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CDF4 promotes leaf senescence by regulating ABA and reactive oxygen species pathways in Arabidopsis

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1st Editorial Decision 23rd Sep 2019

Dear Dr. Xu,

Thank you for the submission of your manuscript to EMBO reports; I am very sorry for this unusual delay in getting back to you. Referee 2 promised several times to send her/his report, but we still have not received it, and I will make a decision now based on the 2 enclosed reports we have in order not to lose more time.

As you will see, both referees acknowledge that the findings are potentially interesting. However, both also point out that the phenotypes are not sufficiently convincing, that the genetic evidence is weak, and that more mechanistic insight should be provided. Referee 3 further pinpoints inconsistencies, and notes that better evidence needs to be provided to support the statement that CDF4 regulates senescence through its effects on ABA biosynthesis and ROS.

Given these comments from 2 experts in the field, it is clear that publication of your study by EMBO reports cannot be considered at this point. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in

the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

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4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors reported that the DOF family transcription factor CDF4 is a positive regulator of leaf senescence. Knockdown of CDF4 delays leaf senescence, while overexpressing lines display an earlier senescence phenotype. Moreover, the authors found that CDF4 promotes leaf senescence by increasing ABA content through directly regulating NCED2/3, and suppressing ROS scavenging enzyme. However, the senescence-associated phenotypes of the key mutant lines were not evident and the genetic evidence was weak. Although this manuscript provides some new information on the functional studies of a senescence-assoicated transcriptional regulation, it is lack of mechanistic insight to deepen our understanding of the regulatory mechanism of leaf senescence.
Specific comments:
1. In Fig. 1D, the NS (non-senescent) leaf displays a yellowing phenotype.
2. Although no T-DNA insertion mutants for CDF4 were available in the public seed collections, CRISPR/Cas9-based knockout lines of CDF4 should be generated for the senescence phenotype assays.
3. Given that 35S::CDF4 plants show a serious alteration of development phenotype, thus the effect of CDF4 on leaf senescence is ambiguous. Furthermore, the growth or development of wild type controls is also abnormal (Fig. 4E).
4. EMSA should be performed to test the binding of CDF4 to its targets.
5. Why did the authors generate the DEX-induced transgenic plants CDF4GR and β-estradiol induced lines to analyze the leaf senescence phenotype? What is the difference?
6. In Fig. 3C and 3F as well as Fig.6D and 6E, the numerical value of the ordinate is too large, which impairs the display of experimental results, and needed to be adjusted.
7. The English language of this paper needs to be dramatically improved.

Referee #3:

Review:
MS: "Transcription factor CDF4 promotes leaf senescence and floral organ abscission by regulating abscisic acid and reactive oxygen species pathways in Arabidopsis"

In this study, Xu et al. demonstrated that CDF4, a member of the Dof family of transcription factors, is involved in the regulation of leaf senescence by modulating ABA and ROS levels. Overexpression of CDF4 (under both constitutive and estradiol inducible promoters) resulted in precocious leaf senescence while knocking down the gene (via RNAi and amiRNA technology) delayed leaf senescence. Further studies indicated that the early senescence phenotype of CDF4 overexpression plants was caused by CDF4 stimulation of ABA biosynthesis by directly regulating the expression of NCED1 and NCED2, two ABA biosynthetic genes. Conversely, knocking out

NCED1 and NCED2 was able to suppress the early senescence phenotype of CDF4 overexpression.
Further, CDF4 was shown to regulate H2O2 level by directly and negatively regulating the expression of CAT2. Overexpression of CAT2 suppressed the early senescence phenotype of CDF4 overexpression.
Accumulation of ABA and ROS is a common feature of developmental and stress-induced senescence. Collectively Xu et al data show that CDF4 controls leaf senescence by simultaneously regulating ABA biosynthesis and ROS accumulation.
Furthermore, Xu et al demonstrated a role for CDF4 in the regulation of floral organ abscission and that it regulates the PGAZAT gene.

The data are interesting. However, there are a number of issues that need to be addressed.

• Figure 2: There is an inconsistency between the phenotype shown in Figure 2C and the Chl data presented in Figure 2E. Leaf numbers are not indicated in Figure 2C, but if we follow the general rule for leaf numbering, the third and fourth leaves of 25-day-old vector control and pER8-CDF4 plants look quite yellow (Figure 2C). However, looking at their Chl content (in Figure 2E) those leaves maintain more than 80% Chl content (almost in all the genotypes) at 25 days after planting. The inconsistency must be clarified.
• Figure 3A: ABA level was measured in the third and fourth leaves of 32-day-old transgenic plants after 4 days of Est treatment. It is curious to know how the plants look like (particularly in terms of senescence phenotype) at this stage? Based on the phenotype of the vector control presented in Figure 2C, I assume there should be a visible phenotypic difference among the genotypes, in terms of leaf yellowing or senescence at this stage. If so, differential accumulation of ABA may not be a direct effect of alteration of the CDF4 level.
The authors must provide evidence that the levels of ABA were measured in leaves before showing a significant difference in visible yellowing. Otherwise, no wonder to detect higher ABA in overexpression lines than RNAi lines (simply due to developmental age difference)

• It is known that treatment with ABA induces leaf senescence. Is ABA-induced leaf senescence affected in CDF4 transgenic lines? More importantly, does ABA treatment restore the delayed senescence phenotype of CDF4 knock-down lines?

