01g66120.1 | Achr3T18990 | ATA1 (ANAC002) Drought stress responses (Hu et al. 2006); ATA1-2 (ANAC002-081), repressor of pathogenesis-related proteins (Delessert et al. 2005; Wang et al. 2009); OsNAC6 (SNAC2), 5, 52 (Os01g66120, Os11g08210, Os5g34830) Abiotic stress (Ohnishi et al. 2005; Nakashima et al. 2007; Hu et al. 2008; Takasaki et al. 2010; Gao et al. 2009) |
|                   | VvNAC39     | ANAC032     | Os05g34830.1 | Achr6T17720 |          |
|                   | ANAC081     |             | Os11g08210.1 | Achr6T18720 |          |
|                   | ANAC102     |             |           | Achr6T25380 |          |
|                   |             |             |           | Achr7T23250 |          |
|                   |             |             |           | Achr10T04570 |          |
| 3b                | VvNAC44     |             | Os01g60020.1 | Achr4T02390 | OsNAC19 (Os3g60080), response to infection by M. grisea (Lin et al. 2007); OsNAC1 (Os3g60080) drought stress (Hu et al. 2006) |
|                   |             |             | Os03g60080.1 | Achr4T0310 |          |
|                   |             |             | Os07g12340.1 | Achr5T07590 |          |
|                   |             |             |           | Achr6T32330 |          |
|                   |             |             |           | Achr7T21780 |          |
|                   |             |             |           | Achr10T29200 |          |
| 3c                | VvNAC60     | ANAC047     | Os01g01430.1 | Achr3T18010 | AtNAP (ANAC029), leaf senescence (Guo and Gan 2006); OsNAC10 (Os1g03300) drought tolerance (Jeong et al. 2010); Os11g03300, Os12g03040, response to Magnaporthe grisea infection, (Sun et al. 2013) |
|                   | VvNAC26     | ANAC029     | Os03g21060.1 | Achr4T02380 |          |
|                   |             |             | Os05g34310.1 | Achr6T32320 |          |
|                   |             |             | Os07g48450.1 | Achr7T21770 |          |
|                   |             |             | Os11g03300.1 | Achr9T04960 |          |
|                   |             |             | Os12g03040.1 | Achr10T12860 |          |
|                   |             |             |           | AchrUn_randomT17360 |          |
| 3d                | VvNAC03     | ANAC018     | Os07g37920.1 | Achr1T08860 | Os07g37920, senescence (Distelfeld et al. 2012) |
|                   | VvNAC43a    | ANAC025     |             | Achr9T19520 |          |
|                   | VvNAC18     | ANAC056     |             |             |          |
| 3e                | VvNAC17     | ANAC072     |             |             | ANAC072 (RD26), 019 and 055, Drought tolerance (Tran et al. 2004); ANAC019, ANAC055, Defense disease, Jasmonate pathway (Bu et al. 2008) |
|                   | ANAC019     |             |             |             |          |
|                   | ANAC055     |             |             |             |          |
| 4a                | VvNAC64     | ANAC028     | Os03g02800.1 | Achr1T02820 | RIM1 (Os3g02800) virus resistance; Jasmonate pathway signalling (Yoshii et al. 2010) |
|                   | ANAC045     |             |             | Achr8T13430 |          |
|                   | ANAC086     |             |             | Achr11T01400 |          |
|                   |             |             |             | Achr11T16860 |          |
|                   |             |             |             | AchrUn_randomT08190 |          |
Table 1 continued

