Two recently duplicated maize NAC transcription factor paralogs are induced in response to *Colletotrichum graminicola* infection

Anna-Maria Voitsik1, Steffen Muench2, Holger B Deising2 and Lars M Voll1*

**Abstract**

**Background:** NAC transcription factors belong to a large family of plant-specific transcription factors with more than 100 family members in monocot and dicot species. To date, the majority of the studied NAC proteins are involved in the response to abiotic stress, to biotic stress and in the regulation of developmental processes. Maize NAC transcription factors involved in the biotic stress response have not yet been identified.

**Results:** We have found that two NAC transcription factors, ZmNAC41 and ZmNAC100, are transcriptionally induced both during the initial biotrophic as well as the ensuing necrotrophic colonization of maize leaves by the hemibiotrophic ascomycete fungus *C. graminicola*. ZmNAC41 transcripts were also induced upon infection with *C. graminicola* mutants that are defective in host penetration, while the induction of ZmNAC100 did not occur in such interactions. While ZmNAC41 transcripts accumulated specifically in response to jasmonate (JA), ZmNAC100 transcripts were also induced by the salicylic acid analog 2,6-dichloroisonicotinic acid (INA). To assess the phylogenetic relation of ZmNAC41 and ZmNAC100, we studied the family of maize NAC transcription factors based on the recently annotated B73 genome information. We identified 116 maize NAC transcription factor genes that clustered into 12 clades. ZmNAC41 and ZmNAC100 both belong to clade G and appear to have arisen by a recent gene duplication event. Including four other defence-related NAC transcription factors of maize and functionally characterized Arabidopsis and rice NAC transcription factors, we observed an enrichment of NAC transcription factors involved in host defense regulation in clade G. In *silico* analyses identified putative binding elements for the defence-induced ERF, Myc2, TGA and WRKY transcription factors in the promoters of four out of the six defence-related maize NAC transcription factors, while one of the analysed maize NAC did not contain any of these potential binding sites.

**Conclusions:** Our study provides a systematic *in silico* analysis of maize NAC transcription factors in which we propose a nomenclature for maize genes encoding NAC transcription factors, based on their chromosomal position. We have further identified five pathogen-responsive maize NAC transcription factors that harbour putative binding elements for other defence-associated transcription factors in the proximal promoter region, indicating an involvement of the described NACs in the maize defence network. Our phylogenetic analysis has revealed that the majority of the yet described pathogen responsive NAC proteins from all plant species belong to clade G and suggests that they are phylogenetically related.

**Keywords:** NAC transcription factor, Maize, *Colletotrichum graminicola*, Biotic stress response, Phylogeny, NAC domain, DNA binding element

* Correspondence: lvoll@biologie.uni-erlangen.de
1 Division of Biochemistry, Friedrich-Alexander-University Erlangen-Nuremberg, Staudtstrasse 5, D-91058, Erlangen, Germany
Full list of author information is available at the end of the article

© 2013 Voitsik et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Background

NAC transcription factors belong to a large family of plant-specific transcription factors that are expressed in different tissues and at various developmental stages. The founding members of the family, NAM from petunia and ATAF1 and CUC2 from Arabidopsis, were described in 1996 and 1997 [1,2], and the initials of these genes were used to derive the name for the newly discovered multigene family. To date, 105 NAC genes have been identified in the Arabidopsis genome [3], 138 in rice [4], 115 in maize [5], 113 in sorghum, 177 in soybean and 148 in poplar but only around 40 in lower plants like mosses and spike mosses [5,6].

The characteristic feature of this group of transcription factors is the presence of a NAC domain at the N-terminus [2], a stretch of ~160 amino acids highly conserved between the members, which consists of five subdomains A – E [3]. This region serves as a platform for DNA binding, and for homo- or heterodimerization with other NAC proteins [7,8]. Determination of the NAC domain structure revealed a novel transcription factor fold; a twisted β-sheet enclosed by α-helices [9], which was recently shown to interact with the major groove of the target DNA [8]. The C-terminal region, in contrary, is variable in sequence and length and serves as a transcriptional activator [10,11] or transcriptional repressor [12].

NAC transcription factors regulate a diverse range of processes in plants. The regulatory role of NACs in the development of plant organs like in the shoot apical meristem [2,13], the axillary meristem [14], the cotyledons [1], lateral roots [11,12], the xylem [15,16] or the secondary cell wall [17,18] has been intensively studied. In addition, it has been described that many members of the NAC transcription factor family coordinate the response to abiotic stress. OsNAC5 and OsNAC6 from rice were shown to be induced by cold, drought and high salinity and to interact with each other and with a third rice NAC transcription factor SNAC1 to induce the expression of stress-responsive genes. Consequently, rice plants overexpressing OsNAC5, OsNAC6, OsNAC10, OsNAC45, SNAC1 and SNAC2 were more resistant to high salt conditions compared to wild type rice plants [19-22]. The expression of OsNAC63 was also strongly induced in rice roots by high salinity and osmotic stress. Arabidopsis plants overexpressing OsNAC63 exhibited a constitutive upregulation of salinity-inducible genes and produced seeds that were more tolerant to both of these stress conditions [23].

Furthermore, NAC transcription factors are involved in the regulation of senescence in Arabidopsis, where overexpression of AtNAP resulted in early senescence of rosette leaves [24], and in wheat, where low transcript levels of TaNAM delayed the onset of senescence [25]. In addition, the Arabidopsis NAC transcription factor RD26 is induced by drought and ABA and plants with reduced RD26 expression were insensitive to exogenous ABA treatment, indicating a role of RD26 in ABA-signalling [26].

In the past decade, NAC transcription factors were also shown to be involved in the regulation of the plant defence network. For instance, the NAC transcription factor ATAF2 acts as a repressor of PR gene expression in Arabidopsis [27], while ATAF1 negatively regulates the defence response to necrotrophic fungi and bacterial pathogens [28]. Furthermore, ANAC019 and ANAC055 were involved in the JA-dependent expression of defence genes in Arabidopsis [29]. OsNAC6 and OsNAC19 were induced in rice upon challenge with the rice blast fungus M. grisea, and the overexpression of OsNAC6 led to increased resistance towards rice blast [21,30]. Finally, one potato NAC gene was induced in leaves after inoculation with Phytophthora infestans [31] and BnNAC1-1, BnNAC5-1 and BnNAC5-7 genes were found to be induced in oilseed rape during flea beetle colonization and Sclerotinia sclerotiorum infection [32].

