Leaf expansion in *Arabidopsis* is controlled by a TCP-NGA regulatory module likely conserved in distantly related species

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The NGATHA (NGA) clade of transcription factors (TFs) forms a small sub-family of four members in *Arabidopsis thaliana*. *NGA* genes act redundantly to direct the development of apical tissues in the gynoecium, where they have been shown to be essential for style and stigma specification. In addition, *NGA* genes have a more general role in controlling lateral organ growth. The four *NGA* genes in *Arabidopsis* are expressed in very similar domains, although little is known about the nature of their putative regulators. Here, we have identified a conserved region within the four *NGA* promoters that we have used as a bait to screen a yeast library, aiming to identify such *NGA* regulators. Three members of the TCP family of TFs, named after the founding factors TEOSINTE BRANCHED 1, CYCLOIDEA and PROLIFERATING CELL FACTOR 1 AND 2), were recovered from this screening, of which two [TCP2 and TCP3, members of the CINCINNATA (CIN) family of TCP genes (CIN-TCP) subclade] were shown to activate the *NGA3* promoter in planta. We provide evidence that support that CIN-TCP genes are true regulators of *NGA* gene expression, and that part of the CIN-TCP role in leaf development is mediated by *NGA* upregulation. Moreover, we have found that this TCP-NGA regulatory interaction is likely conserved in angiosperms, including important crop species, for which the regulation of leaf development is a target for biotechnological improvement.

Introduction

NGATHA (*NGA*) genes have a key role in the specification of style and stigma in the gynoecium, which has been shown to be conserved in distantly related species within the eudicot clade of Angiosperms (Alvarez et al. 2009, Trigueros et al. 2009, Fourquin and Ferrándiz 2014). *NGA* genes were first characterized in *Arabidopsis thaliana*, where they form a small subfamily

of four highly related B3 transcription factors (TFs), *NGA1*, *NGA2*, *NGA3* and *NGA4* (Araki and Komeda 1993, Bleecker 1998, Alvarez et al. 2009, Trigueros et al. 2009). *NGA* factors group together with other members of the RELATED TO AP2 AND VP1 (RAV) clade in this family and are characterized by the presence of a single B3 DNA-binding domain and three C-terminal motifs known as NGA-I motif, RAV-repressive domain and NGA-II motif (Fourquin and Ferrándiz 2014). The four

Abbreviations – BAC, Bacterial Artificial Chromosome; CDS, coding sequence; CIN, CINCINNATA; Col-0, columbia-0; CUC, CUP-SHAPED COTYLEDON; GUS, β -GLUCURONIDASE; LA, LANCEOLATE; LUC, LUCIFERASE; miRNA, microRNA; NGA, NGATHA; NGAL, NGATHA-LIKE; ORF, Open Reading Frame; PCR, polymerase chain reaction; RAV, RELATED TO AP2 AND VP1; REN, Renilla; TF, transcription factor; UTR, untranslated region; Y1H, yeast one-hybrid.

NGA genes in *Arabidopsis* are functionally redundant. Loss of *NGA* function results in the absence of the apical tissues within the pistil, which in quadruple *nga* mutants ends as an open cylinder with distal protrusions of valve-like tissue. In addition to this role in gynoecium development, *NGA* genes are important general regulators of lateral organ growth. The leaves of *Arabidopsis* quadruple *nga* mutants are wider and shorter than wild type, and these phenotypes are also observed in other floral organs, specially sepals and petals (Fourquin and Ferrandiz 2014). Conversely, *NGA* overexpression produces small narrow leaves and petals (Alvarez et al. 2009, Kwon et al. 2009, Trigueros et al. 2009).

NGA genes have been related to auxin signaling (Trigueros et al. 2009, Martinez-Fernandez et al. 2014). Thus, it has been shown that *NGA* factors positively regulate local auxin synthesis and also that *NGA* altered expression leads to differential expression of several Auxin Response Factors and auxin transporters (Martinez-Fernandez et al. 2014). Defective auxin signaling has been associated with altered leaf morphology and carpel patterning defects, so, at least partly, the phenotypes observed in *nga* mutants or overexpressors could be related to altered auxin pathways (Østergaard 2009, Sundberg and Ferrándiz 2009, Sundberg and Østergaard 2009, Koenig and Sinha 2010). However, auxin appears to have only little influence on *NGA* gene expression (Trigueros et al. 2009), suggesting that other upstream regulators remain to be identified.

