Expression patterns of *NbrgsCaM* family genes in *Nicotiana benthamiana* and their potential roles in development and stress responses

Dandan Liu & Qiuying Yang

*rgsCaM* has been reported as a calmodulin-like (CML) factor induced by viral infection in *Nicotiana*. There are three CMLs that belong to the *rgsCaM* family in *Arabidopsis thaliana*. In this study, we found a total of 5 *NbrgsCaM* coding sequences in *N. benthamiana* genome. We analyzed transcription patterns of *NbrgsCaMs* in transgenic plants expressing a β-glucuronidase (GUS) under the promoter of *NbrgsCaMs* by histochemistry staining and RT-qPCR. Similar to their *Arabidopsis* homologs, most *NbrgsCaMs* have an overlapping but distinct expression pattern in response to developmental and environmental changes. Specifically, the *NbrgsCaM4* promoter exhibited robust activity and showed distinct regulatory response to viral infection, developmental stages and other abiotic stimuli. Overall, these findings provide clues for further understanding of the *NbrgsCaM* family genes in regulating plant growth and development under biotic stress and environmental stimulation.

Calmodulins (CaMs) are a group of Ca\(^{2+}\) binding regulatory proteins in the signal transduction cascades of eukaryotic cells. CaMs respond to diverse biotic and abiotic stimuli, and modulate the cellular activities according to developmental and environmental changes\(^1\).

Plants have an extended family of CaMs, and the less conserved forms of CaMs are called CaM-related proteins or CaM-likes (CMLs)\(^2\). *Arabidopsis* has a large CML family including about 50 CML genes\(^3,4\). Among them, *AtCML37* (AT5G42380), *AtCML38* (AT1G76650) and *AtCML39* (AT1G76640) fall into a unique subfamily, and function in plant development, respond to stress stimuli such as hypoxia, drought and herbivore feeding\(^5–8\). This unique subfamily of AtCMLs are closely related to the regulator of gene silencing CML protein (*rgsCaM*) in *Nicotiana tabacum* (*NtrgsCaM*). In the first *rgsCaM* report, *NtrgsCaM* was regarded as a suppressor of virus-induced gene silencing (VIGS), which counters gene silencing effect from plants to promote viral amplification\(^9\). Another more recent report stated that a homologous CML in *N. benthamiana*, *NbrgsCaM*, also was required for suppressing VIGS through interaction with a viral suppressor of RNA silencing factor (VSR), βC110. However, in publications by Tadamura\(^7\) and Nakahara\(^11\), *NtrgsCaM* was concluded to take an antiviral role by sequestrating the RNA silencing suppressors (RSSs) through binding of the dsRNA-binding domains of viral RSSs, acting as an antiviral pattern recognition receptor (PRR).

Morphology changes of *Nicotiana* transgenic lines over-expressing *rgsCaMs* were described to be similar to the deformities caused by viral infections in several reports, such as formation of tumors at the root-stem junction, curly leaves with wrinkles, necrosis and dwarfing\(^8,10,12\). However, no such changes were observed in *N. benthamiana* over-expressing *NtrgsCaM*\(^13\). Although these findings are rather complicated, all evidence pointed to the importance of rgsCaMs in viral infection and plant development. Furthermore, the induction of *rgsCaMs* expression by viral infection was noticed in both tobacco and *Arabidopsis*\(^5,10,11\), which drives us to explore their intrinsic regulation during viral infection, environmental stress and plant development, and also, the possible

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\(^1\)State Key Laboratory for Plant Disease and Insect Pest, Institute of Plant protection, China Academy of Agricultural Sciences, Beijing, 100193, China. 2State Key Laboratory of Biocatalysis and Enzyme Engineering, College of Life Sciences, Hubei University, Wuhan, 430062, China. 3Email: qiuying.yang@hubu.edu.cn
existence of their close homologs. Thereby, we chose \textit{N. benthamiana}, the most widely used model plant in the virology research field\textsuperscript{14}, to begin our study of the rgsCaMs regulation.

**Results**

**Analysis of the sequences of rgsCaM family.** Although several CMLs that belong to the rgsCaM family have been found in \textit{Arabidopsis}, there was only one \textit{Nt}rgsCaM reported in \textit{N. tabacum} and one \textit{NbrgsCaM} in \textit{N. benthamiana}\textsuperscript{15-17}. As an allotetraploid\textsuperscript{18-19}, \textit{N. benthamiana} usually has more homologs than does \textit{Arabidopsis}. Based on this hypothesis, we analyzed the genome of \textit{N. benthamiana} (https://solgenomics.net/organism/Nicotiana_benthamiana/genome)\textsuperscript{20} in order to find all of the \textit{rgsCaM} family genes. We found a total of 7 homologs through blasting against the \textit{N. benthamiana} draft genome with the already known \textit{NbrgsCaM}. These homologs were named \textit{NbrgsCaM 1–7} according to the increasing order of scaffold serial numbers where these homologs reside (Supplementary Data S1). Due to the primitiveness of the current draft genome, some of these homologs have un-sequenced gaps. To fill these gaps, we carried out polymerase chain reactions (PCRs) with homolog-specific primers that were designed according to the available genome resources\textsuperscript{18}, and confirmed/corrected the full-length sequences of these homologs (Supplementary Data S2). Amino acid sequences predicted from these homologs are listed in Supplementary Data S3. The newly discovered \textit{NbrgsCaM1}, 3, 5, 7 have no predictable intron within the coding region, which is similar to the already published \textit{AtCML37}, 38, 39, \textit{Nt}rgsCaM (\textit{Nt}rgsCaM4 in this study is the previously reported \textit{NbrgsCaM}). However, \textit{NbrgsCaM2} and \textit{NbrgsCaM6} contain only 2 fragments of the consensus \textit{NbrgsCaM} open reading frames (ORFs). The two fragments of \textit{NbrgsCaM2} and \textit{NbrgsCaM6} have ORFs less than 100aa in length (Supplementary Fig. S1 and Supplementary Data S3). There is no predicted intron-exon junction between the two fragments to bridge them into longer coding sequences (predicted on NetGene2 server, http://www.ncbi.nlm.nih.gov/projects/gorf/\textsubscript{21}).