| Orthologous group | V. vinifera | A. thaliana | O. sativa | M. acuminata | Function |
|-------------------|-------------|-------------|------------|--------------|----------|
| 4b VvNAC57        | ANAC057     | Os09g38000.1 | Achr3T13880 |              |          |
|                   |             | Os09g38010.1 | Achr9T23940 |              |          |
| 4c VvNAC13        | ANAC071     | Os10g42130.1 | Achr3T18070 |              |          |
|                   | ANAC011     |              | Achr5T03360 |              |          |
|                   | ANAC096     |              | Achr5T18140 |              |          |
|                   |             |              | Achr11T17780 |              |          |
| 4d VvNAC20        | ANAC050     | Os02g57650.1 | Achr2T04010 |              |          |
| VvNAC21           | ANAC051/052 | Os08g44820.1 | Achr9T20400 |              |          |
|                   | ANAC053     |              | Achr9T23580 |              |          |
|                   | ANAC077     |              | AchrUn_randomT07620 | |          |
| 4e VvNAC48        | ANAC082     | Os05g35170.1 | Achr7T23330 |              | VNI1 (ANAC082), vascular development (Yamaguchi et al. 2010) |
|                   | ANAC103     | Os_AK068153  | Achr10T04530 |              |          |
|                   |             |              | Achr11T09000 |              |          |
| 4f VvNAC69        | ANAC020     |              |            |              |          |
| 4g VvNAC15        | ANAC013     | Os09g32040.1 | Achr8T24280 |              |          |
|                   | ANAC016     |              | AchrUn_randomT11980 | |          |
|                   | ANAC017     |              |            |              |          |
| 5a VvNAC01        | ANAC041     | Os01g70110.1 | Achr2T21110 |              | VNI2 (ANAC083), vascular development, salt tolerance, leaf senescence (Yamaguchi et al. 2010; Yang et al. 2011; Seo and Park 2011) |
| VvNAC07           | ANAC084     | Os08g42400.1 | Achr3T18680 |              |          |
| VvNAC73           | ANAC097     | Os09g33490.1 | Achr6T16560 |              |          |
|                   | ANAC083     | Os11g31330.1 | Achr6T20870 |              |          |
|                   |             | Os12g29330.1 | Achr6T23840 |              |          |
|                   |             |              | Achr7T22480 |              |          |
|                   |             |              | Achr10T16940 |              |          |
|                   |             |              | Achr10T11910 |              |          |
|                   |             |              | AchrUn_randomT24680 | |          |
| 5b VvNAC25        | ANAC014     | Os06g01230.1 | Achr2T11810 |              | TIP (ANAC091), virus interacting (Ren et al. 2000); NTL6 (ANAC062) drought stress (Kim et al. 2012); ntm2 (ANAC069) salt stress (Park et al. 2011); NTL9 (ANAC__At4g35580) salt stress (Yoon et al. 2008) |
| VvNAC51           | ANAC062     | Os08g06140.1 | Achr3T00330 |              |          |
| VvNAC52           | ANAC091     |              | Achr4T29170 |              |          |
| VvNAC53           | ANAC__      |              | Achr11T07800 |              |          |
| VvNAC54           | At4g35580   |              | AchrUn_randomT04060 | |          |
| VvNAC55           |             |              |            |              |          |
| VvNAC71           |             |              |            |              |          |
| 5c + MS VvNAC67   | ANAC040     | Os01g15640.1 | Achr5T23620 |              | NTL8 (ANAC040), regulation of salt-responsive flowering (Kim et al. 2007) |
|                   | ANAC060     |              | Achr6T03200 |              |          |
|                   | ANAC089     |              |            |              |          |
| 6a VvNAC40        | ANAC034     | Os01g66490.1 | Achr7T23650 |              | LOV1 (ANAC034) Cold response, photoperiod pathway (Yoo et al. 2007) |
|                   |             | Os05g34600.1 | Achr3T21690 |              |          |
|                   |             | Os08g02160.1 | Achr5T17060 |              |          |
|                   |             |              | Achr10T04320 |              |          |
|                   |             |              | Achr10T10690 |              |          |
|                   |             |              | Achr11T08970 |              |          |
|                   |             |              | Achr11T22590 |              |          |
|                   |             |              | Achr11T26450 |              |          |
| 6b VvNAC10        | ANAC009     | Os08g33910.1 | Achr6T02680 |              | FEZ (ANAC009), Root cap maturation (Willemsen et al. 2008); ONAC063 (Os08g33910) salt stress (Yokotani et al. 2009) |
| VvNAC27           | ANAC094     | Os02g51120.1 | Achr10T08120 |              |          |
in the OG 1 h). Similarly, cluster 5 (containing eleven V. vinifera NAC sequences) was subdivided into three OGs: 5a (containing VvNAC01, VvNAC07 and VvNAC73), 5b (VvNAC25, VvNAC51-VvNAC55 and VvNAC71) and 5c (VvNAC67). Two of these groups did not contain any A. thaliana sequence (3b, 7f), whereas three other groups did