To date, data on the expression profile and possible function of maize NAC transcription factors are limited. ZmNAM1 (ZmNAC70 in this report) and ZmNAM2 (ZmNAC35) are expressed in the shoot apical meristem during embryo development, suggesting that they play a similar role as their Arabidopsis and petunia orthologues. Transcripts of ZmNAC4 were detected in developing endosperm, while ZmNAC5 and ZmNAC6, putative paralogues, were expressed in the coleorhiza [33]. Transcripts of two other NAC transcription factors, NRP-1 and Apn-1 were found in the endosperm, the transcript of Apn-1 was also detected in the developing embryo [34,35]. A group of four NAC transcription factors was shown to be involved in secondary cell wall biosynthesis in maize: ZmSWN1, ZmSWN3, ZmSWN6 and ZmSWN7 were able to complement the phenotype of the Arabidopsis snd1/ nst1 double mutant, which lacks the secondary cell wall in xylem fibers. Overexpression of each of these four maize NAC transcription factors in Arabidopsis wild type led to the ectopic deposition of secondary cell wall, resulting in a curly leaf phenotype similar to that observed for SND1 overexpressing plants, indicating that ZmSWNs are functional orthologues of SND1 [36].

Although evidence for the involvement of NAC transcription factors in plant defence accumulates, no such data are available for maize yet. Therefore, our aim was to characterize two members of the NAM gene family which we found to be induced in maize leaves challenged with Colletotrichum graminicola. C. graminicola (Cesati) Wilson [teleomorph Glomerella graminicola (Politis)] is a causal agent of anthracnose leaf blight and
stalk rot, an economically important disease of maize (*Zea mays* L.). The *C. graminicola* infection cycle starts on the leaf surface, where spores germinate. After germination, a specialized infection cell, the appressorium, is differentiated at the tip of the germ tube. The appressorium melanizes and accumulates compatible solutes to develop a high turgor pressure that is subsequently converted into mechanical force to piercing the plant cell wall with the penetration peg. Within the host tissue, the fungus initially produces voluminous primary hyphae that grow biotrophically, i.e. without disrupting the host plasma membrane. This biotrophic phase lasts for approximately 2 days. Subsequently, a switch to necrotrophic growth that involves both a change in lifestyle and hyphal morphology occurs. Spreading of thin, fast growing necrotrophic hyphae, which rapidly colonize and kill the host cells, can be macroscopically observed as extending necrotic lesions. Finally, the pathogen forms acervuli on the surface of the necrotic area, specialized structures mitotically producing conidia, which are distributed to new host tissue by rain splashes [37,38].

In this study, we provide a systematic nomenclature of the maize NAC transcription factor family, which served as the basis to reveal that the two NACs that were induced in the maize – *C. graminicola* interaction and other defense-inducible NAC from maize and other plant species are evolutionary related.

**Results**

Two maize NAC transcription factors are induced in leaves infected with *Colletotrichum graminicola*

In order to investigate which host genes respond to *C. graminicola* infection at the different stages of the interaction, we compared the transcriptome of leaves that were spray-inoculated with $2 \times 10^6$ conidia/mL to mock-treated control leaves during the biotrophic phase at 36 hpi and after the switch to the necrotrophic phase at 96 hpi by microarray analysis (see [39]). At 36 hpi, more than 313 genes were differentially regulated (fold change > 2), of which 251 were upregulated in infected leaves. In this set, two genes encoding the putative NAC transcription factors *ZmNAC41* and *ZmNAC100* were found, which were also induced during the necrotrophic leaf colonization at 96 hpi. To confirm the microarray data, transcript levels of both NAC genes were assessed at 2 and 4 dpi by qRT-PCR (Figure 1). While *ZmNAC100* transcripts were induced 4–5 fold, *ZmNAC41* was induced 7-fold. As spray-inoculation only led to infection of a fraction of the epidermal cells, the induction of both NACs transcripts is likely significantly higher in the infected cells. To determine the induction kinetics at earlier time points of the interaction, we assessed *ZmNAC41* and *ZmNAC100* transcript amounts in dip-inoculated leaves, where the proportion of infected tissue is higher compared to spray-inoculated leaves (see Methods section). We employed both *C. graminicola* wild type (WT) strain CgM2 and mutant strains generated by *Agrobacterium tumefaciens*-mediated transformation (ATMT), which are affected in virulence to different extent. While fungal penetration was reduced by 50% in mutant AT171, which is weakly affected in virulence (see [40]), mutant AT416 was unable to efficiently penetrate host tissue and was strongly affected in virulence (Figure 2A). In WT-infected leaves, *ZmNAC41* was weakly induced already at the pre-penetration stage at 24 hpi, but massive transcript accumulation coincided with the time of the establishment of biotrophy at 36 hpi (Figure 2B). *ZmNAC41* was also induced in the interactions with the two mutants at all tested time points.

![Figure 1 Induction of maize NAC transcription factors upon infection with *C. graminicola* wild type CgM2.](image-url) Relative quantities of *ZmNAC41* and *ZmNAC100* transcripts were analyzed by qRT-PCR and are expressed relative to *ZmHMG* on a log2 scale as means ± SE (n = 4). Black bars – mock-treated control leaves, grey bars – infected leaves. Error bars represent the standard error. Asterisks indicate significant differences (P-value < 0.05) to the respective mock control.
and the expression level was positively correlated with the virulence of the employed strain (Figure 2B). In contrast, the expression of ZmNAC100 was first induced after successful penetration of the WT and the mutant AT171 strain into the host tissue at 36 hpi. In contrast, mutant strain AT416 failed to induce the ZmNAC100 gene (Figure 2C). The timing of infection was confirmed by microscopic observation of the infected leaves (data not shown). Our data demonstrate that ZmNAC100 is induced only upon successful penetration of C. graminicola into the host tissue, which suggests that this NAC could be a part of the induced defence response. Correlation of the expression level of both NACs genes with fungal virulence suggests that they could be a potential compatibility factors in the interaction of maize with C. graminicola.