Thus, they looked like pseudogenes that derived from full-length ancestral \textit{rgsCaM} genes. Through blasting these ORF fragments to the online NCBI protein library\textsuperscript{22}, we found that the shorter ORFs encode incomplete amino acid fragments of \textit{rgsCaM} family proteins that do not contain any known motifs; while the longer ones encode only one EF-hand superfamily motif (Supplementary Fig. S1). As the CML family proteins typically require a conserved pair of EF-hand superfamily motifs for their function (Supplementary Fig. S1)\textsuperscript{23}, we proposed that \textit{rgsCaM2} and \textit{rgsCaM6} are either pseudogenes that do not encode functional proteins, or encode new proteins of a yet unknown function. Thus we focused on \textit{rgsCaM1}, 3, 4, 5 and 7 only, to elucidate the character of \textit{rgsCaM} family genes in \textit{N. benthamiana}. The rooted phylograms of the coding nucleotide sequences and protein sequences show close similarity within the \textit{NbrgsCaM} family (Fig. 1a,b). To clarify the phylogenetic relationship of these \textit{NbrgsCaM}s with other CMLs, we constructed a phylogenetic tree using protein sequences of CMLs and CMLs in \textit{N. benthamiana} and \textit{A. thaliana} genome. \textit{NbrgsCaM}s group with \textit{AtCML37}, 38 and 39, which probably \textit{AtrgsCaM}s, as previously reported (Fig. 1c)\textsuperscript{4,10,12}. However, \textit{rgsCaM}s do not form a distinct branch that is separated from other CMLs. They form a sub-branch within the CML family in the phylogenetic tree (Fig. 1c).

We also noticed that besides the previously reported \textit{AtrgsCaM}s (\textit{AtCML37}, 38 and 39), \textit{AtCML40} and 41 fall into this subgroup, indicating their close evolutionary relationship.

**Analysis of the promoters of rgsCaM family.** Our analysis (by Softberry TSSP program online prediction, http://linux1.softberry.com/\textsubscript{24}) of the regions between the stop codon of the previous gene and the start codon of \textit{AtCML37}, 38 and 39, which are putative promoters named as \textit{AtrgsCaMp} or \textit{AtCMLps}, revealed promoter and enhancer motifs (listed in Supplementary Data S4) that relate to development, phytohormonal regulation and environmental stresses. The correlation between transcription factors and the predicted transcription factor binding sites/RegSites can be found in the Softberry RegSite database (http://www.softberry.com/berry.pl?topic=regsite\textsubscript{25}). For clarity, we did not list the candidate transcription factors for each promoter/enhancer motifs. Instead, we summarized the transcription factors for these predicted motifs (Supplementary Table S1), consistent with the reported involvement of \textit{AtrgsCaM} in development and stress responses\textsuperscript{3,6,8,24,25}.

The promoters of \textit{NbrgsCaM}s also contain multiple regulatory elements (Supplementary Data S5), many of which are the same type of motifs that exist across the \textit{rgsCaM} family. The transcription factors that are predicted for the recognition of these regulatory elements are listed, together with their specific functions (Supplementary Table S2). According to the description of these transcription factors, \textit{NbrgsCaM}s should be related to multiple developmental, environmental and plant hormonal regulations, and with overlapping but distinct stimuli response patterns among the \textit{NbrgsCaM}s (Supplementary Table S3). Thus, combining the promoter analysis of \textit{AtrgsCaM} (Supplementary Table S1) and the reported function of \textit{AtrgsCaM}s\textsuperscript{3,6,8,24,25}, it can be deducted that \textit{rgsCaM}s are not only homologous in coding sequences, but also respond to similar types of environmental and developmental stimuli in \textit{Nicotiana} and \textit{Arabidopsis}. However, the promoters of \textit{rgsCaM}s are rather conserved in regulatory motifs but not in sequence, and the enhancer motifs do not hold the same positions (Supplementary Data S4 and S5), suggesting that the functionally conserved \textit{rgsCaM} evolved subtle and distinct regulatory niches to carry out precise regulatory work.

**β-glucuronidase (GUS) reporter analysis of the promoters of rgsCaM family genes.** To analyze the expression profile of \textit{rgsCaM}s, we constructed chimeric \textit{rgsCaM-promoter::GUS} (\textit{rgsCaMp::GUS}) reporters. GUS staining of the transgenic \textit{N. benthamiana} with \textit{rgsCaMp::GUS} reporters was carried out at various developmental stages and under specific stress treatments.

The transgene of \textit{rgsCaMp::GUS} reporters (\textit{NbrgsCaMp1::GUS}, \textit{NbrgsCaMp3::GUS}, \textit{NbrgsCaMp4::GUS}, \textit{NbrgsCaMp5::GUS} and \textit{NbrgsCaMp7::GUS}) afflicted no impact on the growth of \textit{N. benthamiana}, indicating that our reporter system didn’t intervene with the physiology of transgenic plants. The GUS staining in these transgenic plants can actually reflect the endogenous promoter activity of these \textit{NbrgsCaMs}. In seedlings of the \textit{N. benthamiana} transgenic lines, GUS expression was detected in \textit{NbrgsCaMp1::GUS}, \textit{NbrgsCaMp3::GUS}, \textit{NbrgsCaMp5::GUS}.
NbrgsCaMp4::GUS and NbrgsCaMp5::GUS, while NbrgsCaMp7::GUS showed no observable staining at all (Fig. 2a). Among the transgenic plants with GUS expression, NbrgsCaMp3::GUS and NbrgsCaMp4::GUS showed darker GUS staining than NbrgsCaMp1::GUS and NbrgsCaMp5::GUS (Fig. 2a). The GUS transcription level was further measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR). The RT-qPCR result affirmed that the GUS staining indeed reflected the transcription level that is the indicator of promoter activity (Fig. 2b). As the seedlings grew older, we found that more GUS staining accumulated in the root of

