The essential role of NGATHA genes in style and stigma specification is widely conserved across eudicots

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SUMMARY

- Carpel development and evolution is a central issue for plant biology. The conservation of genetic functions conferring carpel identity has been widely studied in higher plants. However, although genetic networks directing the development of characteristic features of angiosperm carpels such as stigma and style are increasingly known in *Arabidopsis thaliana*, little information is available on the conservation and diversification of these networks in other species. Here, we have studied the functional conservation of NGATHA transcription factors in widely divergent species within the eudicots.
- We determined by in situ hybridization the expression patterns of NGATHA orthologs in *Eschscholzia californica* and *Nicotiana benthamiana*. VIGS-mediated inactivation of *NGATHA* genes in both species was performed and different microscopy techniques were used for phenotypic characterization.
- We found the expression patterns of *EcNGA* and *NbNGA* genes during flower development to be highly similar to each other as well as to those reported for Arabidopsis *NGATHA* genes. Inactivation of *EcNGA* and *NbNGA* also caused severe defects in style and stigma development in both species.
- These results demonstrate the widely conserved essential role of *NGATHA* genes in style and stigma specification and suggest that the angiosperm-specific *NGATHA* genes were likely recruited to direct a carpel specific developmental program.

Key words: gynoecium, VIGS, *Eschscholzia californica*, *Nicotiana benthamiana*, NGATHA, style and stigma, carpel evolution.

INTRODUCTION

The carpel is the female reproductive organ specific to the angiosperms, or flowering plants. Carpels enclose the ovules, providing numerous benefits in reproductive efficiency over the naked ovules typically present in the gymnosperms These benefits include support for pollination and incompatibility mechanisms, and, after fertilization, fruit development, which in turn protects the developing seeds and ensures seed dispersal. For these reasons, the carpel was probably of vital importance to the success of the angiosperms (Scutt *et al.*, 2006).

Carpels are complex structures comprising several specialized characteristic tissues. At maturity, the carpel is basically divided into an apical stigma, a style and a basal ovary. The stigma is composed of cells specialized in the reception and germination of the pollen grains. The style is a highly vascularized tissue containing at its center a transmitting tract through which the pollen tubes grow to reach the ovules. The basal ovary forms a chamber, with single or multiple locules, that prolongs the transmitting tract towards the ovules that develop from placental tissues. At the base of the ovary, the gynophore, a pedicel-like structure, connects the gynoecium to the flower. Despite this basic plan of organization, there is a great diversity in carpel morphology among the angiosperms. The term gynoecium is most commonly used to name all carpels in a flower, that may occur as single carpels, multiple unfused carpels or multiple fused syncarpic carpels (Ferrandiz *et al.*, 2010).

Regulatory networks underlying gynoecium patterning have been extensively studied in *Arabidopsis*, and many genes required for the development of the different characteristic tissues within the carpel have already been identified in the last few years. Carpel identity in angiosperms is specified by C-function genes in a widely conserved manner, as defined by the ABC model of floral organ identity (Coen & Meyerowitz, 1991), and in *Arabidopsis* this

function is provided by the MADS-box gene *AGAMOUS* (*AG*) (Yanofsky *et al.*, 1990). Once carpel identity is established, the genetic pathways controlling gynoecium patterning and the development of the carpel specific tissues are successively activated (reviewed in Balanzá *et al.*, 2006; Ferrandiz *et al.*, 2010; Reyes-Olalde *et al.*, 2013). Two other transcription factor genes, *CRABS CLAW* (*CRC*) and *SPATULA (SPT)* are required to bring about the full carpel development program (Alvarez & Smyth, 1999). CRC function is required to ensure the correct growth of the carpels, apical gynoecium closure and style development, while SPT function is mainly needed for appropriate development of transmitting tissues. The plant hormone auxin also plays a central role in the establishment of the apical-basal polarity. Nemhauser *et al.* (2000) have proposed that an auxin gradient spans the gynoecium primordium with a maximum at the apex inducing the differentiation of stigma and style, an intermediate level at the central domain promoting the development of the ovary, and a minimum at the bottom specifying gynophore formation. While the exact nature of this gradient has not been experimentally validated yet, it is clear that an apical maximum of auxin present during carpel primordia development is critical to ensure apical gynoecium closure and the development of the style and the stigma (Sundberg & Østergaard, 2009; Larsson *et al.*, 2013). Other transcription factors have also essential roles in the specification of apical tissues. The NGATHA (NGA) factors are required to direct apical gynoecium development in *Arabidopsis* (Alvarez *et al.*, 2009; Trigueros *et al.*, 2009). The *NGA* genes from *Arabidopsis* (referred to as *AtNGA*) form a small subfamily of four members within the RAV clade of the B3-domain transcription factor family and act redundantly to specify style and stigma identity. AtNGA function is intimately linked to auxin as it has been shown that auxin-biosynthetic enzymes of the YUCCA (YUC) family are specifically downregulated in the apical gynoecium domain of *nga* loss-of-function mutants (Trigueros *et al.*, 2009). Also *STYLISH1* (*STY1*) has been identified as a direct activator of *YUC4* in the apical region of the Arabidopsis gynoecium (Sohlberg *et al.*, 2006). STY1 belongs to the *SHI/STY* family of

zinc-finger transcription factors, and while single mutants only show subtle defects in style development, this phenotype is gradually enhanced when combined with mutations in other members of the SHI/STY family, leading to a complete absence of style and stigma in *shi/sty* high-order mutant combinations (Kuusk *et al.*, 2002; Kuusk *et al.*, 2006).

There is a wealth of evo-devo studies across higher plants mainly focused on questions related to the evolutionary origin of the carpel and in particular, the conservation of the major elements of the ABCE model that specify carpel identity (Bowman *et al.*, 1989; Bradley *et al.*, 1993; Pnueli *et al.*, 1994; Mena *et al.*, 1996; Davies *et al.*, 1999; Pan *et al.*, 2010; Yellina *et al.*, 2010; Dreni *et al.*, 2011). However, very little information is available in other angiosperm species about the role of the genetic functions in the lower regulatory hierarchies directing carpel morphogenesis and development. Conducting comparative studies on distant angiosperm species could lead to a better understanding of the different molecular pathways involved in apical carpel development, moreover it could shed some light on the morphological diversity and innovations of gynoecia.

In this work, we have studied the functional conservation of the small NGA gene family, characterizing the expression patterns and the phenotypes caused by the downregulation of the *NGA* genes in two distant species, the basal eudicot *Eschscholzia californica* and in the core eudicot asterid lineage, *Nicotiana benthamiana*. This work represents the first study of the role of the *NGA* genes outside the Brassicaceae and demonstrates a highly conserved NGA function in apical gynoecium development across eudicots. In this context, we also discuss the possible evolution of the different genetic networks known in *Arabidopsis* to take part in the apical gynoecium morphogenesis.

MATERIAL AND METHODS

Plant material and growth conditions

E. californica and *N. benthamiana* plants were grown in the greenhouse, at 22°C (day) and 18°C (night) with a 16-h light/8-h dark photoperiod, in soil irrigated with Hoagland no. 1 solution supplemented with oligoelements (Hewitt, 1966). *E. californica* germplasm used in this study (acession PI 599252) was obtained from the National Genetic Resources Program (USA).

Cloning and sequence analysis

The partial coding sequence of *EcNGA* gene was isolated by RT-PCR on cDNA of young flowers of *E. californica* using the degenerate primers EcNGAdegFOR/EcNGAdegREV designed from the conserved motifs of *NGA* homologs from other species (B3 domain and the NGA-II motif). The 3'end of *EcNGA* was then isolated by RT-PCR using the primers EcNGAFor2 and RT (sequence added to the oligodT primer used for retrotranscription). Finally, the 5'end of EcNGA was amplified by TAIL PCR using the random nested oligos AD1, AD2 and AD3 and the specific nested oligos EcNGATAIL1, EcNGATAIL2, EcNGATAIL3. The full-length CDS sequence has been deposited in Genbank under the accession number KF668646. A BLAST search against *N. benthamiana* draft genome sequence v0.4.4 (solgenomics.net) identified two genomic sequences corresponding to *NGA* homologues that we named *NbNGAa* and *NbNGAb*. The corresponding CDS were cloned, and sequences were deposited in Genbank under the accession numbers KF668647 (*NbNGAa*) and KF668648 (*NbNGAb*). The deduced amino acid sequences alignments were analyzed using the Macvector 12.6 software. See table S1 for primer sequences.

In situ hybridization.

RNA *in situ* hybridization with digoxigenin-labeled probes was performed on 8-μm paraffin sections of *E. californica* and *N. benthamiana* buds as described by (Ferrándiz *et al.*, 2000).