ZmNAC41 and ZmNAC100 are induced by defence signals and during leaf senescence

As both ZmNAC41 and ZmNAC100 responded to biotic stress, we assessed their responsiveness to phytohormones involved in coordinating plant defence response and treated maize leaves with jasmonic acid or 2,6-dichloroisonicotinic acid (INA), an analogue of salicylic acid, or the ethylene precursor 1-aminocyclopropane-1-carboxylic-acid (ACC), a precursor of ethylene. Both transcription factors were induced by jasmonic acid already 10 hours after treatment (hat), and transcripts of ZmNAC100 accumulated further up to 24 hat (Figure 3). Moreover, transcript accumulation of ZmNAC100, but not that of ZmNAC41, was enhanced by exogenously applied INA. These results suggest that ZmNAC41 is specifically induced by JA, and neither ZmNAC41 nor ZmNAC100 responded to ethylene (Figure 3). However, the induction of ZmNAC41 and ZmNAC100 during the compatible interaction with C. graminicola was approx. 100-fold higher as compared to the induction by JA and INA (Figure 3).
Many NAC transcription factors are involved in gene regulation during the senescence program (reviewed by [41,42]), during which defense-related genes are also induced. Transcript levels of both, \textit{ZmNAC41} and \textit{ZmNAC100} increased during leaf development and were about 4-fold greater in senescent leaves, as compared to seedlings (Figure 4A).

\textit{ZmNAC41} and \textit{ZmNAC100} are downregulated during salt stress
Some members of the NAC transcription factor family, such as \textit{OsNAC6}, were described to have overlapping roles in response to both biotic and abiotic stresses [21]. Therefore, we have subjected maize plants to drought or high salinity conditions and evaluated the transcript level

[Figure 3-Induction of \textit{ZmNAC41} and \textit{ZmNAC100} in response to hormone and hormone analog treatments.](image)

Relative quantities of \textit{ZmNAC41} (A) and \textit{ZmNAC100} (B) transcripts in mock-treated control leaves (black bars) and after 0 h (left bracket), 10 h (middle bracket) and 24 h (right bracket) of treatment with 1 mM JA (diagonal hatched bars) or 1.3 mM INA (vertical hatched bars) and after 0 h and 10 h of treatment with 5 mM ACC (cross-hatched bars) were analyzed by qRT-PCR and are expressed relative to \textit{ZmHMG} on linear scale as means ± SE (n = 4). Dissimilar letters indicate significant differences (P-value < 0.05) between the treatments.

[Figure 4-Transcript amounts of \textit{ZmNAC41} and \textit{ZmNAC100} during leaf development and in response to abiotic stress treatments.](image)

Relative quantities of \textit{ZmNAC41} (black bars) and \textit{ZmNAC100} (grey bars) were analyzed at different stages of the leaf development (A) and in leaves upon drought or high salinity conditions (B), as indicated below the graphs. Relative transcript amounts were determined by qRT-PCR and are expressed relative to \textit{ZmHMG} on linear scale as means ± SE (n = 4). Asterisks indicate significant differences (P-value < 0.05) of the seedlings to the mature and senescent leaves (A) or stress-treated leaves to the respective mock-treated control (B).
of the two NACs genes. Both transcription factors were down-regulated during salt stress and the transcripts of ZmNAC100 also declined during drought stress (Figure 4B). These results demonstrate that both ZmNAC41 and ZmNAC100 are distinctly regulated in biotic and abiotic stress conditions.

Additional maize NAC transcription factors are induced during the defense response

We further analysed, whether other maize NACs are also associated with the defence response. As shown by our transcriptome analysis, ZmNAC15 and ZmNAC97 were weakly induced during the necrotrophic stage of C. graminicola infection (Table 1). From the four NAC genes induced in the C. graminicola maize interaction, only ZmNAC41 was also upregulated in response to the fungal biotroph Ustilago maydis [43]. However, the induction of ZmNAC41 by U. maydis has only been observed at 12 hpi, prior to active defense suppression by the smut fungus [43]. In addition, two other NACs, ZmNAC36 and ZmNAC38 were transcriptionally repressed in the interaction with U. maydis upon tumor formation at 4 dpi.

To identify the regulatory circuitry behind the observed regulation of NAC genes in the two pathosystems, we have scrutinized the upstream promoter regions of the identified maize NAC genes for the presence of binding motifs for defence-associated transcription factors to elucidate if certain promoter elements could confer the specific response towards C. graminicola or U. maydis. All promoters contained a core NAC transcription factor binding site that had been predicted from the promoter element analysis of ANAC019 and ANAC092 [7]. The entire NAC-binding motifs identified for the two Arabidopsis NACs could be found in all analyzed promoters in one to five copies, suggesting that other NAC proteins could bind to the promoters of the analysed NACs as homo- or heterodimers (Table 2). Furthermore, the promoters of all except ZmNAC97, contained binding sites for ERF and TGA transcription factors, which regulate the expression of target genes in response to ethylene or salicylic acid, respectively. A Myc2 binding site, present in the promoters of many jasmonic-acid responsive genes, was found in ZmNAC15, ZmNAC38 and ZmNAC41, while a WRKY-binding motif could be detected in ZmNAC15, ZmNAC36, ZmNAC41 and ZmNAC100. Despite considerable conservation of ERF, TGA, Myc2 and WRKY binding motifs, the promoters of the six analysed NAC genes differ in their individual motif composition. In the proximal region 500 bp upstream of the start codon, putative ERF binding motifs were only present in ZmNAC15 and ZmNAC38, while all potential WRKY binding sites were located in this proximal region. Interestingly, a Whirly-binding motif was found only in ZmNAC41 and ZmNAC100, the only members induced during the early interaction of maize with C. graminicola.

In summary, the ZmNAC15, ZmNAC36, ZmNAC38, ZmNAC41 and ZmNAC100 genes all contained potential binding elements for other transcription factors known to be involved in the plant defence network within the proximal promoter region.

Analysis of the family of maize NAC transcription factors

The fact that promoter elements were quite conserved between the six analysed NAC transcription factors prompted us to explore their evolutionary relation. Using the unassembled maize genome information, Shen et al. [6] identified 177 putative maize NAC genes. Since an assembly of the B73 maize reference genome became available [44], we analyzed the NAC transcription factor family based on the assembled B73 genome information.