Figure 1. NbrgsCaMs form a close CML family. (a) The phylogenetic tree of nucleotide sequences of NbrgsCaMs. (b) The phylogenetic tree of amino acid sequences of NbrgsCaMs. c. rgsCaMs are grouped within a sub group in the CaMs and CMLs of A. thaliana and N. benthamiana. AtrgsCaM, NbrgsCaM, rgsCaM subgroups are highlighted by rectangular boxes. The scale bars represent substitutions per sequence position.
**NbrgsCaMps have various levels of activity.** (a) GUS histochemistry analysis revealed various promoter activities of NbrgsCaMs in seedlings of the NbrgsCaM::GUS transgenic N. benthamiana. (b) RT-qPCR results of the GUS expression level confirmed the histochemistry results. The relative GUS expression level of NbrgsCaM7::GUS transgenic N. benthamiana plants was set as 1, as it showed no detectable GUS staining. The relative GUS expression levels of the other NbrgsCaM::GUS transgenic N. benthamiana plants were calculated using that of the NbrgsCaM7::GUS transgenic N. benthamiana plants as control. (c) The promoter activity of NbrgsCaM4 is tissue-specific. The GUS staining accumulates mostly in veins and roots. Bars = 5 mm.

**NbrgsCaMps display tissue-specific activities.** (a) Gus histochemistry analysis revealed tissue-specific activities of NbrgsCaMs in the NbrgsCaM::GUS transgenic N. benthamiana. Bars = 5 mm. (b) RT-qPCR results of the GUS expression levels confirmed the histochemistry results.
β

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NbrgsCaM4

NbrgsCaMp4

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N. benthamiana

or

GUS

NbrgsCaM4

NbrgsCaMp4::GUS

transgenic or wild type

N. ben-

tamiana

or

GUS

NbrgsCaM4

NbrgsCaMp4::GUS

transgenic plants except NbrgsCaMp7::GUS, which showed no detectible expression at all

(Figs. 2a and 3a).

Analysis of NbrgsCaMps activity under salt and PEG treatment. To investigate whether NbrgsCaMps

to environmental stresses, we applied salt and PEG-simulated drought treatment to the transgenic seed-

lings. After transferring the seedlings to media containing additional salt or PEG for 5 days, GUS staining was

carried out to detect the NbrgsCaMps activity. Elevated GUS staining was detected in both salt and PEG treatment

for NbrgsCaMp4::GUS compared to the untreated ones. However, NbrgsCaMp5::GUS responded mainly to salt, and

NbrgsCaMp1::GUS responded mainly to PEG, while the other samples showed no detectable response to the

application of either salt or PEG (Fig. 4).

Analysis of rgsCaMps activity after viral infection. To investigate the response of NbrgsCaMps to viral

infection, we inoculated N. benthamiana plants with DNA A of Tomato yellow leaf curl China virus (TYLCCNV

A), TYLCCNV A together with its β satellite (TYLCCNV A + β), Potato virus X (PVX), Tobacco mosaic virus

(TMV), or inoculation buffer, which was used as a negative control. Then we compared the GUS staining of the

systemic leaves. Because NbrgsCaMps activities are developmental stage-related, we carefully chose the leaves at

the same developmental stage for comparison. TYLCCNV A and TYLCCNV A + β triggered induction of GUS

expression in symptomatic systemic leaves of NbrgsCaMp3::GUS, NbrgsCaMp4::GUS and NbrgsCaMp5::GUS

at 7 days post infection (dpi) (Fig. 5a). The induction difference was observed between NbrgsCaMp4::GUS

infected by TYLCCNV A + β and by TYLCCNV A, and the TYLCCNV A + β infection induced heaver GUS

staining (Fig. 5a), which was also confirmed by RT-qPCR of the expression of NbrgsCaM4 in wild type N. ben-

tamiana plants at 12 dpi (Fig. 5e). However, we failed to observe significant elevation of GUS expression in

NbrgsCaMp4::GUS infected by TYLCCNV A + β comparing to TYLCCNV A and buffer only inoculation at

12 dpi (Fig. 5d). There was a difference in fold change of GUS expression between TYLCCNV A + β and buffer

only inoculation, but was not as high as the previously reported case40. This might be due to the less sensitivity of

gene reporter and large variation between biological repeats. Nonetheless, RT-qPCR of the systemic leaves at

5 dpi (the time when viral symptoms first appear) showed that TYLCCNV A + β and TYLCCNV A triggered

no difference in the expression of either GUS or NbrgsCaM4 in NbrgsCaMp4::GUS transgenic or wild type N. ben-

tamiana plants respectively (Fig. 5b,c). Additional analysis of the systemic leaves of NbrgsCaMp4::GUS infected

by PVX or TMV showed that both viruses triggered induction of GUS expression, as evidenced by the GUS

staining (Fig. 6a,b) and RT-qPCR results (Fig. 6c,d). Additionally, after inoculation with PVX, βC1 (a PVX-based

viral vector for overexpression of geminivirus viral factor βC1), transient overexpression of βC1 induced higher

GUS expression than by inoculation with PVX alone (Fig. 6a,c), resembling the effect of DNAβ in TYLCCNV

infection (Fig. 5a,e).