The RNA antisense and sense probes were generated from a 409 bp fragment of the *EcNGA* cDNA (positions 549 to 957), from a 707 bp of the *NbNGAa* cDNA (positions 472 to 1178) and from a 698 bp fragment of the *NbNGAb* (positions 589 to 1286). *NbNGAa* and *NbNGAb* probes had 58% identity, a low sequence similarity that likely precluded cross-hybridization. Each fragment was cloned into the pGemT-Easy vector (Promega), and sense and antisense probes were synthesized using the corresponding SP6 or T7 polymerases.

Virus-Induced Gene Silencing (VIGS)

The same regions of *EcNGA*, *NbNGAa* and *NbNGAb* coding sequence used for *in situ* hybridization were used for the VIGS experiments. In the case of the single gene constructs, a *Xba1* restriction site was added to the 5' end of the PCR fragment and a *BamH1* restriction site was added to the 3' end. The amplicon was digested by *Xba1* and *BamH1* and cloned into a similarly digested pTRV2 vector. For the double gene construction the fragment of *NbNGAb* coding sequence was introduced into the pTRV2-NbNGAa vector using the *EcoRI* restriction site. The four resulting plasmids, pTRV2-EcNGA, pTRV2-NbNGAa, pTRV2-NbNGAb and pTRV2-NbNGAa-NbNGAb were confirmed by digestion and sequencing, before being introduced into the *Agrobacterium tumefaciens* strain GV3101. The agroinoculation of *E. californica* seedlings was performed as described (Pabon-Mora *et al.*, 2012). The Agroinoculation of *N. bentahamiana* leaves was performed as described (Dinesh-Kumar *et al.*, 2003).

Quantitative RT-PCR

Total RNA was extracted from flowers in anthesis with the RNeasy Plant Mini kit (Qiagen). Four micrograms of total RNA were used for cDNA synthesis performed with the First-Strand cDNA Synthesis kit (Invitrogen) and the qPCR master mix was prepared using the iQTM SYBR Green Supermix (Bio-rad). The primers used to amplify *EcNGA* (qEcNGAFor and

qEcNGARev), *NbNGAa* (qNbNGAaFor and qNbNGAaRev), *NbNGAb* (qNbNGAbFor and qNbNGAbRev) and *NbYUC6* (acc. number NbS00044296g0003.1, qNbYUC6For and qNbYUC6Rev) generated products of 81 bp and did not show any cross-amplification. Results were normalized to the expression of the *ACTIN* gene of *E. californica* (according to Yellina et al, 2010) amplified by EcACTFor and EcCTRev, and to the *Elongation Factor 1* (*EF1*) gene of *N. benthamiana* (accession number AY206004), amplified by qNbEF1For and qNbEF1Rev. The efficiency in the amplification of the genes of interest and the corresponding reference gene was similar. Three technical and two biological replicates were performed for each sample. The PCR reactions were run and analyzed using the ABI PRISM 7700 Sequence detection system (Applied Biosystems). See Table S1 for primer sequences.

Scanning electron microscopy (SEM) and histology

VIGS-treated plants were analyzed by cryoSEM on fresh tissue under a JEOL JSM 5410 microscope equiped with a CRIOSEM instrument CT 15000-C (Oxford Instruments, http://www.oxford-in- struments.com). Young buds were collected for histological analyses, fixed in FAA (3,7% formaldehyde, 5% acetic acid, 50% ethanol) under vacuum and embedded into paraffin. Sections 10 µm thick were stained in 0.2% toluidine blue solution, and observed under a Nikon Eclipse E-600 microscope (http://www.nikoninstruments.com).

For vascular clearing, anthesis gynoecia from wild-type and VIGS-treated lines were fixed, cleared in chloralhydrate, mounted according to Colombo *et al.,* (2010), and viewed under dark-field microscopy.

RESULTS

193 *Identification of NGA genes in E. californica and N. benthamiana*

194 The *NGA* genes of *Arabidopsis* belong to the RAV subfamily within the plant-specific
195 superfamily of B3-domain transcription factors (Swaminathan *et al.*, 2008). The RAV
196 subfamily contains two well-supported classes: Class I genes code for one B3 and one AP2
197 DNA-binding domains, while Class II genes do not possess the AP2 domain. *NGA* genes
198 form a separate clade within class II RAV genes, which also contains a sister clade of three
199 genes named *NGA-like* (Romanel *et al.*, 2009). In addition to the B3 domain, all RAV genes
200 share one conserved motif that has been shown to have repressor activity (Ikeda & Ohme-
201 Takagi, 2009). In addition, the four *NGA* genes are characterized by possessing intronless
202 ORFs that encode two additional conserved motifs flanking the RAV-repressor domain that
203 appear to be specific to the NGA clade and that we have named as NGA-I and NGA-II motifs
204 (Trigueros *et al.*, 2009).

205 To search for the homologues of *NGA* in *E. californica* we designed degenerate primers
206 based on the conserved motifs of *NGA* homologues from other species. One putative *NGA*
207 gene, named *EcNGA*, was amplified from cDNA of young buds of *E. californica*. The
208 complete coding sequence of *EcNGA* was subsequently amplified by TAIL PCR and by the
209 use of an adapted oligodT primer. The predicted EcNGA protein sequence possessed the
210 typical structure of the NGA clade, including a single B3 domain, the RAV-repressor domain
211 and NGA-I and NGA-II motifs (Fig. 1). Repeated attempts to identify additional *NGA* genes
212 from *E. californica* were unsuccesful. This fact, reinforced by the presence in the database of
213 a single *NGA* gene from *Aquilegia caerulea*, a phylogenetically related ranunculid species
214 for which extensive EST databases are available (Kramer & Hodges, 2010), strongly
215 suggested the existence of only one *NGA* gene in the *E. californica* genome.

216 To identify *NGA* homologues from *N. benthamiana* we performed a BLAST search against
217 the draft genome sequence that has been released recently (Bombarely *et al.*, 2012). This

search retrieved two genomic regions that encoded intronless ORFs with the predicted structure of *NGA* genes, comprising the B3 domain, the RAV-repressor domain and the NGA-I and NGA-II motifs. From genomic sequences, primers were designed to isolate the corresponding ORFs from floral cDNA, which we named as *NbNGAa* and *NbNGAb*.

NGA gene expression patterns are highly similar in Arabidopsis, E. californica and N. benthamiana

To characterize the expression pattern of the *NGA* genes identified in *E. californica* and *N. benthamiana* we performed RNA *in situ* hybridization on young flower buds. *EcNGA* transcripts could be detected in the developing flowers from very early stages of development. In stage 3 *E. californica* buds (according to Becker *et al*, 2005), *EcNGA* transcripts were detected in all flower organ primordia except the expanded sepals (Fig. 2a). In stage 5 *EcNGA* accumulated in the apical part of the developing petals, stamens and carpels (Fig.2b). A similar pattern was observed in stage 6 with an additional expression domain in the placental region (Fig.2c). At later stages, *EcNGA* was mostly present in the developing ovules and in the growing apical gynoecium, with a remaining expression at the distal end of the petals (Fig. 2d-e). This expression pattern was very similar to those described for the four *Arabidopsis NGA* genes throughout flower development (Alvarez *et al*, 2009; Trigueros *et al*, 2009).

In *N. benthamiana, NGA* genes showed highly similar expression patterns, which also paralleled those described for *AtNGA* and *EcNGA* genes. In stage 2 buds (as defined in Mandel *et at.,* 1992), the *NbNGA* genes were detected in the floral meristem and more strongly in the distal end of the developing sepal primordium (Fig.2f). This distal expression was observed at later stages in all floral organ primordia. (Fig.2g). At later stages, the

NbNGA expression was mainly detected in the apical gynoecium, the placentae and the anthers (Fig. 2h-i). In the preanthesis mature flower, *NbNGAa* and *NbNGAb* were almost exclusively expressed in the transmitting tract and in the developing ovules (Fig. 2j) (Fig. S1).

Since *AtNGA* genes are also expressed in vegetative tissues, we compared by quantitative PCR the expression levels of *EcNGA*, *NbNGAa* and *NbNGAb* in leaves, young floral buds and anthesis flowers (Fig S2). We found *EcNGA* and *NbNGA* genes to be expressed in all analyzed tissues, similarly to what had been described for *AtNGA* genes (Trigueros et al, 2009)

In summary, *EcNGA*, *NbNGA* and *AtNGA* genes showed remarkably similar expression patterns during flower development, mostly confined to the distal end of developing floral organs, placentae and ovule primordia, and in the apical gynoecium at stages where style and stigma formation takes place.

Silencing of EcNGA in E. californica using VIGS greatly alters style and stigma development

Flower morphogenesis in *E. californica* has been previously described (Becker *et al.*, 2005). The flower at anthesis (stage 11) comprises a first whorl of two sepals fused into a caplike structure, four bright orange petals arranged in two whorls, and several whorls of stamens in variable number (from 18 to 34). The central pistil consists of two fused carpels with placentae developing internally to the carpel fused margins. The pistil comprises a cylindrical ovary, a short style and four long stigmatic protrusions (Fig.3a and Fig. S4). These stigmatic protrusions originate at stage 7, when two stylar lobes start to grow above the valves and then two shorter ones develop above the placentae (Fig. S2). These protrusions elongate

265 rapidly until post-anthesis stages, and are covered by stigmatic papillae, which also line the
266 hollow internal style and the placentae (Fig 3i-j, Fig 4a-g, Fig. S4).