The last column contains information on known functions for genes of the relative groups. Sequences showing best reciprocal Blastp hits are indicated in bold. Os08g33670.1 and Os11g03310.1 were close to the sequences of cluster 1, but they could not be assigned to a specific group. VvNAC42 and VvNAC45 could not be resolved between groups 3c or 3d

| Orthologous group | V. vinifera | A. thaliana | O. sativa | M. acuminata | Function |
|-------------------|-------------|-------------|-----------|--------------|----------|
| 6c                | VvNAC28     | ANAC_       | Os03g56580.1 | Achr4T23030 | ANAC042, heat stress |
|                   | VvNAC29     | At3g12910.1 | Os07g04560.1 | Achr4T32010 | (Shahnejat-Bushehri et al. 2012) and pathogen infection |
|                   | VvNAC30     | ANAC042     | Os12g43530.1 | Achr5T02170 | (Saga et al. 2012); MaNAC2 |
|                   | VvNAC31     |             |           | Achr6T31585 | (Achr6T31585), MaNAC4 |
|                   | VvNAC32     |             |           | Achr7T00860 | (Achr7T00860), banana fruit ripening (Shan et al. 2012); Os10g38834, drought stress (Nuruzzaman et al. 2012) |
|                   | VvNAC36     |             |           | Achr9T10040 |                      |
|                   |             |             |           | Achr10T08420 |                      |
| 7a                | VvNAC34     | ANAC010     | Os01g48130.1 | Achr2T09080 | MaNAC1 (Achr6T27000), banana fruit ripening (Shan et al. 2012) |
|                   | VvNAC37     | ANAC073     | Os05g48850.1 | Achr4T02730 |                      |
|                   |             |             |           | Achr6T27000 |                      |
| 7b                | VvNAC19     | ANAC075     | Os01g09550.1 | Achr2T16590 |                      |
|                   |             | ANAC099     | Os05g10620.1 | Achr4T30940 |                      |
|                   |             |             | Os06g36480.1 | Achr6T05480 |                      |
|                   |             |             |           | Achr7T09510 |                      |
|                   |             |             |           | Achr10T27600 |                      |
| 7c                | VvNAC12     | ANAC008     | Os06g15690.1 | Achr3T07330 |                      |
|                   |             |             |           | Achr6T11230 |                      |
| 7d                | VvNAC59     | ANAC044     | Os04g40140.1 | Achr9T01880 |                      |
|                   |             | ANAC085     | Os02g38130.1 | AchrUn_randomT17050 | |
|                   |             |             |           | Os04g477300 (Os04g40140) boron-toxicity tolerance (Ochiai et al. 2011); Os0238130, viral infection (Nuruzzaman et al. 2010) |
| 7e                | VvNAC47     | ANAC104     | Os02g34970.1 | Achr6T17670 | XND1 (ANAC104), lignocellulose synthesis (Zhao et al. 2007); Os02g34970 drought stress; viral infection (Nuruzzaman et al. 2010; 2012) |
|                   | VvNAC58     | ANAC044     | Os04g35660.1 | Achr6T18640 |                      |
|                   |             |             |           | Achr6T25790 |                      |
|                   |             |             |           | Achr10T10790 |                      |
| 7f                | VvNAC09     | Os10g21560.1 | Achr2T06610 | Achr2T06610 |
|                   |             |             |           | Achr4T07148 |                      |
|                   |             |             |           | Achr7T26050 |                      |
| 8a                | VvNAC04     | ANAC036     | Os03g04070.1 | Achr1T02710 |                      |
|                   | VvNAC41     | ANAC036     | Os06g51070.1 | Achr3T00560 |                      |
|                   |             |             |           | Achr3T14720 |                      |
|                   |             |             |           | Achr5T19060 |                      |
|                   |             |             |           | Achr7T04030 |                      |
|                   |             |             |           | Achr11T01320 |                      |
|                   |             |             |           | AchrUn_randomT08220 | |
| 8b                | VvNAC46     | ANAC061     | Os01g64310.1 | Achr6T19400 | Os11g05614, virus infection (Nuruzzaman et al. 2010) |
|                   | VvNAC62     | ANAC090     | Os05g37080.1 | Achr8T01410 |                      |
|                   | VvNAC74     | ANAC090     | Os11g45950.1 | Achr9T29750 |                      |
|                   |             |             | Os11g05614.1 | Achr10T04720 |                      |
|                   |             |             | Os11g45950.1 |                      | |