Our results confirmed that inoculation with the viruses listed above (TYLCCNV A, TYLCCNV A + β,

PVX, TMV, PVX, βC1), all induced GUS staining in symptomatic systemic leaves of NbrgsCaMp4::GUS. In both the

TYLCCNV A + β-infected plants and the PVX, βC1-infected plants, the existence of βC1 triggered higher

NbrgsCaMp4 activity and NbrgsCaM4 expressions after the initial display of symptoms. The exacerbating effect of

βC1 manifested its function as a viral pathogenicity factor. However, the TYLCCNV A + β inoculated plants

failed to display increase of GUS induction compared to TYLCCNV A when the early symptoms displayed at

5 dpi. It is still unclear whether the presence of βC1 induced the activation of NbrgsCaMp4 directly or through

accumulated pathogenicity of the virus.

Noticeably, in both seedlings and mature plants, the GUS staining particularly accumulated in the veins for all of

the NbrgsCaMp::GUS transgenic plants except NbrgsCaMp7::GUS, which showed no detectible expression at all

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accumulated pathogenicity of the virus.
Analysis of NbrgsCaMps activity in βC1 transgenic N. benthamiana. To elucidate the effect of βC1 on the activation of NbrgsCaMp4, we directly measured the transcription of NbrgsCaMp4 by RT-qPCR. The transcripts level of NbrgsCaMp4 was higher in βC1 transgenic N. benthamiana than in WT (Fig. 7a,b). As the βC1 transgenic N. benthamiana takes much longer time to grow (usually more than a year to reach the flowering stage) than the WT (50 days to reach the flowering stage), we tried to rule out the influence of leaf age on NbrgsCaM4 expression. The results showed that, after the seedling stage, the activity of NbrgsCaMp4 increased as the leaves aged even in the same plant (Fig. 7c–d). Thus, the influence of age on the activity of NbrgsCaMp4 between the βC1 transgenic and WT N. benthamiana becomes more important. To clarify this factor, we further tested the NbrgsCaMp4 expression in seedlings of the βC1 transgenic and WT N. benthamiana at 10 days after germination (Fig. 7h). The RT-qPCR

Figure 5. The activities of NbrgsCaMps were induced by TYLCCNV inoculation. (a) GUS histochemistry analysis revealed activities of the NbrgsCaMps induced by TYLCCNV inoculation at 7 dpi in the NbrgsCaMp::GUS transgenic N. benthamiana plants. In the first column, representative viral symptoms of the inoculated N. benthamiana plants at 7 dpi are shown. TYLCCNV A inoculation triggered mild induction of GUS expression in NbrgsCaMp3::GUS, NbrgsCaMp4::GUS and NbrgsCaMp5::GUS, whereas TYLCCNV A + β inoculation triggered stronger induction of GUS expression in these plants. The GUS staining in NbrgsCaMp4::GUS was most responsive to TYLCCNV A + β infection. Bars = 5 mm. (b,c) RT-qPCR results of the GUS and NbrgsCaM4 expression levels showed that there was no significant elevation of promoter activity for NbrgsCaMp4 in plants with TYLCCNV A and TYLCCNV A + β inoculation at 5 dpi. (d) Compared to the buffer-only inoculation, no significant elevation of GUS expression was detected in TYLCCNV A and TYLCCNV A + β inoculated plants at 12 dpi. (e) Significant elevation in NbrgsCaM4 expression was detected in WT N. benthamiana at 12 dpi. P < 0.05. Error bars indicate S.D.
results showed that the activity of NbrgsCaMps is higher in βC1 transgenic N. benthamiana (Fig. 7i). However, the βC1 transgenic N. benthamiana is deformed with needle-like leaves (Fig. 7a). This type of needle-like leaves mostly are composed of veins due to the pathogenicity of viral factor βC1 (Supplementary Fig. S2). As GUS staining accumulated mainly in veins of the NbrgsCaMp::GUS (Figs. 2a, 3a, 4 and 5a), it is difficult to exclude the influence of tissue expression bias to draw a conclusion that the up-regulation of NbrgsCaMp4 activity is directly generated by βC1 in βC1 transgenic N. benthamiana.

**Analysis of NbrgsCaMps activity under wounding treatment.** Viral symptoms progressed as the infection persisted, and necrotic spots and yellowing usually accumulated in the infected leaves as the symptoms aggravated. For N. benthamiana, the infection by TYLCCNV A + β can induce higher activity of NbrgsCaMp4 than the infection by TYLCCNV A after prolonged infection, but not at the early stage, i.e. at 12 dpi (Fig. 5e) instead of at 5 dpi (Fig. 5c). This made us wonder whether the accumulated damaging effect of TYLCCNV combined with βC1 is the direct trigger of NbrgsCaMp4 activity, rather than the expression of βC1 itself. To investigate the damaging effect on NbrgsCaMp4 activity, we infiltrated the inoculation buffer to the leaves of NbrgsCaMp4::GUS transgenic N. benthamiana plants to impose mechanical damage without introducing viral infection. DAB staining carried out at 1 h after infiltration revealed dark spots around the injection sites, indicating the presence of damages (Fig. 7j). RT-qPCR results showed that the transcription of GUS in buffer-only-infiltrated leaves elevated up to 200 fold, compared to that of the untreated control at 1 h post infiltration (Fig. 7l). This transcription elevation subsided quickly. At 3 h post infiltration, it was still about 10 fold higher than the control but not that dramatic (Fig. 7l). On the other hand, in the GUS staining results, the leaves were stained more heavily at 3 h than at 1 h, a little postponed, which might be due to the accumulation of GUS expression (Fig. 7k). These results indicated that NbrgsCaMp4 responded instantly to damage stress. As viral infection can afflict damages to plants, the up-regulation of NbrgsCaMp4 activity can be attributed to the side effect of viral symptoms.