267 The expression pattern of *EcNGA* suggested that this gene could have similar roles in flower
268 development to those of *AtNGA* genes in *Arabidopsis*. To investigate the function of *EcNGA*
269 in *E. californica* we used Virus Induced Gene Silencing (VIGS) to reduce its transcript levels.
270 This method results in transitory downregulatation of a specific gene via modified plant
271 viruses and it has been shown to efficiently direct the degradation of endogenous mRNAs in
272 *E. californica* and other species (Ratcliff *et al.*, 2001; Constantin *et al.*, 2004; Hileman *et al.*,
273 2005; Wege *et al.*, 2007).

274 For our analyses, a total of 120 *E. californica* seedlings were inoculated with the TRV2-
275 EcNGA construction (Table 1). To evaluate the efficiency of the VIGS treatment we
276 measured by quantitative RT-PCR the level of expression of *EcNGA* on flowers from 5
277 different treated plants showing altered phenotypes. In these plants expression of *EcNGA*
278 was reduced to 15-50% of wildtype level (Fig. 3o), indicating that the VIGS treatment was
279 highly effective. To detect morphological abnormalities associated with *EcNGA*
280 downregulation, we chose the three first flowers of each inoculated plant. No visible
281 phenotypes were observed in sepals, petals or stamens. However, 18% of the flowers
282 displayed pistils with defects in style and stigma development. The weakest phenotypes
283 corresponded to gynoecia with one or two small supplementary stigmatic protrusions (Fig.
284 3h). Intermediate phenotypes were characterized by the reduction and altered distribution of
285 the stigmatic papillae (Fig. 3c,l) and the frequent proliferation of misshapen stylar protrusions
286 of irregular length that precluded stylar closure (Fig.3d,e,f,k). The most severely affected
287 gynoecia completely lacked stigmatic tissue and most stylar lobes, with ovaries terminating
288 apically in irregular indented structures (Fig.3g,h,m,n). Histological sections of EcNGA-VIGS
289 pistils showing intermediate to strong phenotypes revealed that the papillar cells lining the

internal style were severely reduced, leaving an open gap along the style, but were not significantly affected in the placental domains (Fig 4h,n). Vascular strands, which in wildtype pistils run as parallel veins along the stigmatic protrusions, were defective in the VIGS-treated gynoecia, bifurcating abnormally at the distal end of the apical lobes (Fig 4o-q).

NbNGAa and NbNGAb are both involved in carpel and perianth development

We used again VIGS-mediated downregulation of gene expression to investigate the role of the two *NGA* genes identified in *N. benthamiana*. We generated three different TRV constructs designed to either specifically inactivate *NbNGAa, NbNGAb* or both genes simultaneously. Twelve plants were inoculated with each construct (Table 1). To evaluate the efficiency and the specificity of each treatment, we measured the level of expression of *NbNGAa* and *NbNGAb* by quantitative RT-PCR on flowers from five different treated plants. *NbNGAa* was strongly reduced in the NbNGAa-VIGS flowers whereas the expression of *NbNGAb* was not significantly altered, indicating that the TRV2-NbNGAa construct was gene specific (Fig 5o). Likewise, the expression of *NbNGAb* was significantly reduced in the NbNGAb-VIGS flowers compared to the wild type, whereas the expression of *NbNGAa* was practically unaffected. Quantitative RT-PCR in flowers of NbNGAa-NbNGAb-VIGS plants confirmed that both genes were strongly downregulated (Fig. 5o).

The wild type flower of *N. benthamiana* at anthesis is composed of a first whorl of five sepals, a second whorl of five white petals fused in a long tubular corolla, a third whorl of five stamens whose long filaments are adnately fused to the petals, and finally a bicarpellate gynoecium in the central fourth whorl. The *N. benthamiana* mature pistil comprises a short bilocular ovary with central placentation and a very long and thin style of approx. 3 cm, capped by a round wet stigma (Fig.5a,e,j).

In plants inoculated with the TRV2-NbNGAa vector, 63% of the flowers displayed an abnormal phenotype in the apical part of the pistil (Fig. 5b,f,k; Table 1). Style length was reduced (from a few mm to 1 cm shorter) and the stigma was not properly fused leaving a small central hole. In some cases the top part of the style and the stigma were bent (Fig. 5f,k). In histological sections we could observe that the transmitting tissue of the internal style was greatly reduced, leaving a hollow canal (Fig. 6c,d). Vascular development in the affected styles was also perturbed, with stylar veins terminating below the stigma instead of reaching the apical end as in the wildtype pistil (Fig. 6h,i). TRV2-NbNGAb treatment produced similar effects in gynoecium development, only stronger and in a higher proportion (Table 1). 74% of the NbNGAb-VIGS flowers showed affected pistils (Fig 5c,h). Style length was greatly reduced, up to 30-50% of its wildtype length, strong style and stigma fusion defects were frequent, and the transmitting tract at the internal style was severely reduced (Fig.5h, Fig 6e). Stylar veins showed irregular patterning and terminated at varying lengths within the style (Fig 6j) Finally, in plants where both *NbNGA* genes were downregulated, the gynoecium phenotype was greatly enhanced. 83% of the NbNGAa-NbNGAb-VIGS gynoecia completely lacked style development and the ovary directly terminated in a distorted stigma at the apical end (Fig. 5j,l. Fig. 6f,g; Table 1).

In addition to gynoecium phenotypes, the NbNGA-VIGS treatment also affected the development of other floral organs. The three TRV2-NGA contructs produced similar defects in sepal development, with around 60% of the flowers in the treated plants developing serrated sepals (Fig. 5n). In the NbNGAb-VIGS plants an additional phenotype was observed in 38% of the flowers: at anthesis, petal length was reduced and the corolla did not fully expand (Fig. 5c). This phenotype was greatly enhanced by TRV2-NbNGAa-NbNGAb treatment, as 53% of the flowers at anthesis displayed a closed corolla with short greenish petals (Fig. 5d).

In summary, the phenotypes of NbNGAa-NbNGAb-VIGS flowers strongly resembled those of *nga* quadruple mutants in *Arabidopsis*, which also lack style and stigma development and develop short green petals and short sepals (Alvarez *et al.*, 2009; Trigueros *et al.*, 2009).

To test whether *NbNGA* downregulation also produced similar effects on putative NGA targets identified in *Arabidopsis*, we analyzed the expression of a *N. benthamiana YUC* gene in the apical domain of NbNGAa-NbNGAb-VIGS pistils. *YUC* genes encode flavin monooxigenases involved in auxin synthesis and it has been previously reported that *nga* mutants fail to activate the expression of several *YUC* genes in the apical gynoecium (Trigueros *et al.*, 2009), which likely affects auxin synthesis and accumulation in *nga* pistils. Through BLAST search in the *N.benthamiana* genome, we identified a sequence highly related to *AtYUC2* and *AtYUC6*. Quantitative RT-PCR on cDNA extracted from excised styles and stigmas of preanthesis *N. benthamiana* flowers showed a significant reduction of the expression of this *YUC* gene in the NbNGAa-NbNGAb-VIGS apical gynoecia (Fig. 6k), strongly supporting the conserved role in style morphogenesis of *NGA* genes and their downstream effectors involved in auxin synthesis.

DISCUSSION

In this work, we have studied the functional conservation of a small gene family which has been shown to be essential for style and stigma development in Arabidopsis, characterizing the expression patterns and the phenotypes caused by the downregulation of the *NGA* genes in two distant species, the basal eudicot *E. californica* and the solanaceaeous core eudicot *N. benthamiana*. This work represents the first study of the role of the *NGA* genes outside the Brassicaceae and demonstrates a highly conserved NGA function in apical gynoecium development.

NGA function is conserved across eudicots.

The four *NGA* genes of *Arabidopsis* act redundantly to direct the development of the apical domain of the gynoecium, as *nga* quadruple mutants completely lack stigma and style and have very reduced transmitting tissues in the apical regions. This redundancy is likely based both in the similar activities of the NGA proteins, as revealed by the equivalent phenotypes caused by the overexpression of any of them in *Arabidopsis*, and the similarity of their spatio-temporal expression patterns throughout plant development (Alvarez *et al.*, 2009; Trigueros *et al.*, 2009). In this study we have characterized a single *NGA* gene from the basal eudicot *E. californica* and two *NGA* genes in the core sudicot *N. benthamiana*, *NbNGAa* and *NbNGAb.* Our studies have shown a remarkable conservation of *NGA* expression patterns in flowers of these two species and of *Arabidopsis*, each of them belonging to a different clade within the eudicots, mainly associated with the distal domains of growing floral organ primordia, the ovules and the apical domain of the gynoecium in the preanthesis flower. These observations strongly indicate that the regulatory regions of these genes, even across distant taxa and after several independent gene duplication events, have not diverged significantly and probably contain multiple redundant elements that confer robustness to the corresponding expression patterns.