We employed the conserved NAC domain of ZmNAC41 and ZmNAC100 as a query to search against the peptide database (release 5b.60) deposited at http://maizesequence.org. Moreover, gene models for putative maize NAC transcription factors, deposited at Grassius Grass Regulatory Information Server (http://www.grassius.org/index.html), were blasted against the assembled maize genome. As an outcome of both surveys, 116 putative maize NAC genes (excluding alternative splice variants)

Table 1 Maize NAC transcription factors differentially regulated in the interaction with C. graminicola and U. maydis

| NAC gene | Colletotrichum graminicola | Ustilago maydis |
|----------|---------------------------|-----------------|
|          | 36 hpi | 96 hpi | 12 hpi | 96 hpi | 192 hpi |
| ZmNAC100 | 3.3    | 0.19   | 204    | 2.8·10^6 |
| ZmNAC41  | 3.4    | 0.02   | 45     | 3.5·10^4 |
| ZmNAC15  | 3.2    | 0.05   |
| ZmNAC97  | 2.2    | 0.006  |
| ZmNAC38  | 3.7    | 0.09   | –2.4   | 0.003  |
| ZmNAC36  |        |        | –2.4   | 0.07   |

Linear fold change values (f.c.) of gene expression are given; negative values represent a down regulation. Only fold changes > 2 are displayed. Data were extracted from the microarray analysis of the maize transcriptome [39] and p-values (p-val) were calculated using bioconductor (http://www.bioconductor.org).
have been identified, which were renamed using the acron- 
mym ZmNAC and ascending Arabic numbers due to 
chromosomal localisation as a suffix (starting with the short 
arm of chromosome 1, see Additional file 1: Table S1). Mul-
tiple alignment performed on the whole set of putative 
NAC protein sequences served for the construction of a 
phylogenetic tree, which revealed that the family can be di-
vided into 12 clades (Figure 5). Phylogenetic trees generated 
from entire NAC sequences (Figure 5) were very similar 
to those obtained from an alignment of the NAC domains 
only (not shown), indicating that the NAC domains allow 
for most of the distinction between individual clades.

An alignment of the consensus sequences generated 
for each clade revealed the typical domain architecture 
of the NAC proteins. The N-terminal part of the pro-
teins, which includes the NAC domain, was well con-
served between the clades, while the C-terminal region 
was highly divergent even between the members of the 
same clade (Additional file 2: Figure S1). As described 
for the Arabidopsis and rice NAC transcription factor 
families [3,4] and in two surveys using genomic informa-
tion from 9 and 11 different plant species [5,6], respect-
ively, five highly conserved subdomains A-E, separated 
by about 10–20 aa, have been distinguished in the NAC 
domain (Figure 6). To identify consensus sequences of 
the subdomains A-E for all clades, the NAC domains of 
all maize NAC transcription factors were screened with 
MEME (http://meme.sdsc.edu/meme/cgi-bin/meme.cgi, 
[45]). All clades except clade A shared common motifs 
(cut off p-value 1 ∙ e-10) within the NAC domain (Figure 7).

More detailed analysis of these common motifs revealed 
that the conservation of each motif was higher within 
the same clade than in the comparison to other clades. 
As shown for subdomain D (Figure 8), single amino acid 
residues differed between the individual NAC clades, ex-
cept for clades C and D, which cannot be distinguished 
by subdomain D. A high similarity between the NAC 
subdomains A, D and E was evident between clade A 
and the other clades, while the NAC subdomains B and 
C were divergent. Some NAC proteins contained add-
itional motifs in front of the NAC domain. For instance, 
four members of the clade C shared one leading motif, 
while another leading motif was present in six members 
of the clade G and six NACs from other clades.

In the C-terminal part of the protein sequences, in total 
twenty four distinct motifs (cut off p-value 1 ∙ e-10) have 
been identified (Figure 7 and Additional file 3: Table S2), 
some of which were specific to certain clades and subclades as described in the following paragraph. The C-
terminus of clade F was distinct from all other clades.
First, 6 of 7 clade members contained the QYGAPF motif (motif 12), which is also present in six rice NACs (representing motif 39 in [4]), and in addition, two different kinds of long C-terminal extensions were present in these members. Furthermore, subfamily G has even been divided into three subclades, based on the presence of motif SYDDIQ (motif 10) in subclade G1 and of motif NLDDLQ (motif 27) in subclade G2, which were both absent from the C-terminus of the third subclade. Similarly, one subclade of clade E consisted of six members that all carried a long C-terminal extension that contained three different motifs: ARS (motif 24), IDELS (motif 35) and KIWDWNP (motif 21). Furthermore, 11 members of four different subclades from clade C contained motif TDW (motif 13) and LPLE (motif 30). The latter motif is also present in the C-terminal domains of Arabidopsis and rice NACs (and corresponds to motif iii in [3]). Finally, the 50 aa motif MAAESNL (motif 9) was specific for four members of clade A, which share between 83% (ZmNAC73 vs. ZmNAC75) and 99% (ZmNAC74 vs. ZmNAC75) homology and appear to be the result of recent duplications.

However, some motifs were shared between members of different clades. Motif 36 (CFS) was present in five...
members of clades K and J and in some Arabidopsis and rice NACs (representing motif ix/x in [3]). Furthermore, motif EGSPT (motif 40) was common to members of clades F and K. Motif QT (motif 23) was shared between clades A, B and E, while motif HH/QHH (motif 34) was common to members of clade X, B, C, D and E. The HH/QHH motif is also present in four rice NACs (representing motif 31 in [4]). Some members of clade X contained motifs that were also found in clade A and B, respectively, which reflects the phylogenetic position of clade X between A and B.