The last column contains information on known functions for genes of the relative groups. Sequences showing best reciprocal Blastp hits are indicated in bold. Os08g33670.1 and Os11g03310.1 were close to the sequences of cluster 1, but they could not be assigned to a specific group. VvNAC42 and VvNAC45 could not be resolved between groups 3c or 3d

a Putative pseudogenes
b Os11g03310.1 was assigned to the group 1g based on the phylogenetic analysis results

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not contain any monocot sequences (3e, 4f, and 5c). An additional OG was obtained with one *O. sativa* and two *M. acumininata* very closely related sequences; this monocot-specific group was named MS (Table 1). Four sequences (Os08g33670.1 and Os11g03310.1 close to the sequences of OGs 1; VvNAC42 and VvNAC45 close to OGs 3c or 3d) could not be assigned due to very similar Blastp scores with sequences of different OGs.

**OrthoMCL analysis**

To verify whether our orthologous inferences could be reproduced automatically in such a very large gene family as the NAC, OG sequences were submitted to OrthoMCL clustering, using a panel of growing inflation parameters (from 1.5 to 14) that increase the cluster stringency. With the lowest stringency, six clusters were obtained (Fig. 2 and Online Resources 5) containing NAC members of variable numbers (from 4 to 10) of those OGs determined by expert comparison. None of these OGs was split in different OrthoMCL clusters. The increase in stringency did not allow OG differentiation, but with higher inflation parameters, single NAC sequences were isolated or OGs split in different clusters (Fig. 2, Online Resources 6-10). Only the cluster containing all NAC sequences of OGs 7a-d was maintained regardless of stringency level (Fig. 2, Online Resources 5-10).

**Structural analysis of NAC genes**

In addition to the sequence similarity, the exon/intron structure of the NAC genes was comprehensively analysed. Almost all NAC genes of OGs 1, 2, 3, 6 and 8 were found to have very similar structures, with three exons that aligned well (Fig. 3). The first exon contains A and B subdomains (Kikuchi et al. 2000) and ends at the first nucleotide of the first codon after the B subdomain; the second exon contains the C and D subdomains and ends at the third nucleotide of a codon; the third exon begins with the E subdomain and contains all the C-terminal region of the gene that includes the TRR. A few exceptions to this typical structure were observed in *A. thaliana* and *O. sativa* due to intron loss or, in other words, exon merging. In *A. thaliana*, only ANAC066 and ANAC101 genes are composed of two exons, because the second intron has been lost. In *O. sativa*, Os06g23650.1, Os08g40030.1 and Os11g05614.1 lost the first intron, Os03g42630.1, Os01g01470.1 and Os01g29840.1 lost the second intron, and Os12g05990.1 and Os06g51070.1 lost both introns. Moreover, all the three NAC members of the 3b group lost the first intron, whereas only the Os07g12340.1 gene lost also the second intron resulting in a mono-exonic structure.

Genes included in OGs 4, 5 and 7 have more variable numbers of exons. OGs 4a-h and 5a-c have the typical position for first two introns, but additional introns could be present in the TRR, varying from none (OGs 4b and 5a) to four (OG 5b). In monocot sequences of OG 4d, the first intron was lost and, consequently, the first exon contains the first four subdomains (Fig. 3).

OGs 7a-d have particular features. E subdomains are not recognizable and the beginning of the gene has a different structure. OGs 7a-b genes have an additional exon at the beginning, so A-B subdomains are included in the second exon (that ends at the same position as the first exon of other groups), and third exon contains the C-D subdomains (Fig. 3). These two OGs can be differentiated according to their structure. Genes of OG 7a have only three exons; in OG 7b, genes are longer and have three additional exons in the TRR. Genes in OGs 7c and 7d have two additional exons at the beginning, the first with a very small coding region (3–4 amino acids). Consequently the A-B subdomains are in the third exon, whereas the C-D subdomains are in the fourth one. The fifth and sixth exons contain the TRR (only Os04g40140.1 has 7 exons due to an additional intron in the fourth exon). Finally, genes of OG 7e have the typical structure with three exons and genes of group 7f have two additional exons located in the TRR (with the exception of GSMUA_Achr2T06610.1 that merged the last two exons).