Silencing of the *NGA* homologues by VIGS technology further demonstrates their conserved key role in style and stigma development both in *E. californica* and *N. benthamiana*. The psitils of *E. californica* VIGS-EcNGA plants displayed a range of phenotypic defects that strongly affected the development of the apical domain and in extreme cases caused the absence of stigma, style and apical transmitting tissues and produced altered vascular development. Very similar phenotypes were observed in *N. benthamiana* plants when the two *NbNGA* genes were downregulated. Thus, *NGA* loss-of-function in *Arabidopsis, E.*

388 *californica* and *N. benthamiana* precluded the development of the same set of tissues in the
389 gynoecium, despite the evolutionary distance of these three eudicot species.

390 In addition to their role in gynoecium development, the *NGA* genes in Arabidopsis have a
391 more general function in the regulation of lateral organ growth. In the *nga* quadruple
392 mutants, sepals and petals are shorter and wider than in wildtype flowers and rosette leaves
393 are also wider and more serrated, consistent with the reported expression of *AtNGA* genes
394 in the distal domain of growing leaves and floral organs (Alvarez *et al.*, 2009; Trigueros *et*
395 *al.*, 2009). In this work, we have shown that the expression of *NGA* genes in distal floral
396 organ primordia is conserved in *E. californica* and *N. benthamiana*. Moreover,
397 downregulation of *NbNGA* genes resulted in shorter serrated perianth organs, indicating that
398 the role of *NGA* genes in floral organ growth is also conserved. No phenotypic defects were
399 observed in the leaves of *N. benthamiana* VIGS-treated plants or in perianth or leaves of *E.*
400 *californica*, which may suggest that *NGA* genes do not share these roles with their
401 Arabidopsis homologues. However, it seems more likely that the residual activity of *NGA*
402 genes in the inoculated *E. californica* or *N. benthamiana* plants could be sufficient to
403 preclude leaf phenotypic defects. Actually, while double or triple mutant combination of *nga*
404 mutations in *Arabidopsis* already display conspicuous phenotypes in style and stigma
405 development, only quadruple mutants have obvious defects in perianth or leaf development,
406 supporting the idea of the gynoecium functioning as the ‘‘canary in a coalmine’’ whereby
407 carpel development could be especially sensitive to minor defects in patterning factors that
408 do not strongly affect the development of other organs (Dinneny & Yanofsky, 2005).

409 We have also shown that the specific inactivation of each of the *NbNGA* genes caused
410 much weaker but similar phenotypic defects, demonstrating the dosage-dependent
411 functional redundancy of *NbNGAa* and *NbNGAb* in *N. benthamiana*, another feature also
412 shared by the *AtNGA* genes. Thus, *NGA* genes in Arabidopsis and *N. benthamiana* appear

413 to have retained both regulatory regions and protein activities, undergoing little or no sub- or
414 neofunctionalization. Published phylogenies of the B3 transcription factor family, the likely
415 presence of a single gene in *E. californica* or *Aquilegia*, as well as the copy number and
416 position of the *NGA* genes in the *Arabidopsis* genome are consistent with the existence of a
417 single ancestor of *NGA* genes previous to monocot/dicot divergence. Interestingly, database
418 mining in species for which genome sequence is available indicate that *NGA* homologues
419 are frequently found in several copies (at least three in *Oryza sativa*, two in *Populus*
420 *thricocarpa* or two in *Vitis vinifera*; Alvarez *et al.*, 2009; Trigueros *et al.*, 2009). The retention
421 of multiple copies of *NGA* genes in different species from distant taxa, together with the high
422 degree of conservation of both regulatory sequences and function across and within species
423 that we have shown in this work, may suggest that maintaining multiple copies of mostly
424 equivalent NGA proteins could be beneficial for reproductive success, maybe buffering
425 against dominant-negative mutations and/or ensuring the availability of NGA factors for
426 participation in multimeric transcriptional complexes.
427

428 *The NGA genes and the evolution of carpel structures.*

429 Carpel identity in angiosperms is specified by C-function genes, as defined by the ABCE
430 model of floral organ identity. Members of the AGAMOUS (AG) lineage of the MADS box
431 gene family have been shown to carry out this function across angiosperms (Bowman *et al.*,
432 1989; Bradley *et al.*, 1993; Pnueli *et al.*, 1994; Mena *et al.*, 1996; Davies *et al.*, 1999; Pan *et*
433 *al.*, 2010; Yellina *et al.*, 2010; Dreni *et al.*, 2011), but putative AG orthologs have also been
434 found in gymnosperms, where they are expressed in reproductive organs like their
435 angiosperm counterparts (Rutledge *et al.*, 1998; Jager *et al.*, 2003; Zhang *et al.*, 2004; Groth
436 *et al.*, 2011). Thus, the C-function appears to be conserved since the common ancestor of
437 seed plants and predates the origin of the angiosperm carpel, suggesting that additional

gene functions must have evolved to specify the distinctive features of this organ.

In this work we have demonstrated the conserved essential role of *NGA* genes in the specification of style and stigma, distinctive characters of angiosperm carpels. Phylogenies of B3 domain transcription factors show that class I genes from the RAV subfamily (those encoding both AP2 and B3 domains) are already present in bryophytes. In contrast, *NGA* genes, which belong to class II and are defined by the single B3 domain and the three characteristic C-t motifs, can only be found in angiosperms and likely originated by duplication of AP2-B3 RAV genes and subsequent loss of the AP2 domain. Interestingly, NGA genes in Arabidopsis have been listed as putative targets of AG (Gomez-Mena *et al.*, 2005). We can thus speculate that angiosperm specific RAV-derived *NGA* genes could have been recruited downstream of the C-function and by neofunctionalization they could have provided a new role in the specification of style, stigma and transmitting tissues.

A conserved program for style and stigma development?

Molecular studies in *Arabidopsis* have shown that apical gynoecium patterning is a complex process where many genes with highly redundant functions are involved, and that at least partially depends on YUC-mediated auxin biosynthesis at the distal end of the growing pistil primordium (Cheng *et al.*, 2006; Sohlberg *et al.*, 2006; Trigueros *et al.*, 2009). Although comparative studies in distant angiosperm species assessing conservation of these relevant gene functions for style and stigma development are still scarce, the results presented here as well as other published studies suggest that the genetic network operating in *Arabidopsis* might be broadly conserved.

First, our work highlights the central role of the *NGA* genes in style and stigma development across eudicots. Moreover, we have shown that in *N. benthamiana YUC* expression in the

462 apical gynoecium is also reduced by *NGA* downregulation, suggesting that the NGA-YUC-
463 auxin module could be part of a conserved network directing style and stigma
464 morphogenesis.

465 Likewise, in Arabidopsis, the *SHI/STY* genes have been shown to participate in these
466 functions and to be intimately related to the NGA factors (Alvarez et al., 2009; Trigueros et
467 al., 2009). In addition to displaying similar mutant phenotypes, *AtNGA* and *SHI*/*STY* genes
468 share similar expression patterns and, when simultaneously over-expressed, *NGA3* and
469 *STY1* are able to direct ectopic style development (Alvarez *et al.*, 2009; Trigueros *et al.*,
470 2009). Moreover, STY1 is a direct activator of *YUC4* (Sohlberg *et al.*, 2006; Eklund *et al.*,
471 2010a), and this connection of *SHY/STY* genes with auxin biosynthesis pathways seems
472 largely conserved among land plants, as it has been demonstrated by the reduction in auxin
473 levels and the reproductive organ developmental phenotypes caused by *SHY/STY*
474 inactivation in the bryophyte *Physcomitrella patens* (Eklund *et al.*, 2010b; Landberg *et al.*,
475 2013). While the specific role of *SHY/STY* in carpel development has not been explored in
476 detail in species other than *Arabidopsis*, it has been described recently in the monocot
477 *Hordeum vulgare* that mutants in the *short awn2* gene, a member of the SHY/STY family,
478 show defects in style and stigma morphology (Yuo *et al.*, 2012). Thus, it would appear that
479 *SHY/STY* genes could also have a conserved function both in driving auxin synthesis as well
480 as style and stigma development.

481 Other factors with putatively conserved roles in apical gynoecium development have also
482 been described. *CRC* orthologs have been identified in a wide range of angiosperm species
483 (Yamaguchi *et al.*, 2004; Fourquin *et al.*, 2005; Lee *et al.*, 2005; Orashakova *et al.*, 2009).
484 *CRC* belongs to the YABBY family, specific to seed plants. Phylogenetic studies on the
485 family are still partial due to the scarcity of gymnosperm sequences, but they suggest that
486 *CRC* genes would be specific to angiosperms (Yamada *et al*, 2011; Bartholmes *et al*, 2012).