The four *NGA* genes in *Arabidopsis* share very similar expression patterns. In leaf primordia, expression is associated with distal marginal tissues in very young primordia, restricted later to the tips of cotyledons and leaves and to leaf hydathodes. In floral organs, *NGA* expression is mainly detected in the distal part of growing primordia, disappearing in expanded sepals and petals. In the gynoecium, *NGA* accumulates in the apical end of the growing gynoecial tube, restricting later to the incipient style and stigma and the placental tissues (Alvarez et al. 2009, Trigueros et al. 2009). The highly similar expression patterns of all four *NGA* genes strongly suggest that they may share common regulators. In this work, we have identified a conserved region among the four *NGA* promoters and hypothesized that it was likely bound by these putative regulators. Using a yeast one-hybrid (Y1H) strategy followed by molecular and genetic analyses, we have shown that some miR319-regulated TCP factors are activators of *NGA* expression and that *NGA* factors partly mediate TCP roles in leaf morphogenesis. Moreover, we have observed that this TCP-*NGA* regulatory module appears to be conserved in distantly related species, including crops such as tomato, rice or common bean.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana plants were grown in cabinets at 21°C under long day (16 h light) or short day (8 h light) conditions, illuminated by cool-white fluorescent lamps (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$), in a 1:1:1 mixture of sphagnum:perlite:vermiculite. To promote germination, seeds were stratified on soil at 4°C for 3 days in the dark. The *Arabidopsis* plants used in this work were in the Columbia-0 (Col-0) background. Mutant alleles and transgenic lines have been previously described: *jaw-D* (Palatnik et al. 2003), *tcp2-1* (SAIL_562_D05) (Schommer et al. 2008), *nga1-4 nga2-3 nga3-3 nga4-1* and *35S::NGA3* (Trigueros et al. 2009).

Sequence analyses

NGA orthologs from rice, common bean and tomato were identified by standard BLAST searches, using *NGA3* coding sequence (CDS) as a query. The flanking genomic sequences for these and for *Arabidopsis NGA* genes were then obtained from the database repositories of genome sequences for each species. Accession numbers of BACs containing the *NGA* genes are listed in Appendix S2, Supporting Information. Pairwise alignments of 1 kb genomic sequence for each gene upstream of the ATG codon were performed with the VISTA bioinformatic tool (Frazer et al. 2004). Following identification of conserved sequences, alignments of the most similar fragments were performed using the ClustalW tool in the Macvector 12.6 software.

Y1H experiments

The fragment used for the Y1H screening was generated by annealing primers oPBF48 and oPBF50 (Appendix S1) and the subsequent synthesis of the reverse-complement strand using a Taq-mediated step of extension. The final product was cloned into pTUY1H (Castrillo et al. 2011), previously digested with *Xba*I and *Xma*I restriction enzymes. For subsequent tests, a second bait construct was generated where a trimeric repetition of the 53 bp sequence used for the screening was cloned into the pTUY1H vector. The trimer was generated by two sequential polymerase chain reactions (PCRs) using in the first step the primers oPBF28 and oPBF41 and as a template the primer CFYtri; the resulting product was reamplified with primers oPBF46 + oPBF47 and the final product was cloned into pCRII vector (Invitrogen, Carlsbad, CA), digested with *Xba*I and *Xma*I restriction enzymes and ligated into pTUY1H. See Appendix S1 for primer sequences.

For the screening of the arrayed yeast library, transformation, growth and mating of yeast cells were performed as described previously (Castrillo et al. 2011, Rueda-Romero et al. 2012). The *Saccharomyces cerevisiae* Y187 and YM4271 strains carrying bait and prey constructs, respectively, were used to test DNA–protein interactions. The screening was performed at 28°.

Quantitative reverse transcription PCR

Total RNA was extracted from *A. thaliana* Col-0, *tcp2-1* and *jaw-D* seedlings with the E.Z.N.A. Plant RNA Kit (OMEGA Bio-Tek, Doraville, GA). Three micrograms of total RNA were used for cDNA synthesis with the First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), and the quantitative PCR master mix was prepared using the SYBR Premix Ex Taq (Takara Co., Tokyo, Japan). Primers used to amplify the *NGA* genes were as follows: OMNG48 and OMNG49 for *NGA1*, OMNG46 and OMNG47 for *NGA2*, OMNG42 and OMNG43 for *NGA3* and OMNG44 and OMNG45 for *NGA4*. Results were normalized to the expression of the *UBIQUITIN-CONJUGATING ENZYME21* (*UBC21*) mRNA, amplified with *UBC21F* and *UBC21R* primers. The PCR reactions were run and analyzed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The Student's *t*-test was used to determine the significance of expression level differences. See Appendix S1 for primer sequences.

GUS histochemical detection

For β -glucuronidase (GUS) histochemical detection, samples were treated for 15 min in 90% ice-cold acetone and then washed for 5 min with washing buffer (25 mM sodium phosphate, 2 mM ferrocyanide, 2 mM ferricyanide and 1% Triton X-100) and incubated from 4 to 16 h at 37°C with staining buffer (washing buffer + 1 mM X-glucuronide). Following staining, plant material was fixed, cleared in chloral hydrate and mounted according to Weigel and Glazebrook (2002) to be viewed under bright-field microscopy. All detections were made in heterozygous lines for the reporter transgene.

To generate the *NGA2::NGA2:GUS* reporter, first the 3' *NGA2* genomic sequence downstream the STOP codon (+898 to +2919) was amplified with primers oMNG24 and oMNG25 and cloned into a pGREENII0029 vector (<http://www.pgreen.ac.uk>). The fragment of *NGA2* genomic sequence spanning approximately 2.5 kp upstream of the ATG and the *NGA2* CDS (–2575 to +12) was amplified with primers (oMNG22 and oMNG23) and subsequently cloned into a pCRII vector previously modified by introducing the *GUS*

CDS from pBI101.2. Finally, the fragment containing *NGA2* upstream sequences, the *NGA2* CDS and the *GUS* CDS, was digested from this intermediate plasmid and cloned into the pGREENII0029 vector already containing the 3' genomic fragment downstream *NGA2* CDS digested with *KpnI* and *SalI*. See Appendix S1 for primer sequences.