**Figure 6.** The activity of NbrgsCaMp4 was induced by PVX and TMV inoculation. (a,b) GUS histochemistry analysis revealed activities of NbrgsCaMps by PVX, PVX:βC1 and TMV inoculation, at 5 dpi and 4 dpi respectively, in NbrgsCaMp::GUS transgenic N. benthamiana with mock-inoculated or untreated plants as controls. Bars = 5 mm. (c,d) RT-qPCR results of the GUS expression level showed that there were significant differences in promoter activity for NbrgsCaMp4 by TMV infection when compared to that of the controls. P < 0.05. Error bars indicate S.D.
Discussion

**NbrgsCaM**s form a unique branch of CML subfamily with multiple members. While rgsCaM was first discovered in *Nicotiana*\(^4\), and had been assigned complicated functions in viral responses\(^7\)–\(^12\), it has been considered the only rgsCaM in *Nicotiana* for about 20 years. Its close homologs have been left in the shadows until this study, in which our analysis revealed 5 coding genes and 2 possible pseudogenes in *N. benthamiana* (Supplementary Data S1–S3). We named these coding genes and pseudogenes *NbrgsCaM*\(^1\)–\(^7\), sequentially according to the scaffold serial numbers they reside in. *NbrgsCaM*\(^2\) and *NbrgsCaM*\(^6\) are counted as pseudogenes because their ORFs show no similarity to other proteins except the CaM family, and even their longest ORFs contain only an incomplete CaM function motif. Coding sequences of *NbrgsCaM*\(^1\), \(^3\), \(^4\), \(^5\) and \(^7\) share identities ranging from 70.4% to 93.3% (amino acid sequences identity ranging from 60.2% to 83.2%) (Supplementary Data S2 and S3). Like AtCML\(^{37,38,39}\), the ArgrsCaMs in *Arabidopsis*\(^4\), NbrgsCaMs form a unique subclass of CMLs in *N. benthamiana* (Fig. 1), with close similarities to each other. In the phylogenetic tree, AtCML\(^{40,41}\) coexist in the same subclass with the reported rgsCaMs (Fig. 1c). These two genes have not yet been thoroughly studied, as had the AtCML\(^{37,38,39}\)\(^5\)–\(^6\),\(^24,25\). Further studies of them might be able to provide more clues to the role of rgsCaMs in plant development and stress responses.
**NbrgsCaMs display differential expression during development and under environmental stresses.** There are about 50 CMLs in the *Arabidopsis* genome, answering to Ca^{2+} fluctuations generated through nearly all environmental, hormonal and developmental stimuli. As a close subfamily of CMLs, not only the resemblance in amino acid sequences, but also the existence of identical enhancer motifs in the promoters of AtCML37, 38 and 39 correlate with their similar but distinct responses to environmental and developmental stimuli (Supplementary Table S11,8,24-25). Similarly, our analysis revealed that *NbrgsCaMs* are enriched with developmental and stress specific enhancer elements, many of which also exist in the promoters of *AtCMLs*, and each *NbrgsCaM* has a specific combination of enhancer motifs (Supplementary Table S2 and S3). The disclosure of the amino acid sequences and enhancer elements of *NbrgsCaMs* indicates that they possess overlapping but non-identical regulatory functions in *N. benthamiana*. *NbrgsCaM*::GUS reporter analysis revealed tissue- and developmental stage-specific promoter activities of *NbrgsCaM1*, 3, 4 and 5 (Fig. 3), corroborating that *NbrgsCaMs* are important developmental regulatory factors. Furthermore, salt and PEG treatment induced elevated GUS expression in most of the *NbrgsCaM*::GUS transgenic *N. benthamiana* (Fig. 4), demonstrating that *NbrgsCaMs* respond to environmental stresses as well. Each of these *NbrgsCaMs* drives a specific GUS expression pattern. In vegetative tissue, the GUS staining is most pronounced in veins of leaves and roots (Figs. 2a, 3a, 4a and 5a), corresponding to the vascular and root development-related promoter motifs: ATHB-2, AtMyb77, RAV1 and Root-specific nuclear factor enhancer elements (Supplementary Table S2). Among the *NbrgsCaMs*, *NbrgsCaMp3* and 4 have the strongest GUS staining in both seedling and mature leaves (Figs. 2 and 3); *NbrgsCaMp4* and 5 responded more to salt stress than the other *NbrgsCaMs* did (Fig. 4); while *NbrgsCaMp4* is the strongest *NbrgsCaM* in roots (Fig. 3a). All in all, *NbrgsCaMp4* is the most highly active promoter among the *NbrgsCaMs* during vegetative growth, and responds actively to salt and PEG stress. In flowers, *NbrgsCaMp4* is also one of the strongest *NbrgsCaMs* (Fig. 3). Thus, there is no wonder that *NbrgsCaMp4* was the first gene discovered in *N. benthamiana* due to its predominant expression level13. On the other hand, although we failed to detect promoter activity in *NbrgsCaMp7* by GUS staining in the *NbrgsCaMp7*::GUS transgenic lines, the expression of other *NbrgsCaMs* are not to be neglected according to their promoter activities that were shown clearly by the GUS staining and RT-qPCR analyses, and undoubtedly they play important roles, considering that the activities of *NbrgsCaMp1*, 3, and 5 are robust during development and under stress treatments (Figs. 2–5). The evidence of *NbrgsCaMs* expression can also be found in the RNA-seq data provided by the Sol Genomics Network (https://solgenomics.net/jbrowse_solgenomics/1,8,26). The RNA_seq reads can be viewed by typing in the scaffold location of specific genes on JBrowse, the Sol Genomics Network. Despite the presence of gaps and assembly incompleteness in genomic region of some *NbrgsCaMs*, it is clear that RNA_seq reads for *NbrgsCaM1*, 3, 4 and 5 are abundant18,26. The RNA_seq reads count for *NbrgsCaM4* is the highest, and for *NbrgsCaM7* is the lowest, which is only about 1/10 of that of the *NbrgsCaM4*.