In general, the characterization of expression patterns and of phenotypic defects associated with downregulation of *CRC* orthologs are consistent with an ancestral role in conferring the identity to carpel specific tissues in angiosperms, including style and stigma, while other roles such as nectary specification could have been derived in eudicots (Yamaguchi *et al.*, 2004; Lee *et al.*, 2005; Fourquin *et al.*, 2007; Ishikawa *et al.*, 2009; Yamada *et al.*, 2011). Finally, in dicot species, some PLE-subclade MADS-box genes also have been recently shown to have a conserved role in style and stigma development (Colombo *et al.*, 2010; Fourquin & Ferrandiz, 2012; Heijmans *et al.*, 2012).

All this evidence suggests that the major factors involved in style and stigma differentiation identified in Arabidopsis may form an ancient module with conserved functions, where some ancestral genetic routes, like the SHY/STY-auxin pathway already present in bryophytes, might have been coopted to direct style and stigma development by the acquisition of angiosperm-specific functions like CRC or NGA, although much deeper comparative analyses of these functions in evolutionary meaningful species will have to be undertaken to confirm this hypothesis.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Fig. S1 *In situ* expression analyses of *NbNGAb* in *N. benthamiana*.

Fig. S2 Expression level by real-time PCR analysis of *EcNGA* and *NbNGAa* in different organs.

Fig. S3 Negative controls for *in situ* hybridizations (sense probes).

Fig. S4 Developmental series of *E. californica* pistils and flowers.

Table S1 Primers used in this work.

FIGURE LEGENDS

Figure 1: Amino acid alignment of the conserved domains in the NGA homologues from different species.

(a) B3 DNA binding domain, (b) NGA-I domain, (c) RAV repressive domain and (d) NGA-II domain. Amino acid sequences of AqNGA (*Aquilegia caerulea* NGA homologue) and AmbNGA (*Amborella trichopoda* NGA homologue) were deduced from the following EST sequences DR951353 and FD442133 respectively.

Figure 2 :*In situ* expression analyses of homologues of *NGA* in *Eschscholzia californica* and *Nicotiana benthamiana* flowers. Control hybridizations with sense probes are shown in Fig S3.

(a to e) Longitudinal sections of *E. californica* flowers probed with *EcNGA*. (a) At stage 3 *EcNGA* transcripts are detected in all flower organ primordia except in the developing sepals (S). (b) At stage 5, *EcNGA* accumulates in the apical part of developing organs in the three inner whorls (arrows) (c) At stage 6, *EcNGA* expression is still present at the top of the petals, stamens and carpels. In addition *EcNGA* transcripts begin to accumulate in the placental region of the carpel where the ovules will further develop (arrow). (d and e) At stage 7, *EcNGA* expression concentrates in the developing style (Sty) and in the ovules (Ov).

(f to j) Longitudinal sections of *N. benthamiana* flowers probed with *NbNGAa*. (f) At stage 2, *NbNGAa* is expressed in the floral meristem (FM) and more strongly at at the tip of the developing sepals (S, arrows). (g) At stage 6, *NbNGAa* transcripts are detected at the apical region of each developing floral organ (arrows). (h) At the moment of style inception, *NbNGAa* expression accumulates in the apical gynoecium (arrow), in the placenta (P) and in the stamens (Stm). (i and j) In mature flowers, *NbNGAa* is strongly expressed in the inner part of the style (transmitting tract, TT) and in the developing ovules (Ov). Scale bar: 100 µm.

Figure 3. Phenotypes of *Eschscholzia californica* plants inoculated with pTRV2-EcNGA.

(a) Wild type pistil at anthesis, comprising a cylindrical ovary, a short style and four long yellow stigmatic protrusions. (b to h) EcNGA-VIGS pistils at anthesis displaying an alteration of their apical region development. (b) A weak phenotype characterized by the development

of a supplementary stigmatic protrusion (arrow). (c to f) Examples of intermediate phenotypes: (c) Modification of the distribution of the stigmatic tissue along the four protrusions, the green zones correspond to style tissue (arrows); (d and e) Presence of an increased number of stigmatic protrusions with irregular lengths; (f) EcNGA-VIGS pistil presenting an enlarged and opened style with a great reduction of stigmatic tissue (f). (g and h) Strong phenotypes: total lack of stigmatic tissue. (i to n) Scanning electron microscope pictures of apical regions of pistils at anthesis. (i) Wild-type stigmatic protrusions entirely covered by papillae cells. (j) Tip of a wildtype stigmatic protrusion fully covered by the typical globular stigmatic cells. (k) EcNGA-VIGS pistil displaying an intermediate phenotype with the presence of several stigmatic protrusions of different shape and size partially covered by the papillae cells. (l) Tip of a stigmatic protrusion from a EcNGA-VIGS pistil displaying an intermediate phenotype revealing the absence of stigmatic tissue. (m and n) Two examples of EcNGA-VIGS pistil with strong phenotypes, note the total lack of stigmatic protrusions and of papillae cells. (o) Expression level by real-time PCR analysis of *EcNGA* in TRV2-EcNGA flowers. The error bars depict the s.e. based on two biological replicates. (***) indicates significantly different ($P < 0.005$) from WT control according to a t-test . Scale bar: 500 µm; except in (j) and (l) scale bar: 100 µm.

Figure 4: Phenotypic characterization of *Eschscholzia californica* pistils from plants inoculated with pTRV2-EcNGA.

(a to g) Consecutive sections of *E. californica* wild-type pistil at anthesis. (a to f) Transversal sections from the stigmatic protrusions to the ovary. (g) Longitudinal sections of the whole wild-type pistil. Note the presence of the four stigmatic protrusions with stigmatic papillae developing adaxially (arrow) (a and b), of a dense transmitting tract inside the style (c, d and g) which continues inside the ovary (e, f and g). (h to n) Consecutive sections of EcNGA-

VIGS pistils at anthesis. (h-m) Transversal sections of an EcNGA-VIGS pistil displaying an intermediate phenotype. Note the disorganization of the stigmatic tissue (arrow) (h and i) and the greatly reduced transmitting tract present inside the style and leaving an opened canal (j and k); the ovary shows a similar structure to the wild-type (l and m). (n) Longitudinal section of an EcNGA-VIGS with a weak phenotype and showing the limited amount of transmitting tract developing inside the style. (o to q) Observation of the vasculature in cleared whole mount pistils. (o and p) In the wild-type pistil the vasculature strands run as parallel veins along the stigmatic protrusions, (p) note the closer view of a protrusion final tip. (q) Three EcNGA-VIGS pistils with intermediate phenotypes displaying abnormal bifurcations of the veins at the distal end of the apical lobes (arrows). Scale bar: 500 µm.

Figure 5 : Phenotypes of *Nicotiana benthamiana* plants inoculated with pTRV2-NbNGAa, pTRV2-NbNGAb, or pTRV2-NbNGAa-NbNGAb.

(a-d) Top view of *N. benthamiana* flowers. (a) Wild-type flower at anthesis, note the five expanded white petals and the central stigma (arrow) surrounded by the five stamens. (b) NbNGAa-VIGS flower. Note the absence of stigma in the centre. (c) NbNGAb-VIGS flower with reduced petals and no stigma visible. (d) NbNGAa-NbNGAb-VIGS flower displaying abnormal sepals and greenish not fully developed petals. (e to i) Gynoecium of *N. benthamiana* flowers. (e) Wild-type gynoecium characterized by an ovoid ovary, a long style and a flat stigma. (f) NbNGAa-VIGS pistil presenting a reduced style and a bended stigma. (g) Close-up of the wild-type ovary and beginning of the style. (h) NbNGAb-VIGS gynoecia presenting a short style not fully fused and an abnormal opened stigma. (i) Examples of NbNGAa-NbNGAb-VIGS gynoecia with extremely reduced styles and deformed stigmas. (j to l) *N. benthamiana* stigma. (j) Top view of a wild-type stigma: symmetrical, circular and flat. (k) Top view of an asymmetric and not fully fused NbNGAa-VIGS stigma. (l) Lateral view of a

NbNGAa-NbNGAb-VIGS stigma showing an opened and highly deformed structure. (m and n) Top view of *N. benthamiana* sepals. (m) wild-type sepals. (n) NbNGAa-NbNGAb-VIGS very serrated sepals. (o) Expression level by real-time PCR analysis of *NbNGAa* and *NbNGAb* in TRV2-NbNGAa, TRV2-NbNGAb or TRV2-NbNGAa-NbNGAb flowers. The error bars depict the s.e. based on two biological replicates. (*) indicates significantly different ($P < 0.05$) and (***) significantly different ($P<0.005$) from WT control according to a t-test . Scale bar: 500 µm.