Genotyping

Genotyping of *nga* mutants was performed as previously described (Trigueros et al. 2009). For *tcp2-1* genotyping, guidelines from SIGNAL webpage were followed (<http://signal.salk.edu>). In brief, primers LP_SAIL_562_D05 and RP_SAIL_562_D05 were used to amplify the wild-type allele, while primers RP_SAIL_562_D05 and LB1 amplified the mutant allele. See Appendix S1 for primer sequences.

Luciferase assays

To generate promoter:LUC fusions for transient expression assays in *Nicotiana benthamiana* leaves, a 2.7-bp *NGA3* promoter fragment (–2722 to ATG) was amplified from Arabidopsis Col-0 genomic DNA with primers A904-GUSd and oPBF11, cloned into pCRII vector (Invitrogen), digested with *HindIII* and *NcoI* restriction enzymes and ligated into pGREEN_LUC (Hellens et al. 2005). To generate the *NGA3Δ270::Luc* reporter, *NGA3* promoter fragments were amplified by a two-step sequential PCR. First, the fragments flanking the 270 bp region were amplified using two primer combinations (A904-GUSd/oPBF53 and oPBF83/oPBF11) that produced two fragments with overlapping ends. An equimolecular mixture of both fragments was used as a template for a final PCR with primers A904-GUSd+oPBF11, and the final product was cloned into pCRII vector (Invitrogen), digested with *HindIII* and *NcoI* restriction enzymes and ligated into pGREEN_LUC (Hellens et al. 2005). Finally, the *S/NGAa* promoter was amplified from *Solanum lycopersicum* cv. M82 genomic DNA with primers oPBF123 and oPBF124, cloned into pCRII vector (Invitrogen), digested with *SmaI* and *NcoI* restriction enzymes and ligated into pGREEN_LUC (Hellens et al. 2005). The effector constructs were generated by amplifying the corresponding ORFs of *TCP1*, *TCP2*, *TCP3* and *LA* from cDNA of Arabidopsis Col-0 leaves (*TCP1*, *TCP2* and *TCP3*) or *S. lycopersicum* cv. MoneyMaker leaves (*LA*), that were subsequently cloned into PCR8/GW/TOPO (Invitrogen) and then transferred by Gateway reactions into the pMCD32 destination vector (*TCP1*, *TCP2* and *TCP3*) (Curtis and Grossniklaus 2003) or the pEARLEY100

destination vector (LA)(Earley et al. 2006). See Appendix S1 for primer sequences.

The transient expression assays were performed by *Agrobacterium*-infiltrated transient transformation of *N. benthamiana*, which was carried out as previously described (Espley et al. 2009). Briefly, *N. benthamiana* plants were grown until about 5 cm in height. Approximately, 300 µl of *Agrobacterium* containing the reporter or/and effector plasmids were infiltrated into a young leaf at three points. Firefly luciferase and Renilla luciferase were assayed 3 days after infiltration using the Dual-Luciferase Reporter Assay System (Promega). Data were represented as the ratio of LUC/REN. Background controls were obtained by infiltrating only the reporter construct. At least three plants at the same developmental stage were used for each treatment, and the experiment was repeated three times. The Student's *t*-test was used to determine the significance of relative LUC activity differences.

Results

TCP TF bind a conserved region in *NGA* promoters

The four Arabidopsis *NGA* genes are expressed in highly similar domains, suggesting that they may share common regulators (Alvarez et al. 2009, Trigueros et al. 2009). To identify *cis*-regulatory elements that could mediate this regulation, we compared 1 kb of 5' upstream promoter sequences of the four *NGA* genes by in silico analyses with the VISTA bioinformatic tool (Frazer et al. 2004). A region of approximately 270 bp with significant similarity was identified around positions −300 to −30 upstream of the transcription start site in all *NGA* genes, where similarity was higher in *NGA1/NGA2* and *NGA3/NGA4* pairs. These regions contained a fragment of approximately 55 bp in proximal positions that showed the highest level of conservation (Fig. 1). To identify putative TFs that could bind to these regions, we used a 55 bp fragment of the *NGA3* promoter spanning this highly conserved sequence to perform a Y1H screening against an arrayed yeast library of ca. 1200 Arabidopsis TFs which has been previously described (Rueda-Romero et al. 2012). Seven positives were recovered from the screening, representing TFs from different families. Positive interactions were confirmed by two independent experiments using the same bait construct and a second construct where the 55 bp sequence was cloned as a trimer upstream of the reporter genes to minimize false negatives by reinforcing positive DNA–TF interactions (Fig. 2). In these experiments, only three positives were unequivocally confirmed, all members of the class-II of the TCP family: TCP2 and TCP3, which belong

Fig. 1. Position of the conserved sequences in *NGA* promoters used in this study. A schematic representation of the four genomic regions for the Arabidopsis *NGA* genes is shown. The transcribed regions are noted with wide boxes where gray color corresponds to untranslated regions and blue color to coding sequences. Upstream of the transcription start, the position of the conserved sequences in the four promoters has been color-coded: red corresponds to the 55 bp sequence showing highest similarity among the four promoters; pink corresponds to the fragment showing the highest similarity between the *NGA1* and *NGA2* promoters and orange to the fragment showing higher similarity between the *NGA3* and *NGA4* promoters. The regions represented in pink and orange are also similar to each other.

to the CIN-clade of the TCP family and are targets of the miR319; and TCP1, a member of the CYC/TB1 clade and not regulated by microRNA (miRNA) (Martín-Trillo and Cubas 2010).