• Suppressing ABA or ROS level delays leaf senescence. Therefore, it is not surprising to see that the accelerated leaf senescence phenotype of CDF4-overexpression is compromised upon suppressing ABA biosynthesis (in pER8:CDF4/nced2nced3) or enhanced ROS scavenging (SAG12:CDF4/35S:CAT2). Therefore, further experiments are required to conclude that the regulation of senescence by CDF4 is indeed through a specific regulatory function of CDF4 on ABA biosynthesis or ROS accumulation. This includes, for example overexpressing ABA biosynthesis genes in CDF4-RNAi lines or treating those lines with ABA in order to assess the restoration of the phenotype (delayed senescence).

• One of the main mechanisms through which ABA regulates leaf senescence is controlling stomatal movement and thus water loss during leaf senescence. Therefore, measuring stomatal conductance and leaf water loss of CDF4 transgenic plants would provide a more in-depth insight into the role of CDF4 for ABA-mediated regulation of leaf senescence.
• Do pER8:CDF4 and pER8:CDF4-RNAi show any floral abscission phenotype? And if so, does it alter upon knocking down NCED genes (in pER8:CDF4/nced2,nced3)?

• Supplementary Figure S4B: already GFP alone is nuclear-localized, which is well known from many

other experiments (small size of GFP, moving into the nucleus). Here, also GFP-CDF4 goes to the nucleus, seen in the figure. Thus, as both GFP alone and GFP-CDF4 fusion enter the nucleus, there is no evidence that CDF4 has a specific capacity to enter the nucleus.

Minor:

- Figure 1: Lacks statistical analysis and information about replication of experiments (1B)
- Line 214: To me it is unclear on what basis the authors conclude that "the knockdown transgenic lines did not affect the expression of DOF genes obviously". As far as I see, only COG1 (and no other DOF) expression has been checked here. I would therefore replace "Dof" with "COG1"
- Figure 2E: Chlorophyll content is presented as percentage. Please describe what the percentage indicates.
- Quantification of the data presented in Figure 8A is missing.
- Supplementary Figure S8. Asterisks are not shown in the graph while they are described in the legend.

Referee #1:

The authors reported that the DOF family transcription factor CDF4 is a positive regulator of leaf senescence. Knockdown of CDF4 delays leaf senescence, while overexpressing lines display an earlier senescence phenotype. Moreover, the authors found that CDF4 promotes leaf senescence by increasing ABA content through directly regulating NCED2/3, and suppressing ROS scavenging enzyme. However, the senescence-associated phenotypes of the key mutant lines were not evident and the genetic evidence was weak. Although this manuscript provides some new information on the functional studies of a senescence-associated transcriptional regulation, it is lack of mechanistic insight to deepen our understanding of the regulatory mechanism of leaf senescence.

Reply: Thank you very much for your comments, and we have carefully revised the paper according to your opinions.

Specific comments:

1. In Fig. 1D, the NS (non-senescent) leaf displays a yellowing phenotype.

Reply: According to your advice, we repeated the experiment and the phenotype of NS leaf is shown in new Fig 1D.

2. Although no T-DNA insertion mutants for CDF4 were available in the public seed collections, knockout lines of CDF4 should be generated for the senescence phenotype assays.

Reply: Although we did not get the CRISPR/Cas9-based knockout lines until now due to time constraints. We obtained two mutants, CS91480 and CS87649, from the Nottingham Arabidopsis Stock Centre (NASC) mutant repository. It was found that each of them had a single amino acid substitution, at the 90aa and 105aa positions, respectively, in the conserved DOF domain (Appendix Fig S10A). Compared with the wild-type control, leaf senescence was delayed in both mutants (Appendix Fig S10B). We complement the CS91480 mutant line which contains amino acid substitution within the DOF domain, by transforming wild type CDF4 genomic DNA. The mutant leaf senescence phenotype was fully reverted in the complementary line. These results confirm the function of *CDF4* in promoting leaf senescence. The results were provided in Lines 226-232 and Appendix Fig S10.

Appendix Fig S10. [Figures for referees not shown.]

3. Given that 35S::CDF4 plants show a serious alteration of development phenotype, thus the effect of CDF4 on leaf senescence is ambiguous. Furthermore, the growth or development of wild type controls is also abnormal (Fig. 4E).