**Phylogenetic analyses**

A phylogenetic analysis was performed with all the sequences included in the OGs (84, 69, 92 and 162 for *A. thaliana*, *V. vinifera*, *O. sativa* and *M. acumininata*, respectively) and a gene tree was obtained (Fig. 4 and Online Resources 11) based on 86 aligned amino acids, all belonging to the NAC domain. Twenty-six clusters were observed that contain all the NAC members of the OGs determined by expert comparison. On the other hand, a number of sequences relationships were not well defined. Two OGs, MS and 5c (specific to monocots and dicots, respectively), were consistent with the phylogenetic results, but these clusters were closely related and formed a unique cluster (aLRT = 0.876). Consequently these two OGs were merged into the 5c OG for subsequent analysis.

In order to verify whether the phylogenetic resolution could be improved using a set of more closely related sequences, we performed additional phylogenetic analyses with the sequences of OGs 2a–f (51 sequences) and 7a–d (33 sequences) which, in the global NAC gene tree, are clustered and sharply isolated from all other NAC but not well resolved in clear sub-clusters (Online Resources 11). The trees were obtained based on 163 and 175 aligned amino acids, respectively, and they contained also positions
outside the NAC domain. The phylogenetic trees were perfectly consistent with expertly determined OGs, being all the sequences in OG-specific clusters, with aLRT values spanning between 0.790 and 0.997 (Online Resources 12).

Finally, phylogenetic analyses were performed for each expertly identified OG; since *A. thaliana* or monocots NAC sequences were lacking in four OGs (3b, 3e, 4f and 7f), only thirty-six trees were built (Online Resources 13). Among the 36 phylogenetic trees obtained, 31 showed the expected dicot and monocot clustering. In two additional trees, a sequence of *M. acuminata* (*Achr7T17500, OG 1h*) and two sequences of *A. thaliana* (ANAC084 and *ANAC085*) were included.
ANAC097, OG 5a) occupy unexpected positions; all these three sequences were characterized by very long branches (Online Resources 13). In the remaining three trees, one was unresolved (3c) whereas in the other two (3d and 1d) monocot and dicot sequences were not separated into phylum-specific clusters. For a given species, when more than one sequence is included in an OG, species-specific clusters are often observed, which indicates gene amplifications took place after the lineage divergence. On the other hand, in few cases sub-clusters containing dicot and/or
monocots (e.g. group 2a, Online Resources 13) could indicate gene duplications that occurred before the *Arabidopsis-Vitis* and/or *Musa-Oryza* lineage divergence.

**Discussion**

The phylogenetic position of *M. acuminata* (order Zingiberales, a sister group to the well-studied Poales order) offers the opportunity for a deeper exploration into monocot evolution and into plant genome evolution in general. The present study analysed the evolution of NAC genes, a high copy TF family in the monocots and dicots, the two major lineages of angiosperms.

**Musa acuminata** NAC

The availability of the *M. acuminata* genome sequence allows global analysis of large gene families such as NAC transcription factors. Automatic gene annotation provides an initial panorama of abundance and chromosome distribution of the members of this gene family. As automatic gene structure annotation can be imprecise, we performed a revision of the members of this large family. We considered 167 genes to be potentially functional, and eliminated from the analyses five pseudogenes predicted by the presence of mutations modifying the typical gene structure and by the lack of observed transcription. However, due to the limited availability of transcriptome data, the lack of functionality of these genes needs to be confirmed, as well as the effective activities of the potentially functional genes. Compared to other species, the *M. acuminata* genome contains a high number of NAC genes. Twelve ancestral genomic regions pre-dating the last two WGDs could be reconstructed by analysis of gene collinearity among *Musa* chromosomes (D’Hont et al. 2012). When the NAC genes were positioned in these ancestral groups, at least 43 genes could be inferred as derived from duplications during one of the last two *Musa* WGDs.

Orthologous grouping of NAC genes from different species

The orthologous grouping of NAC sequences belonging to four distinctly divergent species was based on similarities inferred by Blastp analysis. Even if Blastp does not provide a distance measure between two sequences, a better Blastp score was assumed to indicate a greater similarity between compared sequences.

Among all the NAC sequences available, a number of sequences showed a very low Blastp score with any NAC of other species. The Blastp score is influenced by the distance between the protein sequences, which in turn is influenced by the genetic distance between the analysed species. The latter in turn is determined by the phylogenetic position and evolution rate of the analysed species. For example, a lower genetic distance is expected between two monocot species than between a monocot and a dicot. Given this, empirically set thresholds of Blastp scores were used to exclude highly divergent sequences from the comparative analysis. Moreover, these highly divergent sequences showed similar Blastp scores with sequences assigned to several OGs that render these sequences unassignable to any OG. Sequences showing low Blastp scores with other species could be the result of species-specific sequence evolution or degradation due to the relaxation of any purifying selective pressure (pseudogenization).