TCP2 and TCP3 are able to activate the *NGA3* promoter

To explore the functional relevance of TCP binding to the conserved sequence in *NGA* promoters, we tested the response of the *NGA3* promoter in vivo to each of the three TCP factors identified in the screening. We used the Luc/Ren system described in Hellens et al. 2005. This system allows one to monitor the response of a given promoter to a TF in transient assays by measuring the activity of a *LUCIFERASE* (*LUC*) reporter fused to the promoter under test. The construct also contains a *Renilla* (*REN*) gene under the control of the CaMv35S promoter to estimate the extent of the transient expression, which therefore serves as an internal standard when the activity of the promoter is represented as the ratio of LUC to REN activities (Luc/Ren) (Hellens et al. 2005). For our experiment, approximately 2.7 kb of *NGA3* 5' genomic sequence (−2722 to ATG) were cloned into the pGREENII 0800-Luc vector (*NGA3::Luc*). This promoter fragment was previously shown to recapitulate the native *NGA3* expression in a *NGA3::GUS* reporter (Trigueros et al. 2009).

We expressed the *NGA3::Luc* construct in *N. benthamiana* leaves by agroinfiltration and found that in

Fig. 2. TCP1, TCP2 and TCP3 bind to the 55 bp conserved sequence in *NGA* promoters. A yeast strain containing a trimer repeat of the 53 bp sequence used in the Y1H screening cloned in the pTUY1H plasmid was mated to strains containing the activation domain (AD)-GFP (negative control), AD-TCP1, AD-TCP2, AD-TCP3 and AD-TCP10 constructs. Diploid cells were grown on non-selective minimal medium without Leu and Trp (-L-W) or selective minimal medium without Leu, Trp and His (-L-W-H). Three serial dilutions of diploid cells were spotted. Only cells containing the TCP1, TCP2 and TCP3 factors were able to activate reporter expression, while the strains containing the negative control (GFP) or the related TCP10 factor were not able to grow in selective medium, supporting the specificity of the observed binding.

the absence of effectors, a significant basal level of LUC activity was observed, suggesting the presence of factors able to activate *NGA3* transcription in the leaves of *Nicotiana*. Coinfiltration of the reporter with 35S::TCP2 or 35S::TCP3 produced upregulation of *LUC* activity (Fig. 2), whereas coinfiltration with 35S::TCP1 did not change the basal level of *LUC* activity conferred by the *NGA3* promoter alone (Fig. 3). Next, we assayed a *NGA3Δ270*::Luc reporter where the 270 bp fragment showing similarity among the four *NGA* promoters was eliminated. The *NGA3Δ270* promoter also conferred some basal level of activity, although much lower than the full *NGA3* promoter, suggesting that this region was important for *NGA3* regulation. We found that *NGA3Δ270*::Luc activation in response to TCP2 or TCP3 was significantly reduced, but still higher than the basal level of *LUC* activity driven by the *NGA3Δ270* fragment (Fig. 3). These results strongly suggested that TCP2 and TCP3 could directly bind to this 270 bp fragment to activate *NGA* expression *in vivo*, and that, likely, there were other redundant regions of the *NGA3* promoter responsive to TCP2 and TCP3 outside this 270 bp domain. Interestingly, a GGNCCC sequence that has been previously described as the TCP core-binding site

Fig. 3. Transient assays of *NGA3*::LUC expression. *NGA3*::LUC-35S::REN and *NGA3Δ270*::LUC-35S::REN reporter constructs were transiently expressed in *Nicotiana benthamiana* leaves together with control vector, 35S::TCP1, 35S::TCP2 or 35S::TCP3. The expression of *REN* was used as an internal control. Luc/Ren values represent means and standard errors ($n = 6$). Letters above bars indicate a significant difference according to Student's *t*-test ($P < 0.05$) from (a) the values obtained when the *NGA3*::LUC-35S::REN reporter was infiltrated alone; (b) the values obtained when the full *NGA3*::LUC-35S::REN reporter was infiltrated with the corresponding effector and (c) the values obtained when the *NGA3Δ270*::LUC-35S::REN reporter was infiltrated alone.

was found in all four *NGA* promoters at the 3' end of the 270 bp fragment (Fig. 1; Kosugi and Ohashi 2002).