Reply: The significant leaf senescence phenotype caused by the constitutive overexpression of *CDF4* is accompanied by the plant dwarfing phenotype, which is related to the fact that the

overexpression of *CDF4* inhibits the expression of a series of cell wall elongation factors. Previous reports have also shown that overexpression of many DOF family transcription factors inhibits cell expansion and leads to dwarf plants. This indicates that the phenotype of DOF in inhibiting cell expansion is relatively conservative, while their other biological functions are diverse. Similar to *CDF4*, the transcription factor *JUB1* (a member of the NAC family of transcription factor) strongly inhibits leaf senescence while inhibiting cell expansion (Wu et al., 2012. Reactive Oxygen Species-Responsive NAC Transcription Factor, Regulates Longevity in Arabidopsis. 24(2):482-506). All in all, we came to this conclusion that *CDF4* is an important TF involved in regulating leaf senescence mainly from the following aspects of evidence rather than the 35S::CDF4 phenotype.

1. In 35S::*CDF4* transgenic plants, leaf senescence marker genes were up-regulated by tens to hundreds of times.

2. Induced overexpression of *CDF4* significantly promoted leaf senescence.

3. Knockdown of *CDF4* delays natural and H_2O_2-induced leaf senescence.

4. Two mutants CS91480 and CS87649, which had a single amino acid substitution at the position of the conserved DOF DNA binding domain showed delayed leaf senescence phenotype.

In addition, the growth of wild type control is modified in the new Figure 4E.

4. EMSA should be performed to test the binding of CDF4 to its targets.

Reply: According to your suggestions, we performed EMSA experiment and the data was provided in Appendix Fig S13. These results further confirmed the interaction between CDF4 protein and the selected downstream target genes promoter regions. We have added the results in lines 280-281, 345-346, 397-398 and 645-650 of manuscript.

Appendix Fig S13 [Figures for referees not shown.]

5. Why did the authors generate the DEX-induced transgenic plants CDF4GR and β-estradiol induced lines to analyze the leaf senescence phenotype? What is the difference?

Reply: At the beginning, we obtained these two inducible overexpression systems from different laboratories, and we conducted induction experiments at the same time. We found that both of them could obviously induce the expression of *CDF4* gene. As far as I'm concerned, there's no difference between the two.

6. In Fig. 3C and 3F as well as Fig.6D and 6E, the numerical value of the ordinate is too large, which impairs the display of experimental results, and needed to be adjusted.

Reply: We have changed according to your suggestion.

7. The English language of this paper needs to be dramatically improved.

Reply: According to your advice, the manuscript was revised by an English native speaker to improve the writing.

Referee #3:

MS: "Transcription factor CDF4 promotes leaf senescence and floral organ abscission by

regulating abscisic acid and reactive oxygen species pathways in Arabidopsis" In this study, Xu et al. demonstrated that CDF4, a member of the Dof family of transcription factors, is involved in the regulation of leaf senescence by modulating ABA and ROS levels. Overexpression of CDF4 (under both constitutive and estradiol inducible promoters) resulted in precocious leaf senescence while knocking down the gene (via RNAi and amiRNA technology) delayed leaf senescence. Further studies indicated that the early senescence phenotype of CDF4 overexpression plants was caused by CDF4 stimulation of ABA biosynthesis by directly regulating the expression of NCED1 and NCED2, two ABA biosynthetic genes. Conversely, knocking out NCED1 and NCED2 was able to suppress the early senescence phenotype of CDF4 overexpression. Further, CDF4 was shown to regulate H2O2 level by directly and negatively regulating the expression of CAT2. Overexpression of CAT2 suppressed the early senescence phenotype of CDF4 overexpression. Accumulation of ABA and ROS is a common feature of developmental and stress-induced senescence. Collectively Xu et al data show that CDF4 controls leaf senescence by simultaneously regulating ABA biosynthesis and ROS accumulation. Furthermore, Xu et al demonstrated a role for CDF4 in the regulation of floral organ abscission and that it regulates the PGAZAT gene. The data are interesting. However, there are a number of issues that need to be addressed.

Reply: Thank you very much for your positive comments, and we have carefully revised the paper according to your suggestions.

• Figure 2: There is an inconsistency between the phenotype shown in Figure 2C and the Chl data presented in Figure 2E. Leaf numbers are not indicated in Figure 2C, but if we follow the general rule for leaf numbering, the third and fourth leaves of 25-day-old vector control and pER8-CDF4 plants look quite yellow (Figure 2C). However, looking at their Chl content (in Figure 2E) those leaves maintain more than 80% Chl content (almost in all the genotypes) at 25 days after planting. The inconsistency must be clarified.

Reply: Thank you very much for comments. After checking, it was found that we actually chose the sixth and seventh rosette leaves but not the third and fourth leaves to measure the chlorophyll content. To verify the results, we repeated the induced senescence experiment for three times, and the representative figure and results are shown in new Figure 2C&E.