Clade G is enriched in defence-associated NAC transcription factors

Protein sequence comparison showed that ZmNAC41 and ZmNAC100 are closely related; sharing 78% similarity of the whole sequence and 87% in the NAC domain. Thus, these two transcription factors belong to clade G, as revealed by a phylogenetic analysis (Figure 5). Checking the gene duplication data available for maize [44] further revealed that the two NACs have arisen from segmental duplication between long arms of chromosome 3 (NAC41) and chromosome 8 (NAC100). We were interested to know if the other maize NACs that were associated with defence responses towards the fungal pathogens *C. graminicola* and *U. maydis* (see Table 1) are related to ZmNAC41 and ZmNAC100. Phylogenetic analysis revealed that ZmNAC15 and ZmNAC38 were also members of clade G, while ZmNAC36 and ZmNAC97 were divergent from all of the other five proteins, respectively (Figure 5). Including all functionally characterized Arabidopsis and rice NACs to the phylogenetic tree of maize NACs, we found that four Arabidopsis defence-associated NACs also clustered into clade G (Figure 9). Arabidopsis ATAF1 was reported to be involved in the defence response against bacterial pathogens and necrotrophic fungi [28], while the closely related ATAF2 was shown to regulate the expression of *PR* genes [27]. ANAC019 and ANAC055 were described to be involved in the regulation of the JA-dependent defence response [29]. However, the two only rice NACs that are known to be involved in the response towards biotic stress, OsNAC6 and OsNAC19, were found outside clade G (Figure 8). In summary, eight out of twelve defence-associated NACs from maize, rice and Arabidopsis are members of clade G, while the four other were clustering to the separate families. As of our current knowledge, clade G seems to be enriched in transcription factors involved in response to biotic stress, suggesting that an ancestral NAC of clade G might have acquired its role in defence regulation earlier than NAC proteins from different clades of the family. However, this interpretation is limited by the functional characterization of orthologs of the relevant NAC clades.
Discussion
The involvement of NAC proteins in the plant defence response network

In this study we have characterised two maize NAC transcription factors; ZmNAC41 and ZmNAC100, which are induced during the interaction of maize with C. graminicola. The accumulation of ZmNAC41 transcript preceeded fungal penetration of the host tissue, suggesting that this transcription factor is activated as a part of the basal defence response. A similar induction pattern was described for the HvNAC6 from barley [46], which was transcriptionally induced in epidermal cells shortly after inoculation with Blumeria graminis f. sp. hordei (Bgh). Silencing of HvNAC6 reduced penetration resistance and the number of papilla formed in response to fungal penetration [46]. A deletion of ATAF1 in Arabidopsis, an HvNAC6 orthologue, compromised non-host resistance to Bgh, which was shown to be predominantly associated with papilla formation Jensen et al. [46]. Based on these observations, the ZmNAC41, HvNAC6 and ATAF1 orthologs are hypothesized to integrate the early transcriptional events upon PAMP recognition during the basal defence response.

However, the highest accumulation of ZmNAC41 was reached upon successful penetration of C. graminicola into the maize tissue, while the transcription of the other maize NAC transcription factor, ZmNAC100, was exclusively induced during the post-penetration stage of the infection. These data further suggest that both transcription factors described in this study are also associated with induced defence responses at later stages of infection. Induced defence reactions are controlled by phytohormones like jasmonic acid, salicylic acid and ethylene. We have revealed that both maize NAC transcription factors described here are responsive to jasmonic acid, while transcription of ZmNAC100 was also enhanced by salicylic acid, indicating that both transcription factors are indeed involved in phytohormone triggered defence responses. Similarly, it was shown that OsNAC5 and OsNAC6 from rice are strongly induced by methyl jasmonate, although transcripts of both genes accumulated to a similar level as during drought and cold stress [22]. Two Arabidopsis genes coding for NAC transcription factors, ANAC019 and ANAC055 were also responsive to methyl jasmonate, in a COI1- and AtMYC2-dependent manner. Moreover, studies with the anac019/ anac055 double knock-out and
Figure 8 (See legend on next page.)
Figure 8 Divergence of the NAC subdomain D consensus sequence. Comparison of the subdomain D aa consensus sequences from individual NAC clades after analysis with MEME Multiple Em for Motif Ellicitation. At the top, the consensus aa sequence for subdomain D of all 116 NAC sequences is given and below consensus sequences for the indicated clades are given according to the letter left to the motifs. For clade H, the middle part of subdomain D consists of a stretch of variable amino acid residues. Amino acid residues specific for individual clades are highlighted by asterisks.

Figure 9 Phylogenetic relationship of NAC proteins associated with the plant defence response. In comparison to the pedigree shown in Figure 5, all Arabidopsis and rice NAC proteins with known function were included. Defence-associated NAC proteins are labeled with red (maize) and blue (Arabidopsis, rice) boxes. Clade G was enclosed by dashed blue lines. Bootstrap values are indicated at the nodes, the bar depicts the distance scale for branch length.
overexpression lines revealed that the expression of other JA-responsive genes, like VEGETATIVE STORAGE PROTEIN 1 (VSP1) and LIPOXYGENASE2 (LOX2), is regulated by ANAC019 and ANAC055 [29], suggesting that these two NAC transcription factors are part of a JA feedback loop. The promoter element analysis of six pathogen induced maize NAC transcription factors in our study has revealed response elements for ERF, WRKY, TGA and NAC transcription factors within 500 bp upstream of the ATG in five of the six analysed genes, suggesting an involvement of these five NACs in the transcriptional network controlling the plant defence response.

**Most NAC transcription factors involved in plant defence are phylogenetically related**

Interestingly, ATAF1, HvNAC6, ANAC055, ZmNAC41 and ZmNAC100 as well as the two other pathogen inducible maize NAC transcription factors ZmNAC15 and ZmNAC8 belong to NAC clade G. Taking our data and the recent analyses of rice NACs by Nuruzzaman et al. [47] and Zhu et al. [5] into account, almost two thirds of all studied defence-induced NAC transcription factors belong to clade G. Interestingly, this clade is one of three evolutionary ancient subclades and contains most of the moss and lycophyte NAC representatives analysed [5]. Physcomitrella patens, the most ancient species harboring NAC transcription factors, possesses genes of the oxylipin pathway like allene oxide synthase (AOS, [48]), allene oxide cyclase (AOC, [49]) and lipooxygenase (LOX, [50]). However, jasmonates have not yet been detected in this moss. Nevertheless, it appears tempting to speculate that one of the first acquired functions of NAC transcription factors might have been the perception of oxylipins since more than 410 million years ago, a time estimate, which is based on the analyses by Zhu et al. [5].

The comprehensive phylogenetic analysis of 837 NAC transcription factor genes from 9 fully sequenced species of diverse evolutionary position by Zhu et al. [5] has revealed 21 NAC clades, of which 15 contain maize orthologs. In contrast, our analysis has revealed 12 NAC clades. If we take into account that clade C in our analysis can be divided into two subclades and clade G can be divided into three subclades, our analysis has generated an equal number of discernible clades compared to Zhu et al. [5]. However, the number of clades described in the analysis of Arabidopsis and rice NAC transcription factors [3,4,6,51] deviates from study to study. This indicates that the diversity of the employed genome information determines the computation of NAC clades due to the available number of protein sequences.