Expression of all *NGA* genes is reduced in *tcp2* and *jaw-D* mutants

To test the functional relevance of the observed interactions between TCP factors and the *NGA3* promoter in Arabidopsis, and whether they were also extensive to the rest of the *NGA* genes, the levels of all four *NGA* transcripts were quantified in *tcp2* single mutants. In this background, the expression of *NGA2* and *NGA3* was reduced, while *NGA1* and *NGA4* transcript levels were unchanged (Fig. 4A). Because a high degree of redundancy is found among CIN-like genes in Arabidopsis, *NGA* expression was also determined in *jaw-D* mutants, which overexpress MIR319a to simultaneously downregulate *TCP2*, *TCP3*, *TCP4*, *TCP10* and *TCP24* and show phenotypes similar to those caused by multiple combinations of loss-of-function alleles in these *TCP* genes (Palatnik et al. 2003, Koyama et al. 2010, Schommer et al. 2014). All *NGA* genes were expressed at significantly lower levels in *jaw-D* mutants, indicating that *TCP* genes are required for *NGA* regulation in a likely redundant manner (Fig. 4B).

To check if the effect in *NGA* regulation observed in *jaw-D* mutants affected not only the level but also

Fig. 4. Quantitative PCR analysis of the expression of the four Arabidopsis *NGA* genes in wild type (WT), *tcp2-1* and *jaw-D* 10-days seedlings. The error bars depict the standard error based on three biological replicates. Asterisks indicate a significant difference from the WT control according to Student's *t*-test (* $P < 0.05$; *** $P < 0.001$).

the spatial pattern of *NGA* gene expression, reporter transgenes for *NGA2* and *NGA3* were introduced in the *jaw-D* background. The *NGA3::GUS* reporter has been previously described (Trigueros et al. 2009), while the *NGA2::NGA2::GUS* reporter consisted on a translational fusion of *NGA2* and *GUS* CDSs flanked by approximately 2.5 kb of genomic sequence upstream of the START codon and by approximately 2 kb of genomic sequence downstream the STOP codon, and therefore also containing 5' untranslated region (UTR) and 3'UTR sequences, respectively. Both *NGA2* and *NGA3* reporter expression domains were similar in wild-type and in *jaw-D* backgrounds (Fig. 5), although in accordance with the quantitative reverse transcription-PCR results, *GUS* signal appeared to be weaker in *jaw-D* mutants (Fig. 5). These results indicated that TCP regulation of *NGA* genes mainly affected the levels of *NGA* gene expression, but not the spatial distribution of *NGA* transcripts, and that CIN-like TCP genes were not required for the onset of *NGA* expression, but were important to maintain *NGA*

expression to optimal levels. However, because there is residual *TCP2* and *TCP3* expression in *jaw-D* mutants (Palatnik et al. 2003), it was not possible to rule out that these remaining levels of *TCP* expression could be sufficient to activate *NGA* gene transcription

In summary, our data were consistent with *TCP2* and *TCP3* factors being true regulators of *NGA* expression in Arabidopsis, although mainly affecting expression levels and not spatial patterns.

Leaf phenotypes associated with loss of function of *TCP* genes that are targets of miR319a could be partially caused by reduced *NGA* expression

Loss of function of miR319a-target *TCP* genes of the CIN subfamily causes defects in leaf development. Thus, leaves of *tcp2*, *tcp4* or *tcp10* single mutants are wider and slightly more serrated than wild type (Schommer et al. 2008), and when combined, the severity of the phenotype is strongly enhanced, as it also occurs in *jaw-D* mutants (Palatnik et al. 2003, Schommer et al. 2008, Koyama et al. 2010), Fig. 6A, C–D, G–J). These phenotypes partly resemble those found in *nga* quadruple mutants, where leaves are also wider and more serrated than wild type (Alvarez et al. 2009, Trigueros et al. 2009), Fig. 6B, K). Because *NGA* genes appear to be positively regulated by *TCP2* and *TCP3*, it could be possible that the *tcp2* or *tcp3* phenotypes were due to the weakly reduced levels of *NGA* expression in these mutants. If this was true, overexpression of *NGA* genes in these backgrounds should complement the observed *tcp* leaf phenotypes. 35S::*NGA3 tcp2* plants showed phenotypes indistinguishable from those of 35S::*NGA3* lines, supporting this hypothesis (Fig. 6N). We also tested the effect of *NGA3* overexpression in the *jaw-D* background, where the expression of all *NGA* genes is strongly reduced. Because *jaw-D* and 35S::*NGA3* confer dominant phenotypes, we compared leaf morphology in the F1 plants (hemizygous to 35S::*NGA3* and heterozygous for *jaw-D* allele) to heterozygous *jaw-D* and hemizygous 35S::*NGA3* plants. Rosette leaves of 35S::*NGA3 jaw-D* plants were much narrower than the corresponding heterozygous *jaw-D* leaves, indicating that *NGA3* overexpression was able to significantly suppress lateral growth produced by loss of TCP function. Leaf serration was also reduced by *NGA3* overexpression, although it still was more conspicuous than in wild type or in 35S::*NGA3* lines (Fig. 6C–F, H–I, L–M).

The TCP/NGA regulatory module appears to be conserved in crop species

TCP genes have been shown to control leaf morphology in several species, including tomato, rice and legumes.