• Figure 3A: ABA level was measured in the third and fourth leaves of 32-day-old transgenic plants after 4 days of Est. treatment. It is curious to know how the plants look like (particularly in terms of senescence phenotype) at this stage. Based on the phenotype of the vector control presented in Figure 2C, I assume there should be a visible phenotypic difference among the genotypes, in terms of leaf yellowing or senescence at this stage. If so, differential accumulation of ABA may not be a direct effect of alteration of the CDF4 level. The authors must provide evidence that the levels of ABA were measured in leaves before showing a significant difference in visible yellowing. Otherwise, no wonder to detect higher ABA in overexpression lines than RNAi lines (simply due to developmental age difference).

Reply: Thank you for your comments. ABA level was measured in the third and fourth leaves of the 28-day-old transgenic plants after 4 days of β-estradiol treatment. The leaves were green and did not show senescence phenotype at this time. Furthermore, in order to exclude the influence of developmental leaf age, we also selected rosette leaves from 18-day-old plant to conduct the estrogen induction treatment and ABA content measurement. We found that estradiol induction treatment for three days (once every day) also significantly increased ABA content in pER8::*CDF4* plants and down-regulated ABA content in pER8::*CDF4*-RNAi plants. The results are shown in below and new Figure 3A. Therefore, we concluded that *CDF4* positively regulate ABA content.

[Figures for referees not shown.]

• It is known that treatment with ABA induces leaf senescence. Is ABA-induced leaf senescence affected in CDF4 transgenic lines? More importantly, does ABA treatment restore the delayed senescence phenotype of CDF4 knock-down lines?

Reply: Per your advice, we checked the ABA-induced leaf senescence in the *CDF4* transgenic lines. The results showed that compared with the wild-type control, ABA induced senescence was accelerated in detached leaves of *CDF4* overexpressing plants but slowed down in *CDF4* knock-down plants (Appendix Fig 8C). In addition, we found that exogenous ABA treatment partially restored the leaf senescence phenotype of *CDF4* knockdown plants (Appendix Fig 9). Therefore, ABA-induced leaf senescence was also affected in *CDF4* transgenic lines. These results were shown in below and in the manuscript lines 221-223.

Appendix Fig 8C. [Figures for referees not shown.]

• Suppressing ABA or ROS level delays leaf senescence. Therefore, it is not surprising to see that the accelerated leaf senescence phenotype of CDF4-overexpression is compromised upon suppressing ABA biosynthesis (in pER8:CDF4/nced2nced3) or enhanced ROS scavenging (SAG12:CDF4/35S:CAT2). Therefore, further experiments are required to conclude that the regulation of senescence by CDF4 is indeed through a specific regulatory function of CDF4 on ABA biosynthesis or ROS accumulation. This includes, for example overexpressing ABA biosynthesis genes in CDF4-RNAi lines or treating those lines with ABA in order to assess the

restoration of the phenotype (delayed senescence).

Reply: Thank you for your comments. We performed new experiments by treating transgenic pER8:*CDF4* lines with Fluridone (ABA biosynthesis inhibitor) or ROS generation inhibitor DPI (Diphenyleneiodonium) to assess the alteration of the detached leaf senescence phenotype. We found that exogenous Fluridone or DPI treatments delayed the leaf senescence phenotype of *CDF4* overexpressing plants. In addition, we found that ABA treatment partially restored the leaf senescence phenotype of *CDF4* knockdown plants. These results were shown in Appendix Fig S9 and in the manuscript lines 223-226. Therefore, we concluded that the regulation of senescence by *CDF4* is indeed through a specific regulatory function of *CDF4* on ABA biosynthesis and ROS accumulation.

Appendix Fig S9. [Figures for referees not shown.]

•One of the main mechanisms through which ABA regulates leaf senescence is controlling stomatal movement and thus water loss during leaf senescence. Therefore, measuring stomatal conductance and leaf water loss of CDF4 transgenic plants would provide a more in-depth insight into the role of CDF4 for ABA-mediated regulation of leaf senescence.

Reply: Per your advice, in order to provide a more in-depth insight into the role of *CDF4* for ABA-mediated regulation of leaf senescence, we measured the stomatal aperture of vector control and *CDF4* transgenic plants. The results showed that stomatal aperture obviously decreased in

CDF4 overexpression plant leaves and increased in *CDF4* knockdown plants. The change of stomatal aperture may be due to the effect of *CDF4* regulating ABA content in plants. These results indicated that the change of stomatal aperture was involved in *CDF4*-induced leaf senescence. These results are shown in Appendix Fig S20 and in corresponding lines 466-471 and 597-602 in the manuscript.

Appendix Fig S20. [Figures for referees not shown.]

•Do pER8:CDF4 and pER8:CDF4-RNAi shows any floral abscission phenotype? And if so, does it alter upon knocking down NCED genes (in pER8:CDF4/nced2, nced3)?