**Conclusion**

In this study, we have identified six maize NAC transcription factors that are induced upon challenge by fungal attack and in silico analysis revealed the presence of promoter elements that supports an involvement of five maize NACs in the defence transcription network. The two members that responded strongly to penetration by C. graminicola and that were studied in more detail, ZmNAC41 and ZmNAC100, were predominantly JA responsive. We have generated a systematic classification of maize NAC genes. On the basis of our phylogenetic analysis, we could reveal that the majority of those NAC transcription factors that have yet been described to be involved in the defence network of higher plants are monophyletic.

In summary, our study adds to a number of previous reports on the involvement of NAC transcription factors in the Arabidopsis, rice and barley defence response. Thus, an increasingly large number of NAC transcription factors seems to be involved not only in the regulation of developmental processes and abiotic stress responses, but also in the regulation of biotic stress responses.

**Methods**

**Cultivation of plant and fungal material**

Maize plants (Zea mays L.) cv. Nathan were cultivated in phytochambers at a PFD of 400 μE · m⁻² · s⁻¹ in a 14 h/10 h light/dark cycle as described by and Colletotrichum graminicola (Ces.) Wils. [teleomorph Glomerella graminicola (Politis)] was grown as described in [40].

**Infection assays**

For the infection experiments Colletotrichum graminicola wild type isolate CgM2 of C. graminicola and ATMT-generated pathogenicity mutants [40] were used. Spores of C. graminicola were washed off from 2 weeks old OMA plates with 1 ml distilled water and diluted to a final concentration of 2 × 10⁴ (low titer) or 2 × 10⁶ (high titer) spores/ml. As specifically stated in the text, fully expanded fourth leaves of two weeks old maize plants were either sprayed with a spore suspension of a high titer (2 × 10⁶ spores/ml), containing additionally 0.02% (v/v) Tween-20 or dipped in a spore suspension of a low titer (2 × 10⁴ spores / ml) for 24 h. Sprayed plants were kept in 100% relative humidity conditions for the next 24 h. Mock-treated leaves were sprayed with 0.02% (v/v) Tween-20 in Milli-Q distilled water or dipped in pure distilled water, respectively. As evaluated by microscopy of acid fuchsin stained leaf material [40], fungal proliferation was comparable in dip-inoculated and in spray inoculated leaves, although the conidia titer was 100-fold lower in dip-inoculated material. However, dip-inoculation resulted in a much more homogenous infection of the treated leaf segments. Leaves were collected at 24, 36 or 44 h after inoculation, frozen immediately in liquid nitrogen and subjected to further analysis.
Hormone treatment
Hormone treatments were performed with 1 mM jasmonic acid (JA), 1.3 mM of the SA analog 2,6-dichloroisonicotinic acid (INA), and 5 mM of the ethylene precursor 1-aminocyclopropane-1-carboxylic-acid (ACC). Fourth leaves of two weeks old maize plants were cut submerged in distilled water and incubated in 15 ml 0.2% ethanol containing the indicated hormone concentrations or no addition for mock controls. Leaves were collected after 0, 10 or 24 h of treatment, frozen immediately in liquid nitrogen and subjected for RNA extraction.

Abiotic stress assay
Maize plants were grown with regular watering to 100% field capacity every other day. Three weeks old plants were subjected to drought and high salinity by withholding water or continuing irrigation with 200 ml of 200 mM sodium chloride. Mock-treated plants were watered as before. After one week of stress treatment, all leaves of each plant were harvested, pooled and subjected for RNA extraction.

RNA extraction
Frozen plant material was ground with mortar and pestle in liquid nitrogen to a fine powder and extracted according to the method described by Chomczynski and Sacchi [52].

qRT-PCR
1 μg of total RNA was treated with DNase I (Fermentas, St. Leon-Rot, Germany) and RT reaction was performed with Revert Aid™ H Minus Reverse Transcriptase (Fermentas) in total volume of 40 μl according to the manufacturer’s protocol. qRT-PCRs were performed with 1 μl of cDNA from the above RT reactions using 2 × Brilliant II SYBR® Green QPCR Master Mix (Stratagene, St. Leon-Rot, Germany) and RT reaction was performed with the program ClustalW 2.0 [53] and phylogenetic trees were build using the UPGMA method implemented in the program Geneious Pro 5.4.3 [54] with 100 replicates for bootstrap assessment. Protein sequences were screened for common motifs with MEME Multiple Em for Motif Elicitation (http://meme.sdsc.edu/meme/cgi-bin/meme.cgi) [45].

Additional files

**Additional file 1:** Table S1. Chromosomal positions of the 116 identified ZmNAC genes in the maize genome.

**Additional file 2:** Figure S1. Domain architecture of maize NAC proteins. A multiple alignment of the consensus sequences of the whole length NAC proteins from each clade was compiled using ClustalW 2.0. Protein sequences were screened for common motifs with MEME Multiple Em for Motif Elicitation (http://meme.sdsc.edu/meme/cgi-bin/meme.cgi) at a cut off p-value of e-10. The motif location is given as follows: C-/N-term. – C-/N- terminus, NAC – NAC Domain, sd. A-E – subdomain A-E.

**Additional file 3:** Table S2. A list of motifs detected within the 116 NAC proteins. The protein sequences were screened with MEME (Multiple Em for Motif Elicitation, http://meme.sdsc.edu/meme/cgi-bin/meme.cgi) at a cut off p-value of e-10. The motif location is given as follows: C-/N-term. – C-/N- terminus, NAC – NAC Domain, sd. A-E – subdomain A-E.