Figure 6: Phenotypic characterization of *Nicotiana benthamiana* pistils from NbNGA-VIGS inoculated plants.

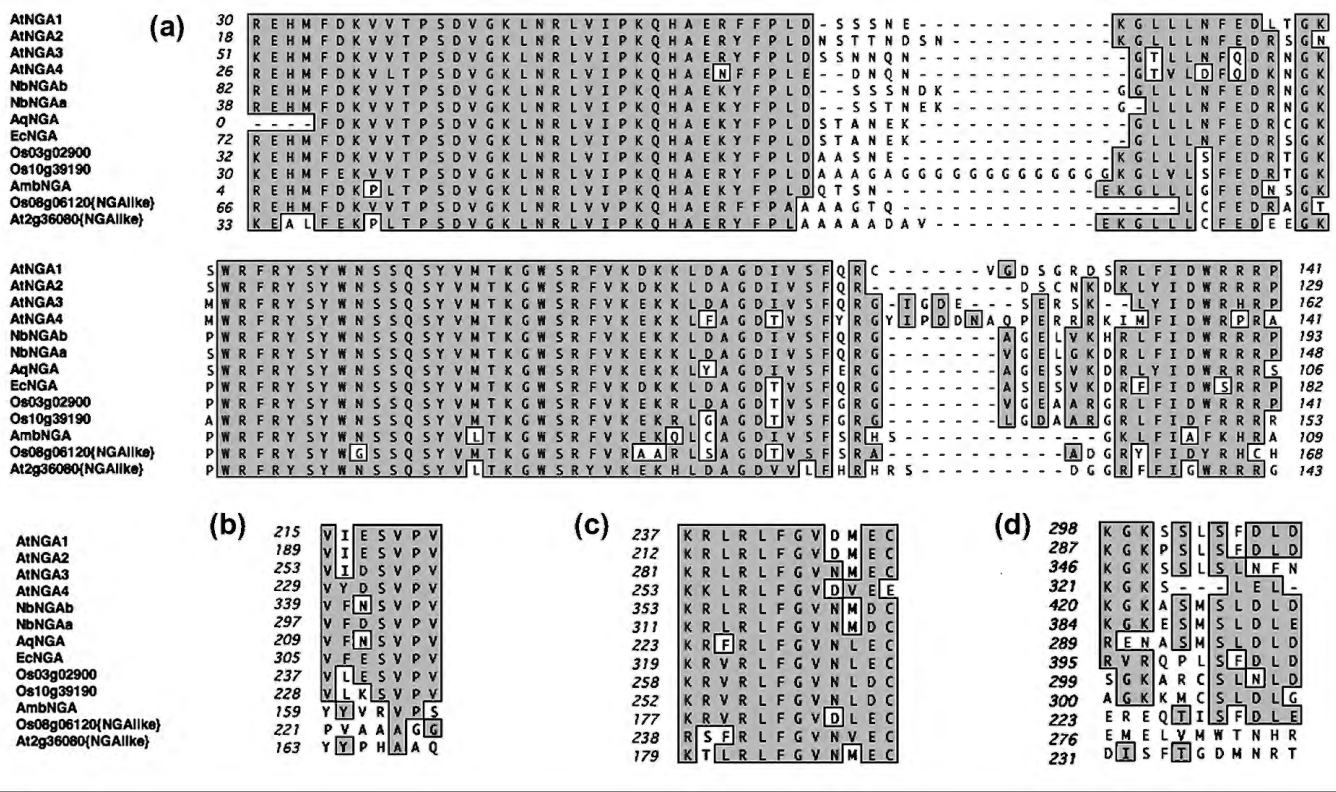
(a and b) Apical part of the *N. benthamiana* wild-type pistil: (a) Stigma and style longitudinal section, (b) Style transversal section. Note the presence of a transmitting tract tissue filling the wild-type style and stigma (arrow). (c and d) Apical part of an NbNGAa-VIGS pistil: (c) Stigma and style longitudinal section, (d) Style transversal section. Note the presence of an opened canal in the NbNGAa-VIGS style and the greatly altered transmitting tract (arrow). (e) Longitudinal section of the apical part of an NbNGAb-VIGS gynoecium revealing the absence of transmitting tract in the unfused style (f-g) Transversal sections of the apical region of an NbNGAa-NbNGAb-VIGS gynoecium displaying highly modified style tissue with a total lack of transmitting tract development. (h-k) Vascular patterning in *N. benthamiana* style and stigma (h) In the wild-type pistil the stylar veins reach the apical stigma (arrow). (i and j) In NbNGAa-VIGS (i) or NbNGAb-VIGS pistil (j) the stylar veins terminate below the stigma (arrows). Scale bar: (e, h-k) 500µm, (a-d, f and g) 100 µm. (k) Expression level of *NbYUC6* gene by quantitative PCR in wild-type and NbNGAa-NGAb-VIGS style tissue. For each biological replicate, style-stigma tissue was excised from more than 50 anthesis flowers from different wild-type or VIGS-treated plants. The error bars depict the s.e. based

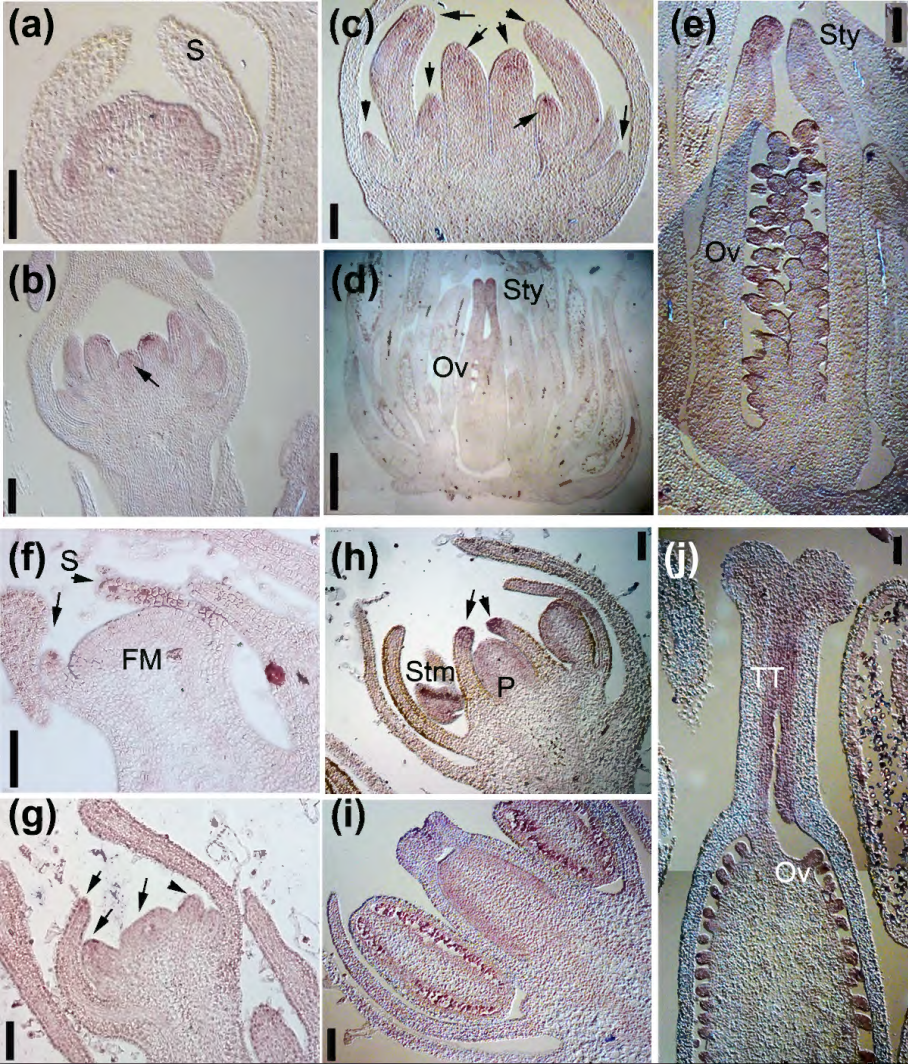
on two biological replicates. Asterisk (*) indicates significantly different ($P < 0.05$) from WT control according to a t-test .