**TYLCCNV infection induces the activity of *NbrgsCaMp3* and *NbrgsCaMp5*, in addition to *NbrgsCaMp4*.** *NbrgsCaMp4* was the first discovered *NbrgsCaM*14. The namesake of “rgs” came from its role as a regulator of gene silencing in virus-plant interaction. “rgs” is by far the pivotal role studied for Nicotiana rgs-CaM, despite the discovery of developmental and stress-related functions for *AtCMLs* in *Arabidopsis*19,20,24,25. In this study, we investigated *NbrgsCaMs* responses to viral infection. Increased induction of GUS expression was detected in the systemic leaves of *NbrgsCaMp3*::GUS for not only *NbrgsCaMp4*, but also *NbrgsCaMp3* and 5 after inoculation with TYLCCNV A and TYLCCNV A + β, compared to those of the untreated plants (Fig. 5a).

βC1 induced the expression of *NbrgsCaM4* through a damaging side effect of its virulence. We detected elevated *NbrgsCaM4* expression in TYLCCNV A + β inoculated wild type *N. benthamiana*, when compared to those inoculated with TYLCCNV A at 12 dpi (Fig. 5e), similar to a previous report20. But quite unexpectedly, we found that TYLCCNV A and TYLCCNV A + β treatment induced equivalent GUS or *NbrgsCaM4* expression levels in systemic leaves of *NbrgsCaM4*::GUS and wild type *N. benthamiana* at 5 dpi (Fig. 5b,c). The time point of 5 dpi is when viral symptoms shown up in the TYLCCNV A + β inoculated systemic leaves. The initial viral symptoms of TYLCCNV A + β infection appeared in our observations as wrinkled curly leaves and bulging veins at 5dpi, demonstrating the presence of βC1 in the leaves that we analyzed at this time point. This suggested that βC1 possibly is not the direct factor for *NbrgsCaM* + β expression in the early stage of viral infection. Furthermore, the GUS expression levels were induced by RNA viruses, PVX and TMV (Fig. 6), similar to the results reported by Chung et al.13, suggesting that the induction of *NbrgsCaMp4* is rather a general response to viral infection, indiscriminate of DNA or RNA viruses. We have used untreated instead of mock-inoculated *N. benthamiana* plants as controls for TMV infection. This is the only place where the untreated *N. benthamiana* plants were used instead of mock-inoculated ones. As the damage induced activity of *NbrgsCaMp4* subsides quickly (Fig. 7l), and no induced upregulation of GUS expression has been detected in the mock-inoculated *NbrgsCaMp4*::GUS transgenic *N. benthamiana* plants after 3 dpi, we consider the untreated plants to be as sufficient a control as the mock-inoculated ones in TMV infection. In addition, the presence of βC1 in TYLCCNV A + β and PVX:βC1 both induced higher activity of *NbrgsCaM4* promoter (Figs. 5 and 6), suggesting that βC1 can promote the expression of *NbrgsCaM4* together with its natural master virus TYLCCNV A or the artificial viral vector PVX.

The application of βC1 together with its master viral DNA – TYLCCNV A, or with a RNA viral vector – PVX, introduced other viral factors that probably interfered with *NbrgsCaM4* expression. To single out βC1 for further analysis, we directly analyzed *NbrgsCaM4* transcription in transgenic *N. benthamiana* for βC1 overexpression. Although in both seedlings and mature plants, the expression level of *NbrgsCaM4* was higher in βC1 transgenic plants than in WT (Fig. 7a,b,h,i), we still cannot solely attribute the induction of *NbrgsCaM4* to βC1 directly, as we can’t rule out the impact of morphology changes and extended vegetative growth stage of the βC1 transgenic plants on *NbrgsCaM4* expression. Our analysis of the expression levels of *NbrgsCaM4* and GUS in WT...
and NbrgsCaM4p::GUS transgenic N. benthamiana plants respectively, provided evidence that the NbrgsCaM4p activity increased greatly according to the advance of leaf aging in mature plants (Fig. 7c to g). Furthermore, the GUS staining accumulated in veins of NbrgsCaM4p::GUS (Figs. 2a, 3a, 4 and 5a), corroborating with the presence of vascular-specific enhancer element in NbrgsCaM4p (Supplementary Table S2). As the deformed leaves of βC1 transgenic N. benthamiana are composed mostly of vascular tissue (Supplementary Fig. 2), and take a very long time to grow, it is hard to justify whether the elevated NbrgsCaM4p activity came from the skewed development caused by βC1 transgene or from the βC1 factor directly. Thus, it is more reliable to analyze the induction of NbrgsCaM4p through TYLCCNV A + β treatment than in βC1 transgenic plants.

TYLCCNV A is a mild virus that induces almost no symptoms to N. benthamiana27,28. On the other hand, βC1 is a pathogenic factor which for the severe viral symptoms generated by TYLCCNV A + β, such as curly leaves29. Thus, it was considered to be the trigger of many physiological changes in the infected plants. The fact that the TYLCCNV A + β and TYLCCNV A triggered divergence in NbrgsCaM4 activity happened several days after the appearance of viral symptoms is largely the reflection of the side effect of βC1 virulence, consistent with the appearance of yellowing in severely infected plants, the signature of damages and aging, which happened at the late stage of infection. It is also worth noticing that the TYLCCNV A + β treatment is not acute in induction of the NbrgsCaM4p activity, compared to the instant and dramatic elevation of NbrgsCaM4p activity triggered by wounding (Fig. 7j to l).

The elevation of GUS and NbrgsCaM4 expression during aging and wounding is dramatic (Fig. 7c to g, and 7 to 7l). But, the increase of their expression during viral infection is rather moderate (Fig. 5). Thus, compared to that during aging and wounding, the promoter activity of NbrgsCaM4 is far less robust under viral infection. As a regulatory factor, efficient responses are necessary for cascading amplification of signals to cope with environmental and developmental fluctuations of the surroundings. The moderate responses of NbrgsCaM4p to viral infection suggest that the inflections from viral factors are not the major situations that evolved to NbrgsCaM4 to viral infection.