Reply: As expected, we found that pER8:*CDF4* and pER8:*CDF4*-RNAi shows altered floral abscission phenotype after a period of EST. induction. However, knocking down NCED genes in pER8:*CDF4*/*nced2nced3* cannot significantly affect floral organ abscission progress compare with pER8:*CDF4* plants. We inferred that the phenotype of *CDF4*-regulated floral organ abscission progress may be mainly through the downstream *PG* genes. Therefore, the involvement of *NCED2* and *NCED3*-mediated ABA synthesis in this area may be limited. These results were presented in Appendix Fig S19 and lines 390-392 of the manuscript.

Appendix Figure S19. [Figures for referees not shown.]

•Supplementary Figure S4B: already GFP alone is nuclear-localized, which is well known from many other experiments (small size of GFP, moving into the nucleus). Here, also GFP-CDF4 goes to the nucleus, seen in the figure. Thus, as both GFP alone and GFP-CDF4 fusion enter the nucleus, there is no evidence that CDF4 has a specific capacity to enter the nucleus.

Reply: GFP proteins can be expressed both in the nucleus and the cytoplasm due to their small molecular size. However, CDF4 fusion GFP protein only locates in the nucleus, as shown in our experimental results. Large molecules require a nuclear localization signal (NLS) for translocation into the nucleus. A previous research demonstrates that an atypical bipartite NLS with a 17 amino acid long linker (underlined in the Figure as below) between its flanking basic regions directs *Arabidopsis thaliana* DOF proteins to the cell nucleus (Krebs et al., 2010. A novel bipartite nuclear localization signal with an atypically long linker in DOF transcription factors. Journal of Plant Physiology, 2010, 167(7): 583-586.). The novel bipartite NLS is highly conserved in plant DOF transcription factors. The nuclear localization of *CDF4* is closely related to its function as a transcriptional regulator. These results are included below and lines 234-235 of the manuscript.

[Figures for referees not shown.]

Minor:

• Figure 1: Lacks statistical analysis and information about replication of experiments (1B).

Reply: Per your advice, statistical analysis and information about replication of experiments are added in Fig 1B and the lines 689-690 of Figure legend.

• Line 214: To me it is unclear on what basis the authors conclude that "the knockdown transgenic lines did not affect the expression of DOF genes obviously". As far as I see, only COG1 (and no other DOF) expression has been checked here. I would therefore replace "Dof" with "COG1".

Reply: Changed.

• Figure 2E: Chlorophyll content is presented as percentage. Please describe what the percentage indicates.

Reply: The percentage indicates the chlorophyll content relative to vector control, and we described in the Figure legend.

• Quantification of the data presented in Figure 8A is missing.

Reply: The quantification of the data was added in Appendix Fig S21 as requested.

• Supplementary Figure S8. Asterisks are not shown in the graph while they are described in the legend.

Reply: Per your advice, asterisks are added in Appendix Fig S8B.

Referee 2:

Xu et al. report a potential involvement of the DOF transcription factor CDF4 in the control of senescence in Arabidopsis thaliana. Experimental evidence suggests an effect of CDF4 on ABA and ROS levels. The data seem to show that CDF4 functions as a positive regulator of senescence, as e.g. evidenced by the effect of CDF4 on the expression of senescence-regulated genes such as SAG12 and SAG13. I have a major overall concern: The overexpression of several DOF TFs inhibits cell expansion, and authors mention the point that overexpression of CDF4 inhibits plant growth (by inhibiting cell expansion). The strong growth inhibitory effect on growth of CDF4 is seen in Figure 2A. Although "early senescence" of leaves appears to happen in the CDF4 overexpressors (see Fig. 2), plant/rosette development is strongly affected as well. Therefore, the "early senescence" seen in such lines appears to be a pleiotropic side-effect triggered by the severe effect on shoot development due to CDF4 overexpression. This is an important point to consider for any valid conclusion made about the function of CDF4.

Reply: As you can see, the significant leaf senescence phenotype caused by the constitutive overexpression of *CDF4* is accompanied by the plant dwarfing phenotype, which is related to the fact that the overexpression of *CDF4* inhibits the expression of a series of cell wall elongation factors. Since the constitutive 35S promoter is very strong, it may lead to a series of inevitable secondary phenotypes. Therefore, in order to further reveal the true function of *CDF4* in regulating leaf senescence, we verified the function of CDF4 by characterizing the phenotype of transgenic plants with overexpressing or knocking down *CDF4* gene expression under the control of estradiol inducible promoter and senescence marker gene SAG12 promoter (Fig 7A). At the same time, we obtained the CDF4 point mutants and found that the leaf senescence process was also affected (Appendix Fig S10). All together, we came to the conclusion that *CDF4* is an important TF involved in regulating leaf senescence mainly from the following aspects of evidence rather than just the overexpression of 35S::*CDF4* phenotype. 1. In 35S::*CDF4* transgenic plants, leaf senescence marker genes were up-regulated by tens to hundreds of times. 2. Induced overexpression of *CDF4* under SAG12 promoter or under estradiol induction system significantly promoted leaf senescence; 3. Knockdown of *CDF4* delays natural and H_2O_2-induced leaf senescence. 4. Two mutants CS91480 and CS87649, which contained a single amino acid substitution at the position of the conserved DOF DNA binding domain showed delayed leaf

senescence phenotype. Combined with the above results, we finally confirmed the function of *CDF4* in promoting leaf senescence.