Fig. 5. The effect of *jaw-D* mutation in the activity of NGA2 and NGA3 reporters. NGA2::NGA2:GUS expression in seedlings of (A) wild type or (B) *jaw-D/+* mutant plants and in anthesis flowers of (C) wild type or (D) *jaw-D/+* mutants. NGA3::GUS expression in seedlings of (E) wild type or (F) *jaw-D/+* mutant plants and in anthesis flowers of (G) wild type or (H) *jaw-D/+* mutants. Detail of NGA3::GUS reporter activity in a young wild-type leaf (I) and a *jaw-D/+* leaf at an equivalent stage (J). In all cases, the expression patterns are similar in wild type or *jaw-D/+* backgrounds, although GUS activity is detected at lower levels in *jaw-D/+* mutants. Bar = 1 mm in A, B, E, F, I and J. Bar = 500 μ m in C, D, G and H.

The conservation of NGA function across angiosperms has not been so extensively explored, although it has been shown that, at least in eudicots, NGA factors have an essential role in style and stigma development in distantly related species. Moreover, *N. benthamiana* flowers with reduced NGA activity showed altered sepal and petal development, suggesting that NGA role in lateral organ expansion is also conserved (Fourquin and Ferlandiz 2014). To test whether the observed interaction between TCP factors and NGA promoters was part of a conserved regulatory module in other species, we compared the promoters of NGA orthologs from Arabidopsis, rice, tomato and common bean. We found that the 53 bp core sequence detected in Arabidopsis promoters was also present in several NGA orthologs in these species, including the putative TCP-binding site, and strongly suggesting that NGA genes could be regulated by TCP factors in these species as well (Fig. 7A).

To validate *in vivo* this suggested interaction, we used again the Luc/Ren system to test whether LANCEOLATE (LA) [the tomato ortholog of *AtTCP3* (Ori et al. 2007)] was able to activate the promoter of *SINGAa* (the tomato ortholog of *AtNGA3*). A fragment of approximately 2 kb of the *SINGAa* promoter was cloned into the

pGREEN-Luc 800 vector. Coinfiltration of the *SINGAa* reporter with 35S::LA resulted in a significant increase of the Luc/Ren ratio, indicating that LA was able to activate the *SINGAa* promoter in planta (Fig. 7B). Likewise, LA could also activate the Arabidopsis NGA3::Luc reporter to a similar extent as its Arabidopsis ortholog, *AtTCP3* (Fig. 7C). Altogether, these results supported the hypothesis of the conservation of a TCP-NGA regulatory module in distantly related species.

Discussion

In this work, we have found that TCP1, TCP2 and TCP3 are able to bind a fragment of the NGA3 promoter in a Y1H experiment, whose sequence is highly conserved within the promoters of the four NGA genes. In addition, we show that TCP2 and TCP3, but not TCP1, are able to activate the transcription of a reporter gene fused to the NGA3 promoter in planta. Moreover, the expression levels of all NGA genes are strongly reduced in the *jaw-D* background, where TCP2, TCP3 and other CIN-like TCP genes are downregulated. The potential role of CIN-like TCP factors in the upregulation of NGA genes had been already suggested by a previous work that showed

Fig. 6. Phenotypes of the different mutants and combinations generated. Rosettes of (A) wild type, (B) *nga1 nga2 nga3 nga4* quadruple mutant, (C) *jaw-D* homozygous mutant, (D) *jaw-D* heterozygous mutant, (E) 35S::NGA3 hemizygous line, (F) an F1 plant derived from the cross of *jaw-D* and 35S::NGA3, therefore heterozygous for *jaw-D* and hemizygous for 35S::NGA3. Dissected leaves from plants of different genotypes at equivalent stages: (G) wild type. (H) *jaw-D* homozygous. (I) *jaw-D* heterozygous. (J) *tcp2* mutant. (K) *nga1 nga2 nga3 nga4* quadruple mutant. (L) 35S::NGA3 hemizygous. (M) *jaw-D* heterozygous, 35S::NGA hemizygous. (N) *tcp2* 35S::NGA3 hemizygous. Bars = 1 cm.

reduced levels of *NGA3* and *NGA4* expression upon estradiol-mediated induction of a ProXVE::TCP3:SRDX transgene (Koyama et al. 2010). Our results, which identify TCP factors as putative regulators of *NGA* genes through a completely different approach, provide further functional data that confirm TCP2 and TCP3 as likely direct activators of *NGA* gene expression.