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NbrgsCaMps have overlapping expression patterns, indicating overlapping functions of NbrgsCaMs. From the non-negligible GUS staining in the NbrgsCaMp3::GUS and NbrgsCaMp5::GUS transgenic N. benthamiana plants, and their overlapping expression patterns that are similar to that of the NbrgsCaMp4::GUS, we deduced that besides NbrgsCaM4, other NbrgsCaMs, such as NbrgsCaM3 and NbrgsCaM5 probably respond redundantly to certain stimuli as a close homologous gene family. Based on our observations, though we did encounter several deformed plants randomly from tissue culture during transgene process, neither transgene of 35S promoter-driven overexpression, nor knockdown of NbrgsCaM4, exhibited obvious transgene-related phenotypes. The overlapping expression patterns and similar functions of the other NbrgsCaMs probably mitigated the changes in expression of NbrgsCaM4 alone, so the overexpression or knockdown of NbrgsCaM4 yielded no obvious phenotype.

In summary, the NbrgsCaMs form a distinct class of CMLs (Fig. 1c). There are more than one rgsCaM in both Arabidopsis and N. benthamiana (Supplementary Data S3, Fig. 1)18. They respond to developmental and environmental changes, particularly salt, drought and wounding stresses, via elevated expression to meet the need of timely regulation, and have overlapping but still distinct expression atlases which have been demonstrated by their promoter activities (Figs. 2–7). NbrgsCaM4 is the most robustly expressed rgsCaM in N. benthamiana according to the promoter activity analysis (Fig. 2–5) and RNA-seq data from the Sol Genomics Network28,26. Fitting its role in regulation, NbrgsCaM4 responds to diverse stresses, including viral infection, though its response to viral infection is mild and probably indirect through damages owing to a side effect of viral symptoms (Figs. 6 and 7). Thus, rather than being induced by a specific viral factor, NbrgsCaM4 is a member of a CML subfamily that respond mostly to general developmental stages and stresses. Despite the importance of NbrgsCaM4, other NbrgsCaMs also respond actively to environmental stimuli, with overlapping expression patterns, and are probably also overlapping in functions with respect to their close homology in protein sequences. The findings of this study are helpful in characterizing not only the expression patterns, but also the relative expression strength of rgsCaM genes, being the first step towards a future understanding of the rgsCaM family's multiple functions.

Materials and Methods

Plant materials and growth conditions. Wild type (WT) and 35S::βC1 transgenic (generated by Qiuying Yang according to the method described before29) N. benthamiana seeds were surface-sterilized with 75% ethanol and 2% bleach for 1 min and 3 min respectively, and then washed three times with sterile water. Sterile seeds were plated on Murashige and Skoog (MS) medium plus 2.0% sucrose and 0.5% phytagel. Plated seeds were placed in a greenhouse set at 24°C, 16-h-light/8-h-dark photoperiod for germination and growth. For culturing of mature plants, seedlings were transferred to soil after 5 days on plates and placed in the same greenhouse as above. Plants were watered as required and supplemented every other week with fertilizer. Seedlings and tissues were harvested at various time points for GUS staining and RT-qPCR analysis.

Construction of alignments and trees. Sequences of CaM and CML proteins were downloaded from the Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org) and the Sol Genomics Network (https://solgenomics.net/organism/Nicotiana_benthamianagenome) and subjected to phylogenetic analysis. All of the
A. thaliana CaMs and CMLs have been listed in the previous publication\(^4\), except for CML51 (At1g73440), which was added in this study. While the NbCaMs and NbCMLs were obtained by blasting the Sol Genomics Network data with A. thaliana CaMs. Alignments were constructed using the alignment mode of ClustalW in MEGA X\(^22\). Note: calcineurin B-like calcium sensor proteins and CMLs that contain domain protein kinases are not included as CMLs in the analyses. Multiple alignment of protein sequences was carried out with the following alignment parameters: gap opening penalty of 10, gap extension penalty of 0.2, and relatively divergent cutoff of 30%. Protein trees were constructed using the maximum likelihood method with bootstrap of 500 embedded in the MEGA X software. Parameters for multiple alignment of nucleotide sequences were: gap opening penalty of 15, gap extension penalty of 6.66, DNA weight matrix IUB, transition weight 0.5, negative matrix off and delay divergent cutoff of 30%.

**Generation of NbrgsCaMp::GUS transgenic N. benthamiana.** The promoter sequences of *NbrgsCaMs* have not been reported yet. We took 1kbp before the start codon of these genes as the putative promoters, as most of the *NbrgsCaMs* promoter elements locate within this region according to the online prediction results in this study. We cloned and constructed the promoter sequences into pBI101.GUS to generate pBI101.NbrgsCaMp::GUS (n = 1, 3, 4, 5, 7, order of the *NbrgsCaM* homologs). Primers used in the construction are listed (Supplementary Table S4). The plant transgene was done by the Bio-run company (http://www.biorun.com/). More than 10 T0 positive transgenic lines were tested for each NbrgsCaMp::GUS transgene, and homozygous T1 lines were obtained through self-fertilization of T0 plants.

**Stress treatments: salt and PEG.** Salt and drought-simulation (using PEG) treatments of the *NbrgsCaMp::GUS* transgenic *N. benthamiana* plants were conducted using 5-day-old seedlings. For each stress treatment, seedlings were carefully removed from the MS plates and transferred to plates supplemented with stress reagents, and grown for an additional 5 days. Salt stress plates were simply the MS plates supplemented with 200 mM NaCl; drought simulation plates were prepared by equilibrating the MS plates with 20% PEG8000 solution (filter sterilized) overnight \(^33,34\). At least three independent transgenic lines of each *NbrgsCaMp::GUS* were analyzed. Samples were collected after the 5 days salt or PEG treatments for GUS staining and RT-qPCR analysis.