The existence of a large group of *O. sativa*-specific NACs was highlighted in the study of Nuruzzaman et al. (2010). The large majority of these sequences resulted in cluster II in the phylogenetic analysis of Fang et al. (2008). In the light of their species specificity, these sequences were not considered in our comparative study.

More than 400 sequences were manually compared for their similarity with a species by species Blastp analysis. Forty OGs containing NAC sequences from at least two species were obtained. In addition to their being lower in number, NAC sequences of *V. vinifera* were chosen as the reference for comparison because of the reported lower evolution rate of this species compared to other dicots (Cenci et al. 2010; 2013; Yue et al. 2010). The phylogenetic analyses performed on NAC sequences of 36 OGs were consistent with these reports, i.e. the branch lengths of NAC sequences being generally shorter in *V. vinifera* than in *A. thaliana* (Online Resources 13).

Using the full set of sequences, phylogenetic analyses confirmed most of the orthologous grouping results: 26 of the 40 OGs were found consistent with clustering, i.e. all the NACs of a given OG were found in an OG-specific cluster. The other groups could not be resolved in OG-specific clusters, although no inconsistencies were noted with the proposed grouping (i.e. no well-supported clusters containing sequences from different OGs were observed). OGs 7a-d appeared very divergent from the other NACs, which is consistent with their particular structure (additional exons at the beginning) and sequence (lack of E subdomains). The NAC of the OGs 2a-f and 7a-d resulted in two well-defined clusters, but the distribution of NAC in specific OGs was not completely resolved. By performing phylogenetic analysis restricted to the sequences of these OGs, we obtained perfectly consistent clusters with the grouping made by the Blastp reciprocal analysis. The number of amino acid positions in the filtered multiple alignment was approximately twice the number of the ones obtained with the whole set of NAC sequences (86 amino acids). It is likely that the phylogenetic signal was clearer and enabled a better resolution of these clusters than the global analysis.
The phylogenetic analyses performed on NAC sequences of 36 OGs showed congruence with the known species phylogeny (i.e. the monocot/dicot classification), even if some sequences had unexpected positions. These exceptions could be the results of specific high sequence-divergence (as suggested by their long branches in the tree) maybe due to purifying selection relaxation in the presence of redundant copies of genes.

Automated clustering by OrthoMCL with the lowest stringency level provided six clusters containing several manually obtained OGs (spanning from 4 to 10). The increase in stringency did not provide a better resolution of OGs, but tended to isolate more divergent sequences (Fig. 2). NAC sequences appear to be problematic for automatic clustering, mainly because of the large number of OGs and of repeated gene duplications inside each lineage.

The phylogenetic analysis of O. sativa NAC (Fang et al. 2008) was extremely consistent with the OGs’ distribution obtained in this study. When several O. sativa NAC sequences are included in a given OG, they also appear clustered in the phylogenetic tree obtained by Fang et al. (2008). For example, groups 1a and 1b coincide with the two main sub-clusters of cluster I-2 (NAC1), and the six O. sativa NAC sequences of group 1 g were in a specific cluster as well as both NACs of the 1 h group. Some exceptions were observed involving OGs 3c, 7b and 5a. Even if NAC sequences of OGs 6a-c and 8a-b were resolved in five OG-specific clusters, their clusters were mixed in our phylogenetic analyses (performed with both the global sample or limited to the 6 + 8 groups). This is consistent with the findings of Fang et al. (2008) limited to O. sativa sequences. In conclusion, no major inconsistency was observed between the outcomes of phylogeny analyses and expert definition of OGs.

NAC gene structure was found to be largely consistent with OGs based on NAC sequence similarity. A few exceptions to the three-exon structure were observed in groups 1, 2, 3, 6 and 8. The most frequent changes are intron losses (i.e. exon mergings). One and two additional exons were observed at the beginning of the genes of OGs 7a–b and 7c–d, respectively. Genes of groups 4, 5 and 7 underwent exon/intron structure changes in the TRR, which is also very variable in its amino acid sequence. Similarity in gene structure was very common within each OG. However some differences could be observed. These differences could be explained by independent evolution of the genes (gain or loss of introns), but also they could be the results of erroneous annotations. In fact, the annotation of these genes is difficult in the very variable C-terminal region, due to lack of similarity with other model genes. Moreover, the level of expression of these genes is often low or tissue/condition specific (Wang et al. 2013), which reduces the representation of NAC genes in transcriptome databases.