CIN-like TCP clade in Arabidopsis comprise five genes regulated by miR319 (*TCP2*, *TCP3*, *TCP4*, *TCP10* and *TCP24*) and three other genes not regulated by miRNAs (*TCP5*, *TCP13* and *TCP17*) (Martín-Trillo and Cubas 2010). These eight genes act in a partially redundant manner to control several aspects of leaf development, as deduced from the mild phenotypes of single mutants in several of these genes and the increasing severity of phenotypic alterations seen in multiple combinations of the different mutants or when simultaneous down-regulation of the eight genes is achieved by overexpression of miR319 and an artificial miRNA targeting *TCP5*, *TCP13* and *TCP17* (Efroni et al. 2008, Schommer et al. 2008, Koyama et al. 2010). The functional

relevance of the regulatory TCP-NGA interaction uncovered by this work is well supported by the phenotypes of *nga* quadruple mutants, which show enlarged serrated leaves resembling low order mutant combinations in CIN-like TCP genes, but are much less severe than those of high order *cin-tcp* mutant combinations. Thus, *nga* leaf phenotypes are a subset of those caused by complete loss of CIN-like TCP function. Likewise, *NGA* overexpression results in phenotypes resembling those caused by overexpression of miR319-resistant versions of *TCP2*, *TCP3* or *TCP4*, which include narrow epinastic leaves, fused cotyledons and smooth leaf margins (Palatnik et al. 2003, Koyama et al. 2007, Trigueros et al. 2009). Moreover, 35S::NGA3 *tcp2* plants were indistinguishable from 35S::NGA3 lines, and overexpression of *NGA3* in a *jaw-D* background suppressed part of the phenotypes associated with miR319 overexpression, especially the lateral growth of the lamina and, to some extent, the exaggerated leaf serration (Fig. 6). Altogether, this genetic evidence suggests that *NGA* genes act downstream of TCP factors to partially mediate their functions.

Fig. 7. Conservation of *NGA* promoter sequence and activity in different species. (A) Alignment of a fragment of *NGA* promoters from different species showing similarity to the 55 bp sequence in *AtNGA3* promoter used in the Y1H screening. The putative TCP-binding site is shadowed. In bold, most conserved bases are shown. Numbers on the right correspond to the relative position of the last base to the START codon of the corresponding gene. Accession numbers are as follows: *AtNGA1* (At2g46870), *AtNGA2* (At3g61970), *AtNGA3* (At1g01030), *AtNGA4* (At4g01500), *PvNGA* (PHAVU_010G120900g), *OsNGAa* (LOC_Os03g02900), *OsNGAb* (LOC_Os10g39190), *SINGAa* (LOC101249569). (B) Transient assays of *SINGAa::LUC* expression. *SINGAa::LUC-35S::REN* reporter construct was transiently expressed in *Nicotiana benthamiana* leaves together with control vector or 35S::LANCEOLATE (35S::LA). The expression of *REN* was used as an internal control. Luc/Ren values represent means and standard errors (n=6). Asterisks indicate a significant difference from the values obtained when the *SINGAa::LUC-35S::REN* reporter was infiltrated alone according to Student's *t*-test (***P* < 0.01). (C) Transient assays of *NGA3::LUC* expression in response to the heterologous TCP factor from tomato LA. *NGA3::LUC-35S::REN* reporter construct was transiently expressed in *N. benthamiana* leaves together with control vector, 35S::TCP3 (the ortholog of LA in *Arabidopsis*) and 35S::LA. The expression of *REN* was used as an internal control. Luc/Ren values represent means and standard errors (n=6). Asterisks indicate a significant difference from the values obtained when the *NGA3::LUC-35S::REN* reporter was infiltrated alone according to Student's *t*-test (**P* < 0.05; ***P* < 0.01).

This is compatible with different and not mutually exclusive scenarios:

1. First, CIN-like TCP factors participate in multiple regulatory interactions and developmental processes that could be genetically separated. For example, they have been shown to regulate leaf shape through negative regulation of boundary genes, namely the *CUP-SHAPED COTYLEDON* (*CUC*) genes. This negative regulation is achieved by parallel convergent pathways, which involve the independent activation by TCP of different factors such as *ASYMMETRIC LEAVES1*, miR164 or auxin-related genes, that in turn repress *CUC* expression (Koyama et al. 2007, Koyama et al. 2010). In addition, CIN-like TCP factors also control leaf development through *CUC*-independent mechanisms such as the control of cell proliferation through activation of miR396,

CYCLIN-DEPENDENT KINASE INHIBITOR/KIP RELATED PROTEIN1 and jasmonate biosynthesis (Schommer et al. 2014), or interfering with cytokinin signaling (Efroni et al. 2013). In this context, we can envision that *NGA* genes would be involved in only part of these multiple pathways, therefore only contributing to a subset of TCP functions. In fact, CIN-like TCP and *NGA* factors have been related to common pathways like auxin signaling (Trigueros et al. 2009, Martinez-Fernandez et al. 2014), cytokinin response (Kwon et al. 2011) or control of cell proliferation (Kwon et al. 2009), and detailed genetic analyses will have to be performed to elucidate the *NGA*-dependent and *NGA*-independent modes of TCP function.

2. The weaker phenotype on *nga* quadruple mutants compared with multiple *cin-tcp* mutants could be due to redundancy of *NGA* genes with the

related three *NGA-LIKE* (*NGAL*) genes also present in the Arabidopsis genome (At3g11580, At5g06250 and At2g36080) (Alvarez et al. 2009). Interestingly, it has been recently reported that *DEVELOPMENT-RELATED PcG TARGET IN THE APEX 4*, which corresponds to *NGAL2*, controls leaf serration in Arabidopsis by negatively regulating *CUC2* (Engelhorn et al. 2012). Likewise, it has been described that the overexpression of any of the *NGAL* genes also causes suppression of leaf margin serration and inhibition of lateral growth of the lamina (Alvarez et al. 2009, Engelhorn et al. 2012, Shao et al. 2012). Thus, it seems likely that *NGA* and *NGAL* factors share redundant functions in leaf development, which should be unveiled by generating multiple mutant combinations in all seven loci that have not been achieved so far. These multiple mutants could also be used to compare the effects of full loss of *NGA/NGAL* function and of *CIN-TCP* function to understand further the extent of their functional relationship.