Another important consideration is the following: Like CDF4, the transcription factor JUB1 (to which the authors refer, and which belongs to another class of TFs, namely NACs) inhibits cell expansion while at the same time it strongly inhibits leaf senescence. The obvious question therefore is: while both TFs (CDF4 and JUB1) inhibit cell expansion, CDF4 promotes senescence when overexpressed, while JUB1 inhibits it. Do the authors have any suggestions about how such differences may be achieved in the plant? This should be discussed in the Discussion part of the manuscript.

Reply: Leaves start from leaf primordia and develop into photosynthetic organs through vegetative growth and maturation, which is completed through the coordination of cell division, expansion and differentiation, and finally enter the senescence stage (Lim et al., 2007). Previous study on components of cytokinin and auxin signaling has demonstrated the relevance between leaf growth and senescence. For example, triple mutations of Arabidopsis HISTIDINE KINASE 2 (AHK2), AHK3, and AHK4 result in a smaller leaf size, as a result of reduced cell proliferation and early leaf senescence (Riefler et al., 2006). Cytokinin response factors (CRFs) have been implicated in the control of leaf growth and senescence in Arabidopsis (Raines et al., 2016). In addition, the AUXIN RESPONSE FACTOR 2 (ARF2) mutation enhances leaf growth and retards leaf senescence (Lim et al., 2010). Our knowledge of the interrelationship between early leaf development and senescence is still limited. Here, we identified that *CDF4* mediates cell expansion and senescence during leaf development. As mentioned here, in addition to the positive leaf senescence regulator *CDF4*, *JUB1* is another TF related to leaf senescence. Both of them have the same effect of inhibiting cell extension and regulating the aging phenotype of leaves. However, the difference is that *CDF4* suppresses H_2O_2 scavenging production by inhibiting the expression of *CAT2*, meanwhile promoting the aging process of leaves by upregulating endogenous ABA levels. But *JUB1* overexpression strongly delays senescence, dampens intracellular H_2O_2 levels, and enhances various abiotic stresses by promoting *DREB2A* expression. The growth of plants is accompanied by the increase of cell volume and the change of cell wall rigidity and the cell wall collapses during the late senescence phase of leaf development. Therefore, the regulation of cell

wall plasticity and cell size is closely related to leaf senescence process. Thus, we believe that *CDF4* might provide us with a good opportunity of investigating the mechanisms involved in mediating leaf development and senescence. This result also implies the relationship between plant regulation of leaf senescence and cell size regulation. Per your advice, this discussion paragraph has been added in the discussion part of the manuscript in lines 436-456.

References

Lim, P. O., Kim, H. J., & Hong, G. N. (2007). Leaf senescence - annual review of plant biology, 58(1):115.

Riefler, M., Novak, O., Strnad, M., & Schmu, T. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell, 18(1), 40-54.

Raines T, Shanks C, Cheng C Y, et al. (2016). The cytokinin response factors modulate root and shoot growth and promote leaf senescence in Arabidopsis. Plant Journal, 85(1): 134-147.

Lim, P. O., Lee, I. C., Kim, J., Kim, H. J., Ryu, J. S., & Woo, H. R., et al. (2010). Auxin response factor 2 (*arf2*) plays a major role in regulating auxin-mediated leaf longevity. Journal of Experimental Botany, 61(5), 1419-1430.

In general, authors used extremely long EST induction times to determine effects (e.g. 4 days (!) for the ABA measurements, see lines 246-248). This is surprising as the EST induction system typically works in the hour range in Arabidopsis thaliana. Such long incubation times have the tendency to lead to many downstream and secondary effects. Authors should check the effect of short-term induction of CDF4 on downstream processes to avoid the induction of secondary effects.

Reply: Our estradiol induction assay conducted by spraying the rosette leaves once a day for four consecutive days and then tested the ABA content in the rosette leaves. Furthermore, in order to avoid the secondary effects caused by prolonged induction treatment, we also selected rosette leaves from 21-day-old plant to conduct the estradiol induction assay and ABA content measurement. We found that estradiol induction treatment for three days (once every day) also significantly increased ABA content in pER8::*CDF4* plants and down-regulated ABA content in pER8::*CDF4*-RNAi plants. In addition, we also conducted a short induction time of 2 h and 4 h to detect the downstream target genes expression. It was found that the expression of *NCED2* and *NCED3* was significantly increased by 2 h or 4 h estradiol induction. These results are all shown in below.