All the sequences in each of the 40 OGs are supposed to derive from an individual ancestral gene that was present in the most recent common ancestor of monocot/dicot species. Most of the group assignations look robust. However, for some more divergent sequences, the assignation remains unreliable and further analysis involving improved annotations or additional species could modify the assignation of these sequences. The observed dicot-specific OGs (3e and 4f) could be a lineage-specific evolution from another group (that could reduce the number of ancestral NAC members) or alternatively, the orthologous genes may have been lost in the monocot lineages. Even if the two dicot-specific OGs do not correspond to an ancestral sequence, at least 38 ancestral NAC sequences pre-dated the divergence of monocot/dicot lineages.

NAC duplicates

In the OG-specific phylogenies, when several sequences of a given species were included in an OG, in most of the cases these genes appeared clustered together suggesting lineage-specific amplifications. In addition, when the gene location of the sequences in their respective genome was considered, some tandem duplications were observed in V. vinifera (VvNAC20–21, 28–32, 51–55) (Wang et al. 2013) and in A. thaliana (ANAC003–005, 016–017, 018–019, 046–047, 048–049, 050–053, 055–056, 064–065, 067–069, 073–074, 077–078, 087–088) as well as in O. sativa (Nuruzzaman et al. 2010). Since the members of these tandem duplications are often in the same OG, it is likely that these duplications occurred after the lineage separation of the analysed species. Alternatively, some tandem duplications could be more ancient. For example, inside the phylogenetic cluster of the OG 4d, VvNAC20 + ANAC078 and VvNAC21 + ANAC077 are in a separated sub-cluster (aLRT >0.8). It is therefore likely that their tandem duplication took place before the divergence of these two dicot species (Online Resources 13). In M. acuminata, none of the detected tandem duplications could be assigned to the same OG, which indicates that their origin predates the divergence between monocots and dicots. It is worth highlighting the tandem distribution of two NAC ancestral sequences (originating OGs 1g and 3c) that was retained during the divergence of monocots and dicots (Fig. 5). These genes are still in tandem in all the four analysed species and they were even multiplied during the O. sativa and M. acuminata segmental duplications: VvNAC61/ANAC46/Os3g21030/Os7g48550/Os11g03370/Os12g03050/GSMUA_Achr3T01820/GSMUA_Achr6T32290 (OG 1 g) and VvNAC60/ANAC47/Os3g21060/Os7g48450/Os11g03300/Os12g03040/GSMUA_Achr3T01810/GSMUA_Achr6T32320 (OG 3c). Between the two NAC members, a RicinB gene is often found (Fig. 5). In
addition to the tandem position, when the 5′–3′ orientation is considered, the 3c copies precede the 1g ones in all the loci except in *V. vinifera* and *O. sativa* chromosome 7, where the 1g copies have undergone inversion (Fig. 5). Since the genes in OGs 1g and 3c occupy very remote positions in the phylogenetic tree of all the species, one can speculate that these genes originated through more ancient tandem duplication than the monocot/dicot divergence. Alternatively, it is possible that a fortuitous rearrangement juxtaposed these genes prior to the monocot/dicot divergence. Nuruzzaman et al. (2010) already pinpointed the duplications of NAC containing regions in chr.3/chr.7 and chr.11/chr.12 in *O. sativa*. These regions coincide with known duplications in the evolution of the *O. sativa* genome (Yu et al. 2005). By contrast, the duplication involving the ancestral regions of chr.3/chr.7 and chr.11/chr.12 was never observed. This duplication could have originated from an ancient WGD or via a segmental duplication involving a limited chromosome region. Similarly, duplication in *M. acuminata* was not coincident with the most recent two tetraploidization cycles (D’Hont et al. 2012) and could have originated during the older WGD suggested by D’Hont et al. (2012) or during a segmental duplication independent of any WGD.