Abbreviations
ABA: Abscisic acid; ACC: 1-aminocyclopropane-1-carboxylic-acid; At: Arabidopsis thaliana; ATMT: Agrobacterium tumefaciens mediated transformation; ERF: Ethylene response factor; ET: Ethylene; hpi: hours post infection; hpt: Hours post treatment; INA: 2,6-dichloroisonicotinic acid; JA: Jasmonic acid; NAC: NAM ATAF1 and CUC2-like transcription factor; MEME: Multiple Em for motif elicitation; Myc2: Myeloid differentiation primary response 2; Os: Oryza sativa; PR: Pathogenesis related; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; Ra26: Response to dehydration; Rd26: Secondary wall-associated NAC Domain; SWN: Swinger; Triticum aestivum; Zm: Zea mays.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AMV, HBD and LMV have conceptualized the research. SM and HBD have generated, isolated and characterized the mutants used in this study and performed the microarray analysis. AMV, SM, HBD and LMV have interpreted the microarray results. AMV has performed the experiments that have not yet been performed.
References

1. Souer E, van Houwelingen A, Kloos D, Mol J, Koes R: The No Apical Meristem gene of Petunia is essential for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell 1996, 85:159–170.
2. Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M: A NAC transcription factor family in Arabidopsis. Plant Cell 1997, 9:841–857.
3. Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Yamamoto Y, Yamamoto K, Kikuchi S: Comprehensive Analysis of NAC Family Genes in Oryza sativa and Arabidopsis thaliana. DNA Res 2003, 10:239–247.
4. Fang Y, You J, Xie K, Xie W, Xiong L: Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice. Mol Genet Genomics 2006, 278:547–563.
5. Zhu T, Nevo E, Sun D, Peng J: Phylogenetic analyses unravel the evolutionary history of NAC proteins in plants. Evol 2012, 66:1833–1866.
6. Shen H, Yin Y, Chen F, Xu Y, Dixon RA: A bioinformatic analysis of NAC genes for plant cell wall development in relation to lignocellulosic bioenergy production. Bioenerg Res 2009, 2:217–232.
7. Olsen AN, Ernst HA, Leggo LL, Skriver K: DNA-binding specificity and molecular function of NAC transcription factors. Plant Sci 2005, 169:785–797.
8. Welner DH, Lindemose S, Grossmann JG, Mollegaard NE, Olsen AN: Insight into the regulation of NAC transcription factors in crop plants. Mol Genet Genomics 2006, 275:929–943.
9. Ernst HA, Olsen AN, Larsen S, Lo Leggo L: Structure of the conserved domain of ANAC, a member of the NAC family of transcription factors. EMBO Rep 2004, 5:297–303.
10. Duval M, Hsieh TF, Kim SY, Thomas TL: Molecular characterization of ATNAM: a member of the Arabidopsis NAC domain superfamily. Plant Mol Biol 2002, 50:237–248.
11. Xie Q, Frugis G, Colgan D, Chua NH: Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes Dev 2004, 14:3024–3036.
12. Hao YJ, Wei W, Song QX, Chen HW, Zhang YQ, Wang F, Zou HF, Lei G, Tian AG, Zhang WK, Ma B, Zhang JS, Chen SY: Soybean NAC transcription factors promote abiotic stress tolerance and lateral root formation in transgenic plants. Plant J 2011, 68:302–313.
13. Lunde D: Molecular characterization of Brachycaudus japonicus NAC domain transcriptional activators induced in response to biotic and abiotic stress. Plant Mol Biol 2003, 53:383–397.
14. Zimmermann R, Peier W: Pattern formation in the monocot embryo as revealed by NAM and CUC3 orthologues from Zea mays. Plant Cell 2005, 17:4669–4685.
15. Guo R, Mupe MA, Danilevskaya ON, Yang X, Hu Z: Genome wide mRNA profiling reveals heterochronic allelic variation and a new imprinted gene in hybrid maize endosperm. Plant Cell Physiol 2003, 44:1552–1558.
16. Zhao C, Aoki L, Grant EH, Hageml CH, Beers EP: XND1, a member of the NAC domain family in Arabidopsis thaliana, negatively regulates ligninocellulose synthesis and programmed cell death in xylem. Plant J 2008, 53:425–436.
17. Zhang R, Demura T, Ye ZH: SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. Plant Cell 2006, 18:3158–3170.
18. Zhang R, Richardson EA, Ye ZH: Two NAC domain transcription factors, SND1 and NST1, function redundantly in regulation of secondary wall synthesis in fibers of Arabidopsis. Plant Cell 2007, 22:1603–1611.
19. Hu H, Dai M, Yao L, Xiao B, Li X, Zhang Q, Xiong L: Overexpressing a NAM, ATA1, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. PNAS 2006, 103:12987–12992.
20. Jeong JS, Kim YS, Baek KH, Jung H, Ha SH, Choi YD, Kim M, Reuzeau C, Kim JK: Root-specific expression of OsNAC10 improves drought tolerance and grain yield in rice under field drought conditions. Plant Physiol 2010, 153:185–197.
21. Nakashima K, Tran LA, Van Nguyen S, Fujita M, Maruyama K, Todaka S, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K: Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsiveness gene expression in rice. Plant J 2007, 51:617–621.
22. Takasaki H, Maruyama K, Kidokoro S, Ito Y, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K, Nakashima K: The abiotic stress-responsive NAC-type transcription factor OsNAC5 regulates stress-inducible genes and stress tolerance in rice. Mol Genet Genomics 2010, 284:173–183.
23. Yokotani N, Ichikawa T, Kondou Y, Matsui M, Hirochika H, Iwabuchi M, Oda K: Tolerance to various environmental stresses conferred by the salt-responsive rice gene ONAC063 in transgenic Arabidopsis. Plant Physiol 2009, 229:1065–1075.
24. Guo Y, Yan S: ATNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J 2006, 46:601–612.
25. Uauy C, Distelfeld A, Fahima T, Blecha F, Dubcovsky J: A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 2006, 314:298–301.
26. Fujita M, Fujita Y, Maruyama K, Seki M, Hirasuka K, Ohtake-Makiguchi M, Tran LS, Yamaguchi-Shinozaki K, Shinozaki K: A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J 2004, 39:863–876.
27. Delesse C, Kazan K, Wilson IW, Van Der Straeten D, Manners J, Dennis ES, DoFen R: The transcription factor ATAF2 represses the expression of pathogenesis-related genes in Arabidopsis. Plant J 2005, 43:745–757.
28. Wang X, Baisirayake BM, Zhang H, Li G, Li W, Nik N, Mengiste T, Song F: The Arabidopsis ATAF1, a NAC transcription factor, is a negative regulator of defense responses against necrotrophic fungal and bacterial pathogens. PMB 2009, 22:1227–1238.
29. Bu Q, Jiang H, Li CB, Zhao Q, Zhang J, Wu X, Sun J, Xie Q, Li C: Role of the Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signalized defense responses. Cell Res 2008, 18:756–767.
30. Lin R, Zhao W, Meng X, Wang M, Peng Y: Rice gene OsNAC19 encodes a novel NAC-domain transcription factor and responds to infection by Magnaporthe grisea. Plant Sci 2007, 172:120–130.
31. Collinge M, Roller T: Differential induction of two potato genes, Stnp2 and SINAP, in response to infection by Phytophthora infestans and to wounding. Plant Mol Biol 2001, 46:521–529.
32. Hegedus D, Yu M, Baldwin D, Gruber M, Sharpe A, Parkin I, Whitwill S, Lydiate D: Molecular characterization of Brassica napus NAC domain transcriptional activators induced in response to biotic and abiotic stress. Plant Mol Biol 2003, 53:383–397.
33. Zimmermann R, Weir W: Pattern formation in the monocot embryo as revealed by NAM and CUC3 orthologues from Zea mays L. Plant Mol Biol 2005, 58:669–685.
34. Guo R, Mupe MA, Danilevskaya ON, Yang X, Hu Z: Genome wide mRNA profiling reveals heterochronic allelic variation and a new imprinted gene in hybrid maize endosperm. Plant Cell Physiol 2003, 44:1552–1558.
35. Verza NC, Figuera TR, Sousa SM, Arruda P: Transcription factor profiling identifies an aleurone-preferred NAC family member involved in maize seed development. Ann Appl Biol 2011, 158:115–129.
36. Zhong R, Lee C, McCarthy RL, Reeves CK, Jones EG, Ye ZH: Transcriptional Activation of Secondary Wall Biosynthesis by Rice and Maize NAC and MYB Transcription Factors. Plant Cell Physiol 2011, 2:1856–1871.
37. Bergstrom GC, Nicholson RL: The biology of corn anthocyanin. Plant Dis 1999, 83:596–608.
38. Mendgen K, Hahn M: Plant infection and the establishment of fungal biotrophy. Trends Plant Sci 2002, 7:352–356.
39. Voll LM, Horst RJ, Voitsik AM, Zajic D, Samans B, Pons-Kühnemann J, Doehlermann G, Münch S, Wahl R, Molitor A, Hofmann J, Schmiedl A, Waller F, Deising HB, Kahrnann R, Kämper J, Kogel KH, Sonnewald U. Common motifs in the response of cereal primary metabolism to fungal pathogens are not based on similar transcriptional reprogramming. Front Plant Sci 2011, 2:99.