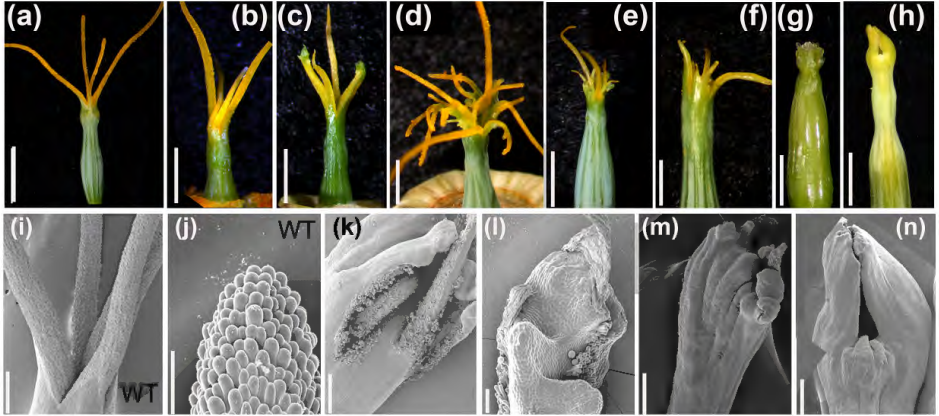
TABLES

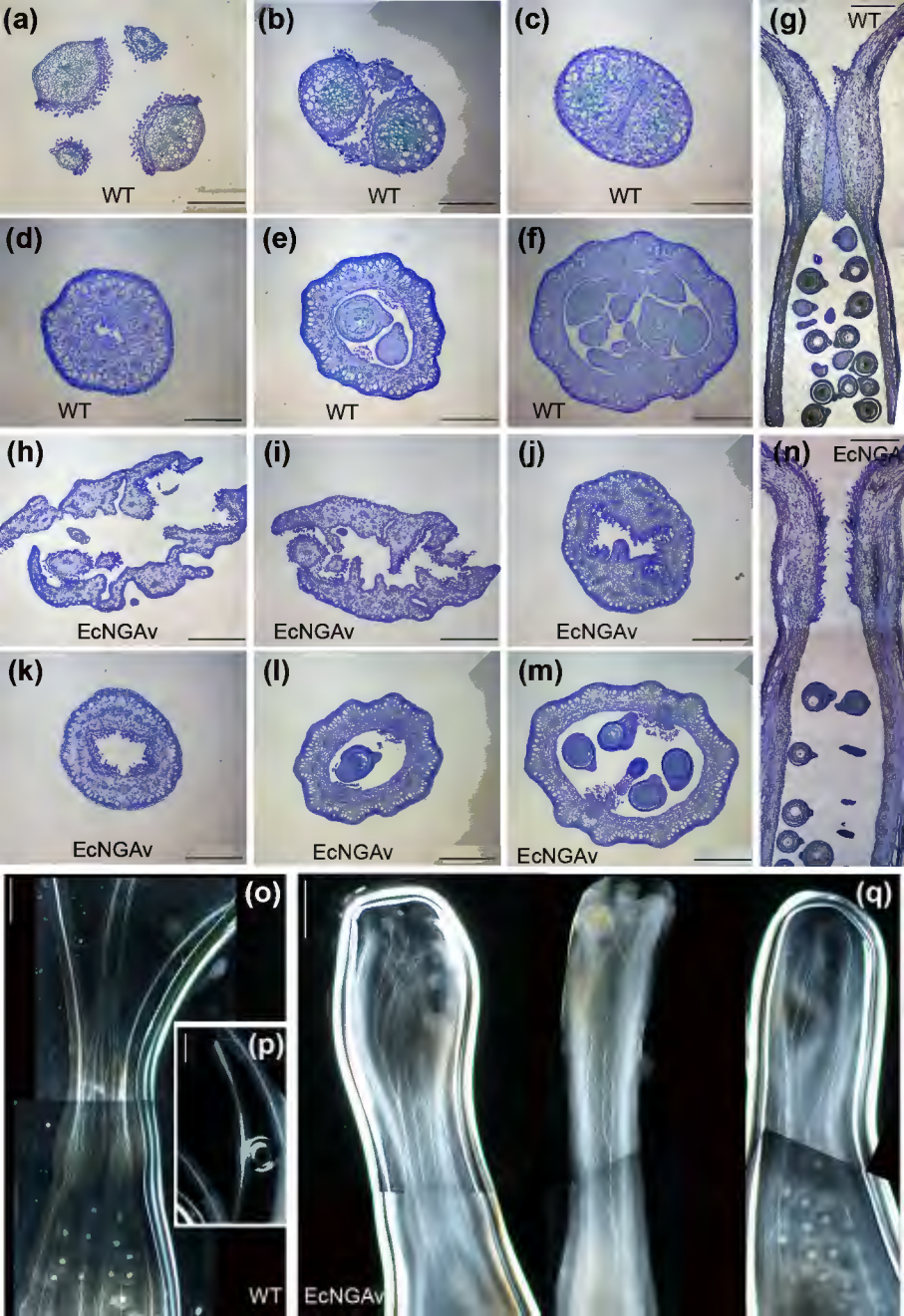
Table 1. Summary of the VIGS experiments on *Nicotiana benthamiana* and *Eschscholzia californica* plants

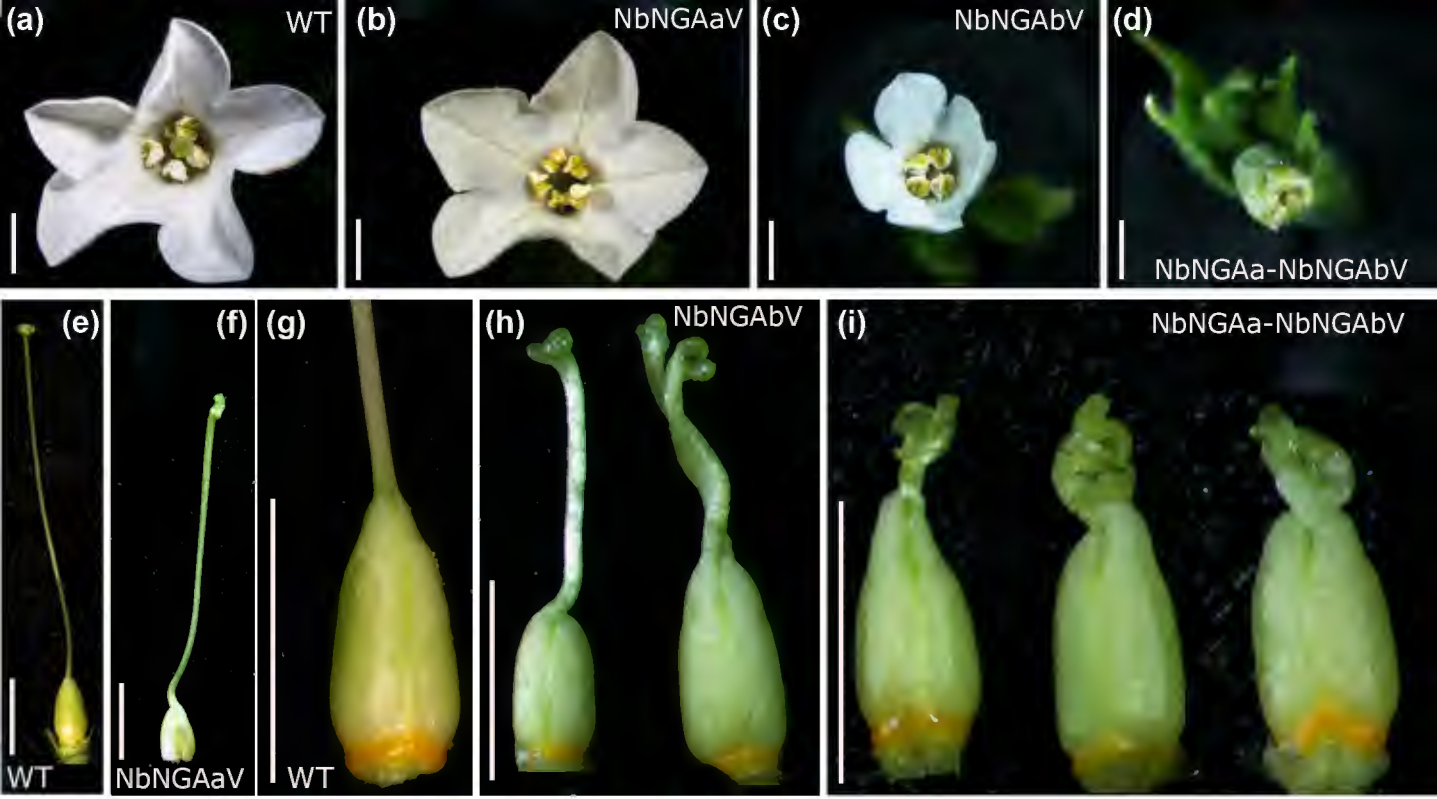
VIGS construct	N° plants inoculated (dead)	N° plants with phenotype	N° flowers/ plant observed	N° flowers/ plant with phenotype	N° total flowers with phenotype	% flowers with phenotype
N. benthamiana						
TRV2-NbNGAa	12 (2)	10	20	9-14	126/200	63%
TRV2-NbNGAb	12 (1)	11	20	11-17	162/220	74%
TRV2-NbNGAab	12 (1)	11	20	14-19	182/220	83%
Empty vector	6 (1)	0	20	0	0/100	0%
E. californica						
TRV2-EcNGA	120 (34)	23	3	0-3	46/258	18%
TRV2-EcPDS	60 (18)	12	-	-	-	-
Empty vector	60 (16)	0	2	0	0/88	0%