A. Measurement of free ABA levels in the third and fourth rosette leaves from 21-day-old transgenic lines with altered *CDF4* expression after estradiol induction for three days (once every day). Values are given as mean ± SD, n=3. *p<0.05 by student's *t* test. Relative expression of (**B**) *NCED2* and (**C**) *NCED3* in 14-day-old pER8::*CDF4* transgenic plants treated with 20 μM β-estradiol or mock treatment for 0, 2, or 4 h. The expression of the corresponding genes in mock-treated plants was set to 1.0. Values are given as mean ± SD, n=3. *p<0.05 by Student's *t* test.

Other points

• In lines 124/125, authors state: ´Among them, CDF4 (AT2G34140) is of particular interest because changing its expression level but not any other cycling DOF family genes can affect leaf senescence.´ However, a reference for this statement is missing. Or do authors indicate that in the following they will demonstrate that CDF4 is a transcription regulator?

Reply: As you're requested, the references were added in line 120 of manuscript.

• Line 234: How long was the EST induction?

Reply: The constitutive CDF4 overexpression lines and wild-type Col-0 rosette leaves were used for this assay.

• Lines 239/240: Authors state ´Compared with the wild type, 1111 genes were downregulated, while 1860 genes were upregulated in 35S::CDF4 rosette leave More importantly, those numbers of repressed or induced genes are likely inappropriate as no repetition of the experiment was performed (line 237: ´so we do not do biological repetition here.´). I am really wondering what the statistical background for the statement of the affected genes is. Considering the not existing statistical support of the statements given, I am also concerned about the gene ontology

enrichment provided in lines 240-243.

Reply: As described in the paper, we did not carry out biological replication due to limited budget, and we screened for the candidate downstream differential genes for subsequent validation and functional analysis experiments. Due to the lack of biological replication, the description of differentially expressed genes lacks the necessary statistical background. Therefore, we have removed this transcriptome data description from the text. However, the lack of such data does not affect the conclusion of the whole article.

Dear Dr. Xu,

Thank you for the submission of your revised manuscript. We have now received the enclosed comments from all referees as well as cross-comments.

As you will see, while the referees acknowledge that the manuscript has been improved, referee 1 points out that a few concerns remain and should be addressed. In their cross-comments, both referees 2 and 3 agree with these points.

The following concerns need to be addressed:

EMSA experiments: competition assays must be added to all experiments.

Figure S10 requires quantification of senescence. Please provide chlorophyll level data and data on the expression of well-known senescence-associated genes (with a suitable number of replicates).

The model needs to be explained better as indicated by the referees.

A few other changes will also be required:

Please send us a conflict of interest statement.

The ORCID ID for Cai is missing. Please add this to your personal profile page in our online manuscript tracking system.

The REFERENCE format lists more than 10 author names and the journal names are not italicised. Please correct. You find the link to our reference style in our guide to authors. Please note that the new link should only be used after the 1st of MAy, the old link can be found below the new link in the guide to authors. We will change our reference style on the 2st of May, hence it is a little confusing at the moment.

Fig 2F is called out after 2D.
Fig 3J+K are called out after 3G.
Fig 7A is called out after 2C.
Fig 7E callout is missing.
Appendix Fig S21 callout is missing. Please correct all.

The APPENDIX file is missing a table of content with page numbers. The tables are incorrectly named as "Table EV3". They should be "Appendix Table S#". Please correct.

Appendix Fig S5B and S12 need to specify "n", error bars and p-values.

I would like to suggest a few changes to the abstract that needs to be written in present tense:

Leaf senescence is a highly complex developmental process that is tightly controlled by multiple layers of regulation. Abscisic acid (ABA) and reactive oxygen species (ROS) are two well-known factors that promote leaf senescence. We show here that the transcription factor CDF4 positively

regulates leaf senescence. Constitutive and inducible overexpression of CDF4 accelerates leaf senescence, while knockdown of CDF4 delays it. CDF4 increases endogenous ABA levels by upregulating the transcription of the ABA biosynthesis genes 9-cis-epoxycarotenoid dioxygenase 2, 3 (NCED2, 3) and suppresses H2O2 scavenging by repressing expression of the catalase2 (CAT2) gene. NCED2, 3 knockout and CAT2 overexpression partially rescue premature leaf senescence caused by CDF4 overexpression. We also show that CDF4 promotes floral organ abscission by regulating [activating or inhibiting? please specify] the polygalacturonase PGAZAT gene. Taken together, we propose that CDF4, ABA and ROS form a tripartite positive feedback loop that connects the upstream signals with the downstream regulatory network executed by ABA- and H2O2-responsive genes during leaf senescence and floral organ abscission.