**Stress treatments: viral infection and wounding.** For viral infection, *N. benthamiana* was grown for 4 weeks after transferring to soil to get ready for viral inoculation. Leaves were agro-inoculated with TYLCCNV A\(^29\), TYLCCNV A + \(^30\), PVX \(^35,36\), PVX:βC1 \(^37\), TMV \(^38\) or mock-inoculated with inoculation buffer as described \(^39\). Systemic leaves from the infected plants, and equivalent leaves from the mock-inoculated ones were harvested for GUS staining and RT-qPCR analysis.

Infiltration of inoculation buffer into the mock-inoculated local leaves can cause mechanical wounding. Local leaves were harvested at specific time points for 3,3’-diaminobenzidine (DAB) staining to detect hydrogen peroxide \(^40\), the signal of damage generated after wounding.

**RNA extraction and RT-qPCR analysis.** Total RNA was isolated using TRIzol method (Invitrogen). RNA concentration and quality were determined by spectrophotometry and gel electrophoresis. For RT-qPCR analysis, total RNA was treated with DNase I (Takara) and reverse transcribed according to the manufacturer’s instructions (EasyScript CDNA Synthesis SuperMix kit, TransGen Biotech). Specific primer pairs for *NbrgsCaM4, GUS* and *GAPDH* (an internal control) were listed in Supplementary Table S4. qPCR was performed using Roche LightCycler 96 with TransStart Green qPCR SuperMix (TransGen Biotech). Primer pairs were validated by cDNA template titration to ensure similar amplification kinetics and a single melting point of quantitative PCR products. Each experiment was performed in triplicate and repeated three times with different biological samples, and the results were analyzed with software supplied by the manufacturer. We used comparative CT method to determine the relative expression level of target gene expression \(^41\). Levels of the housekeeping gene *GAPDH* were used to calculate changes (n-fold) by comparing mean threshold cycle values. P value < 0.05 is used to delimit statistical significance. Error bars indicate S.D. To avoid genomic DNA contamination, a reaction lacking reverse transcriptase was performed in parallel for each sample.

**Histochemical assays: GUS and DAB staining.** For GUS staining, leaves were immersed in GUS staining solution (GUS staining kit, HUAYUEYANG biotechnology co., LTD.) for 12 h at 37°C in darkness, and then washed with 70% ethanol several times to remove background \(^42\). Samples and controls that were to be compared together were always stained in the same batch to eliminate variations caused by altering of conditions during the experiments. Stained samples were observed with Olympus SZX16 microscope (10 X amplification) and photographed by digital camera (Olympus DP72).

For DAB (3,3’-diaminobenzidine) staining, agro-infiltrated leaves were incubated in 1.0 mg/mL DAB-HCl solution in the dark overnight, then destained by boiling in 95% ethanol for 5 min. Dark brown precipitates on the leaves indicate detection of hydrogen peroxide generated after wounding \(^43\).

**Section of the plant leaves.** Leaves of the WT and 35S::βC1 transgenic *N. benthamiana* plants were fixed in FAA fixation buffer (containing 50% EtOH, 5% HAc and 3.7% formaldehyde) and sent to the SanShu Biotech Company (http://www.sanshubio.com) for resin embedded dissection and observation with high-resolution light microscopy according to the protocol \(^44\).

Received: 12 March 2020; Accepted: 20 May 2020; Published online: 15 June 2020
37. Cheng, X., Wang, X., Wu, J., Briddon, R. W. & Zhou, X. betaC1 encoded by tomato yellow leaf curl China betasatellite forms multimeric complexes in vitro and in vivo. *Virology* **409**, 156–162, https://doi.org/10.1016/j.virol.2010.10.007 (2011).
38. Lindbo, J. A. TRBO: a high-efficiency tobacco mosaic virus RNA-based overexpression vector. *Plant physiology* **145**, 1232–1240, https://doi.org/10.1104/pp.107.106377 (2007).
39. Vaghchhipawala, Z. E. & Mysore, K. S. Agroinoculation: a simple procedure for systemic infection of plants with viruses. *Methods in molecular biology* **451**, 555–562, https://doi.org/10.1007/978-1-59745-102-4_38 (2008).
40. Daudi, A. & O’Brien, J. A. Detection of Hydrogen Peroxide by DAB Staining in Arabidopsis Leaves. *Bio-protocol* 2 (2012).
41. Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative C(T) method. *Nature protocols* 3, 1101–1108, https://doi.org/10.1038/nprot.2008.73 (2008).
42. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO journal* **6**, 3901–3907 (1987).
43. Bindichedler, L. V. et al. Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *The Plant journal: for cell and molecular biology* **47**, 851–863, https://doi.org/10.1111/j.1365-313X.2006.02837.x (2006).
44. Chevalier, F., Iglesias, S. M., Sanchez, O. J., Montoliu, L. & Cubas, P. Plastic Embedding of Arabidopsis Stem Sections. *Bio-protocol* 4, e1261, https://doi.org/10.21769/BioProtoc.1261 (2014).

**Acknowledgements**
This research was supported by the National Natural Science Foundation of China (31672006). We thank technical editor, Carl Frederick Rupprecht (Marconi Communications, Inc., USA) for thoroughly reading and editing this manuscript in grammar and words. We apologize for not being able to cover or cite all the achievements in rgsCaM-related research.

**Author contributions**
Q.Y. initiated the work. D.L. and Q.Y. designed and carried out the biological experiments and analyses. Q.Y. and D.L. wrote the manuscript. Both authors approved the final version of this manuscript to be published.

**Competing interests**
The authors declare no competing interests.

**Additional information**
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-66670-x.

**Correspondence** and requests for materials should be addressed to Q.Y.

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