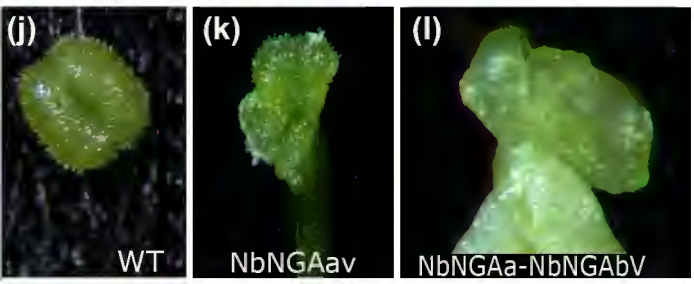

(a)
AtNGA1
AtNGA2
AtNGA3
AtNGA4
NbNGAb
NbNGAa
AqNGA
EcNGA
Os03g02900
Os10g39190
AmbNGA
Os08g06120{NGAlike}
At2g36080{NGAlike}
(b)
(c)
(d)

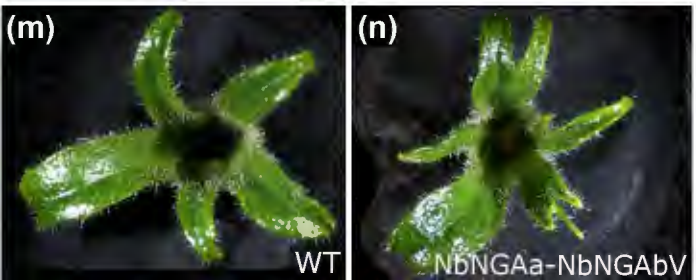

(a)
S
(c)
(e)
Sty
Ov
(b)
(d)
Sty
Ov
(f)
S
FM
(h)
Stm
P
(j)
TT
Ov
(g)
(i)

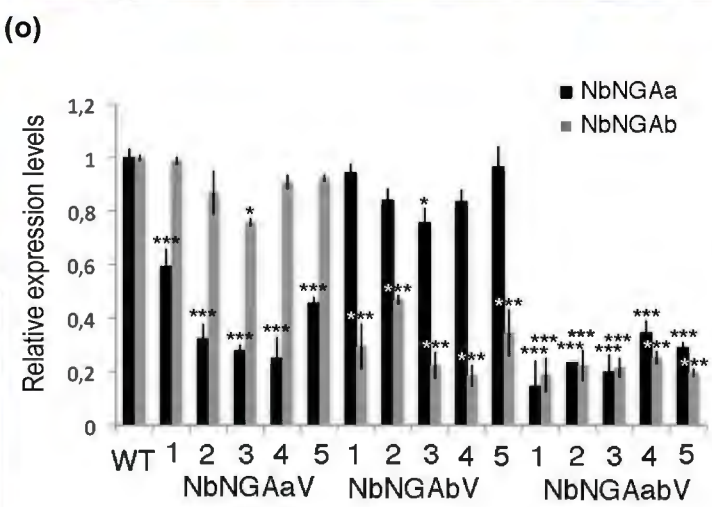

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
WT
(j)
WT
(k)
(l)
(m)
(n)
(o)
Relative expression levels
1,2
1
0,8
0,6
0,4
0,2
0
***
***
***
***
***
WT
1
2
3
4
5
EscNGA expression in flowers
of VIGS plants

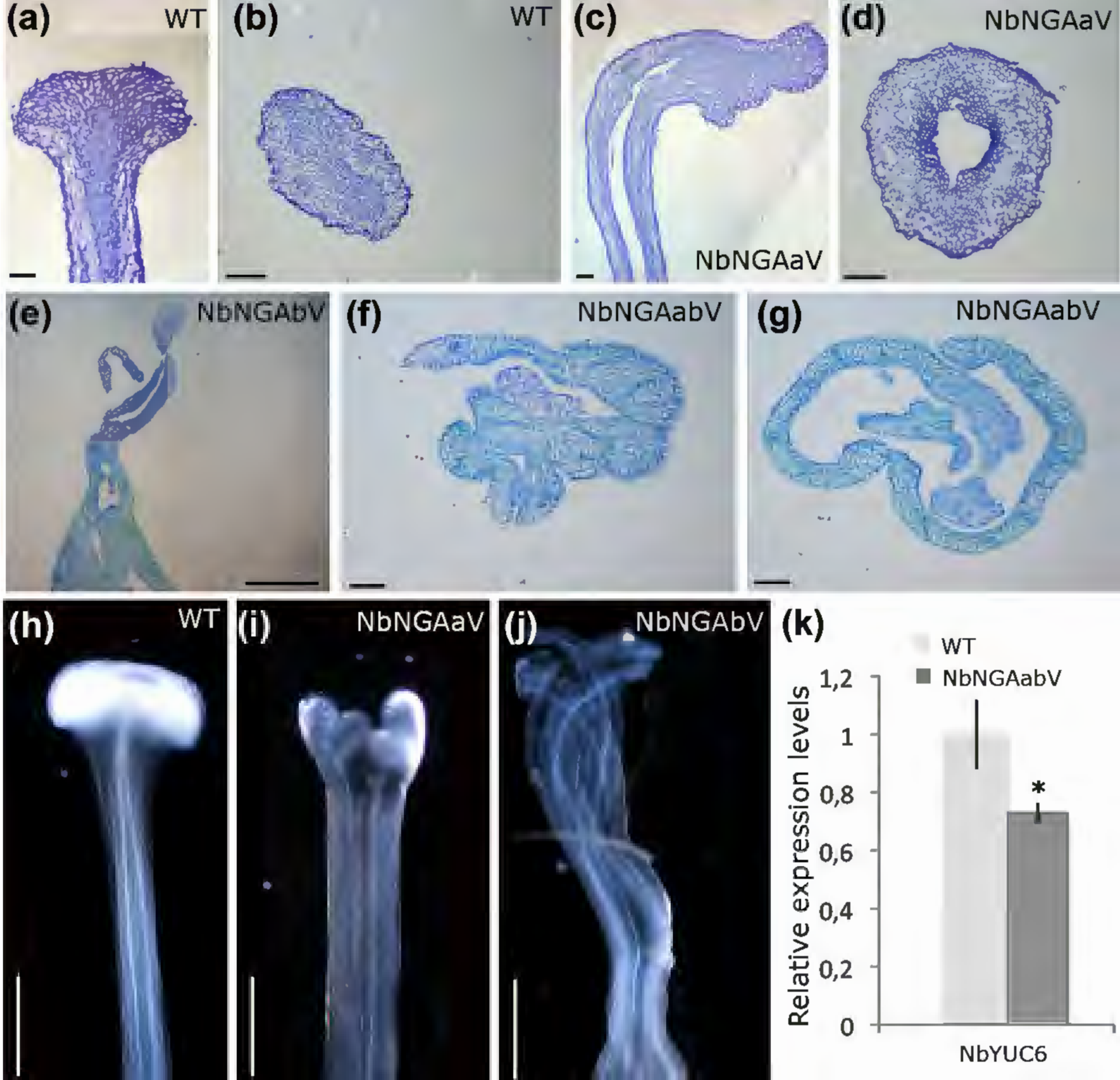

(a)
WT
(b)
WT
(c)
WT
(g)
WT
(d)
WT
(e)
WT
(f)
WT
(h)
EcNGAv
(i)
EcNGAv
(j)
EcNGAv
(n)
EcNGA
(k)
EcNGAv
(l)
EcNGAv
(m)
EcNGAv
(o)
(p)
WT
(q)
EcNGAv


(a)
WT
(b)
NbNGAaV
(c)
NbNGAbV
(d)
NbNGAa-NbNGAbV
(e)
(f)
(g)
(h)
NbNGAbV
(i)
NbNGAa-NbNGAbV
WT
NbNGAaV
WT


(j)
(k)
(l)
WT
NbNGAav
NbNGAa-NbNGAbV


(m)
(n)
WT
NbNGAa-NbNGAbV


(o)
NbNGAa
NbNGAb
Relative expression levels
1,2
1
0,8
0,6
0,4
0,2
0
WT 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
NbNGAaV
NbNGAbV
NbNGAabV


(a)
WT
(b)
WT
(c)
NbNGAaV
(d)
NbNGAaV
(e)
NbNGAbV
(f)
NbNGAabV
(g)
NbNGAabV
(h)
WT
(i)
NbNGAaV
(j)
NbNGAbV
(k)
WT
NbNGAabV
Relative expression levels
1,2
1
0,8
0,6
0,4
0,2
0
*
NbYUC6