3. It could be possible that *NGA* genes are only activated by a subset of *TCP* factors, and therefore only mediate the function of these in leaf development. Thus, *nga* phenotypes could reflect the specific contribution of this *TCP* subset to the process. In fact, the Y1H screening performed in this work only identified three *TCP* factors as able to bind to the 55 bp conserved fragment in *NGA* promoters, even though all members of the *TCP* family except *TCP7* were present in the arrayed collection used for the screening. While we cannot exclude that we obtained some false negatives, these results suggest that not all *CIN-TCP* factors regulate *NGA* expression. In fact, it has been clearly shown that not all *CIN*-like *TCP* factors are fully equivalent: there are clear differences among the expression patterns of the different *CIN*-like *TCP* genes (Koyama et al. 2007, Danisman et al. 2013), their protein–protein interactions (Danisman et al. 2013) or the severity of the phenotypes caused by their overexpression (Palatnik et al. 2003, Koyama et al. 2007, Danisman et al. 2013, Li and Zachgo 2013), so it is conceivable that there are some constraints to the observed *TCP-NGA* regulatory interactions based on these differences. Still, we cannot completely rule out that other *TCP* factors in addition to *TCP2* and *TCP3* regulate *NGA* expression. Our transient expression analyses of *TCP* effect on the *NGA3* promoter suggest that not only *TCP2/TCP3* activation of *NGA* expression is mediated by the conserved region used in the Y1H screening but

that other *TCP*-responsive regulatory elements are also present in different positions of the *NGA3* promoter. To test whether these additional elements could also respond to other members of the *CIN*-like *TCP* clade, it would be interesting to pursue a systematic study in which all *TCP* factors should be tested. These experiments could also add some functional evidence to clarify the differences among related *TCP* factors that act redundantly but are not fully equivalent

In addition to their general role in lateral organ development, *NGA* factors are essential to specify the identity of the apical tissues in the gynoecium, the style and the stigma. According to publicly available microarray data, *TCP2* and *TCP3* are expressed in carpels (Atgenexpress, <http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp>), and we observed reduced *NGA* levels in *jaw-D* gynoecia. However, *jaw-D* mutants only show mild defects in fruit development that mainly affect fruit elongation, but not style or stigma development (Palatnik et al. 2003), suggesting that the *NGA* role in apical gynoecium patterning does not significantly depend on *TCP* activity.

Finally, our work points to the likely conservation of the uncovered *TCP-NGA* regulatory module to control leaf development across angiosperm species. We were able to find the highly conserved sequences identified in the four Arabidopsis *NGA* promoters in *NGA* ortholog genes from distant crop species such as rice (a monocot), tomato (Solanaceae) or common bean (legume). Moreover, we show that one of these promoters (*S/NGAa*) was also activated by the corresponding tomato *TCP3* ortholog in a transient expression assay. Previous reports have shown that *CIN-TCP* genes control leaf development in several species from different clades within angiosperms. Thus, the study of the *cinninnata* mutants in *Antirrhinum majus*, which show severely crinkled leaves, led to the identification of the first *CIN-TCP* gene (Nath et al. 2003); in tomato, a gain-of-function mutation in the *LA* gene causes a extreme reduction of leaf complexity and size, while overexpression of *miR319* results in the increase of leaflet size and the formation of crinkled leaf margins (Ori et al. 2007); likewise, overexpression of *miR319* causes leaf blade expansion in rice or in creeping bentgrass (Yang et al. 2013, Zhou et al. 2013). The well-established general role of *CIN-TCP* genes in leaf development across angiosperms is in contrast with the almost non-existent knowledge about the role of *NGA* genes in leaf development in other species than Arabidopsis. However, a recent study shows that *NGA* genes play similar roles in gynoecium development in distantly related species within eudicots and that, in *N. benthamiana*, other lateral organs such as petals or sepals are

affected by NGA downregulation (Fourquin and Ferrandiz 2014), suggesting that the NGA function in controlling lateral organ growth could also be conserved. In this work, we were able to uncover a likely conserved regulatory interaction of TCP and NGA factors in different species based on the comparison of promoter sequences and transient expression experiments. Even though the functional relevance of this interaction remains to be tested, a simple strategy has allowed us to propose the existence of a regulatory module that could be important to explain leaf development in crop species and therefore justify further work that could provide novel insights in basic questions on leaf morphogenesis, as well as identify new targets for biotechnological crop improvement.

A contributions

P. B. designed and performed most of the experiments, together with M. N. G. P. C. and L. O. S. contributed the Y1H system and helped with these experiments. C. F. conceived the idea, designed most of the experiments, supervised the work and wrote the manuscript.

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