40. Münch S, Ludwig N, Floss DS, Sugui JA, Koszucka AM, Voll LM, Sonnewald U, Deising HB. Identification of virulence genes in the corn pathogen Colletotrichum graminicola by Agrobacterium tumefaciens-mediated transformation, Mol Plant Pathol 2011, 12:43–55.

41. Puranik S, Sahu PP, Prasanna PS, Prasad M. NAC proteins: regulation and role in stress tolerance. Trends Plant Sci 2012, 17:269–381.

42. Singh KB, Foley RC, Orte-Sánchez N. Transcription factors in plant defense and stress responses. Curr Opin Plant Biol 2002, 5:430–436.

43. Döhlemann G, Wahl R, Horst RJ, Voll LM, Usadel B, Poree F, Sitt M, Pons-Kühnemann J, Sonnewald U, Kahrnann R, Kämper J. Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph Ustilago maydis. Plant J 2008, 56:181–195.

44. Schnable PS, Ware D, Fulton RS, Stein JC, Wei F, Pasternak S, Liang C, Zhang J, Fulton L, Graves TA, Minx P, Reily AD, Courtney L, Knuchow SS, Tomilinson C, Strong C, Delehaunty K, Fronick C, Courtney B, Rock SM, Beter E, Du F, Kim K, Abbott RM, Cotton M, Levy A, Marchetto P, Ochua K, Jackson SM, Gillam B, Chen W, Yan L, Higginbotham J, Cardenas M, Waligorski J, Applebaum E, Phelps L, Falcone J. The B73 maize genome: complexity, diversity, and dynamics. Science 2009, 326:1112–1115.

45. Bailey TL, Elkan C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology: AAAI Press, Menlo Park, California, 1994:28–36

46. Jensen MK, Hagedom PH, de Torres-Zabala M, Grant MR, Rung JH, Döhlemann G, Wahl R, Horst RJ, Voll LM, Usadel B, Usadel B, Poree F, Sitt M, Pons-Kühnemann J, Sonnewald U, Kahrnann R, Kämper J. Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph Ustilago maydis. Plant J 2008, 56:181–195.

47. Schnable PS, Ware D, Fulton RS, Stein JC, Wei F, Pasternak S, Liang C, Zhang J, Fulton L, Graves TA, Minx P, Reily AD, Courtney L, Knuchow SS, Tomilinson C, Strong C, Delehaunty K, Fronick C, Courtney B, Rock SM, Beter E, Du F, Kim K, Abbott RM, Cotton M, Levy A, Marchetto P, Ochua K, Jackson SM, Gillam B, Chen W, Yan L, Higginbotham J, Cardenas M, Waligorski J, Applebaum E, Phelps L, Falcone J. The B73 maize genome: complexity, diversity, and dynamics. Science 2009, 326:1112–1115.

48. Bailey TL, Elkan C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology: AAAI Press, Menlo Park, California, 1994:28–36.

49. Hashimoto T, Takahashi K, Sato M, Bandara PKGSS, Nabeta K. Characterization of an allene oxide synthase in Physcomitrella patens. Physcomitrella patens has lipoxygenases for both eicosanoid and octadecanoid pathways. Phytochemistry 2009, 70:460–529.

50. Christensen MW, Holm PB, Gregersen P. Characterization of barley (Hordeum vulgare L) NAC transcription factors suggests conserved functions compared to both monocots and dicots. BMC Res Notes 2011, 4:200.

51. Christiansen MW, Holm PB, Gregersen P. Characterization of barley (Hordeum vulgare L) NAC transcription factors suggests conserved functions compared to both monocots and dicots. BMC Res Notes 2011, 4:302.

52. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156–159.

53. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 1994, 22:4673–4680.

54. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Dusan C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thirer T, Wilson A. Geneious v5.4.1, 2011. Available from http://www.geneious.com.