Globally, a dramatic difference can be observed in the total number of NAC members among the four analysed taxa. Since all these species underwent to independent WGD events, the observed variability could be explained by different levels of the fractionation process (i.e. the loss of duplicated and redundant genes). If this is the main reason for the copy-number differences, similar ratios between NAC and total gene number are expected. When this ratio is calculated, *M. acuminata* showed the highest ratio \((4.7 \times 10^{-3})\), followed by *A. thaliana* \((4.0 \times 10^{-3})\), *O. sativa* \((3.5 \times 10^{-3})\) and *V. vinifera* \((2.7 \times 10^{-3})\). If we take in account that significant numbers of NAC genes of *O. sativa* were excluded from the comparative analysis, it appears that *V. vinifera* has a notably lower percentage of NAC genes than other species. Previous studies showed that, after WGDs, the retention of duplicated genes with regulatory functions such as transcription factors is higher than for other kinds of genes (Blanc and Wolfe 2004; Maere 2005). Consequently, the lower number of WGDs experienced by the *V. vinifera* genome could explain the observed lower genomic representation of NAC genes.

In addition to the comparison of global numbers of NAC copies, the present study allows the analysis of specific OGs. Marked differences in NAC gene number can be observed among OGs, in particular in the *M. acuminata* and *O. sativa* taxa (Fig. 6). These differences suggest that copy retention could vary among different OGs in a same family of transcription factors.

Potential transfer of functional gene annotation

The NAC OGs are supposed to include most of the NAC copies that have evolved from an ancestral copy existing in the most recent common ancestor of monocots and dicots. It is likely that most of these ancestral genes already had their functions, and that these functions were maintained.
in the derived species during the independent evolution. The framework of the NAC OGs should provide a useful tool to predict the best candidates for a given function in species for which less information is available. For example, the sequence database provided in Online Resources 1, containing the NACs of four species representative of monocots and dicots, could be used to classify any NAC sequence of a given species by a simple Blastp analysis.

In particular, NACs involved in abiotic stress-resistance or tolerance could be predicted for newly sequenced genomes such as *M. acuminata*. Based on published functions of characterized NAC genes (Table 1), one can suppose that NAC members belonging to the OGs 3, 5 and 6 are mainly involved in response to biotic and abiotic stresses, even if OGs 1g–h appear to also play a role in this domain. Moreover many OGs do not contain any NAC genes characterized for their functions. Consequently, future results should identify an increasing number of potential genes suitable for improving the adaptability of crops to different environments and to climatic changes.

Fig. 6 Hierarchical clustering of the 40 NAC OGs analysed in the four species (*V. vinifera*, *A. thaliana*, *O. sativa* and *M. acuminata*). The colour gradient from green to red indicates whether a particular group is significantly smaller or bigger based on Z-score for all genes across the four species. The figure was generated using PermutMatrix (Caraux and Pinloche 2004) with the euclidean distance and the McQuitty clustering parameters.
Conclusion

The NAC gene family plays an important role in the regulation of plant development and stress-resistance/tolerance. A better understanding of the complex ancestral gene history may lead to better functional characterization of these genes. The recent sequencing of the *M. acuminata* genome has helped to elucidate the evolution of the NAC TFs in angiosperms.

Our approach to the study of NAC sequences was based on similarity analysis as inferred by a thorough and systematic examination of reciprocal Blastp results (here called expert orthologous grouping) and classical phylogenetic analysis. Phylogenetic analysis with all NAC sequences provided a global view of the reciprocal relations among NACs, but due to very divergent sequences, the resolution was insufficient to resolve some OGs. We have shown that when limiting the analysis to sequences belonging to more restricted groups, the phylogenetic resolution increases with the increase in available informative positions. Globally, phylogenetic analysis confirmed around two-thirds of the OGs based on similarity, but did not invalidate the remaining third of unresolved OGs. Combined with Blastp analysis, we were able to perform a more effective comparison between each pair of sequences with regard to the filtered multiple alignment that may not include divergent but informative sequence regions to resolve unclear OGs.

The OGs resulting from our analysis should provide a reference framework useful for functional gene annotation transfer in the NAC transcription factor family. These orthologous groups provide a curated resource for large-scale protein sequence annotation of NAC transcription factors. The established orthology relationships also provide a useful reference for NAC function studies in newly sequenced genomes such as *M. acuminata* and other plant species.

Acknowledgments This work was supported by the Belgian Directorate-General for Development Cooperation (DGDC) and the CGIAR Research Program on Roots, Tubers and Bananas (RTB). The authors would like to thank Vincent Johnson for his careful editing and helpful comments.

Conflict of interest The authors declare that they have no conflict of interest.

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