It is unclear (to me at least) to which upstream signals you refer in the last sentence. Can you please clarify or may be re-write? Thank you.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I am looking forward to receiving a newly revised manuscript as soon as possible. Please let me know if you have any questions or comments.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In comparison with previous version, the revised manuscript has not been greatly improved. Most of the issues have not been fully addressed. The sections of Introduction and Result are still confusing and need to be reorganized. EMSA experiments were carried out to confirm the binding of CDF4 to targets, but lack of the competitive probe experiment groups. The images of newly added leaf senescence phenotypes are not clear, and lack of senescence-associated parameters, such as chlorophyll content and senescence marker genes. In Fig.9, the feedback loop model needs more data to support it. Given that CDF4 expression was induced by ABA and H2O2 treatment (Lines 485-486), solid lines instead of dashed lines should be used. In addition, there is no picture attached to the text, which brings great inconvenience to the review process.

Referee #2:

Authors have improved their manuscript.

There are a few minor points that should be addressed.

• Line 129: should read „GAL4 DNA-binding domain" (instead of GAL4 DN-binding domain)
• Lines 252/253: authors suddenly speak about ´the CDF4GR transgenic plants´, but these plants were not introduced before. Shortly explain, what these are.
• Line 450: authors state that JUB1 ´enhances various abiotic stresses by promoting DREB2A expression´. This statement is not correct; it has been reported that JUB1 enhances stress tolerance and the induction of DREB2A expression supports this.
• Line 598: authors ´The assay was conducted as previously described with slight modifications.´ However, a reference where this assay was described previously is missing. Please add.

Referee #3:

The revised manuscript EMBOR-2019-48967V2 by Xu et al has been improved significantly.

I have no further comment.

Cross-comments from referee 2:

I had a look at the manuscript again. Overall, it looks good. Regarding the comments raised by reviewer 1 I would comment as follows:

• I do not find the organization of the Introduction and Results confusing. For me, those parts are easily followable and also a non-expert of the field should be able to follow them.
• Regarding the EMSA experiments: competition assays are missing in all experiments. In the way the data are presented, authors cannot conclude that CDF4 binds to a specific cis-binding site. Authors should be requested to include data on competitive probes.
• Figure S10 has been newly added to the manuscript. Unfortunately, however, a quantification of senescence is completely missing. Authors should provide chlorophyll level data and data on the expression of well-known senescence-associated genes (with a suitable number of replicates). Otherwise, the data provided in Figure S10 are not convincing.
• Figure 9 shows a model; and like all models, also this one has its limitations. At least, authors should explain (in the legend) what the solid and dashed lines indicate. It must also be explained what arrow- and T-ending lines, respectively, mean (although this might intuitively be clear to the trained reader). Unravelling all the details and intricacies of the model by lab experiments will require much more time, and this perhaps is not needed for the time being. However, a good model (with its legend) should highlight the open points to be investigated in the future. I think, authors should elaborate on this to some extent.

Minor point:
• Lines 280, 345, 397: "EMAS" must be replaced with "EMSA".

Cross-comments from referee 3:

Overall, I think the authors did a good job of addressing both the referee's comments and revising the manuscript. Among the referee #1's comments, I fully agree with two of them, which you perhaps consider before making a final decision.

Appendix Figure S13: It is necessary to perform a competitive binding assay to confirm the specificity of protein binding. EMSA without competitive assay is not conclusive.

Appendix Figure S10: senescence phenotypes must always be quantified, at least by measuring Chl content and the expression of a few senescence marker genes.

Providing more detailed data for the feedback loop model (Figure 9) requires a fair amount of time and several additional experiments and I am not sure if it necessary for this manuscript.

Referee #1:

In comparison with previous version, the revised manuscript has not been greatly improved. Most of the issues have not been fully addressed. The sections of Introduction and Result are still confusing and need to be reorganized. EMSA experiments were carried out to confirm the binding of CDF4 to targets, but lack of the competitive probe experiment groups. The images of newly added leaf senescence phenotypes are not clear, and lack of senescence-associated parameters, such as chlorophyll content and senescence marker genes. In Fig.9, the feedback loop model needs more data to support it. Given that CDF4 expression was induced by ABA and H2O2 treatment (Lines 485-486), solid lines instead of dashed lines should be used. In addition, there is no picture attached to the text, which brings great inconvenience to the review process.

Reply: Thank you for your comments, and we have carefully revised the paper according to your opinions as well as cross-comments. Specially, we performed additional experiments and provided new data in the revised version. The competition assays were added. 5-, 20- or 50-fold excesses of unlabeled cold probes were used in the competition assay to all the EMSA experiments in new Appendix figure S13. The chlorophyll level data and the expression level of the senescence-associated gene *SAG12* in rosette leaves at various development stages were added in

new Appendix figure S10. We have also added the explanation paragraph of the model in lines 835-837 of manuscript. I hope you are satisfied with the results.

Referee #2:

Authors have improved their manuscript.

Reply: Thanks!

There are a few minor points that should be addressed.

• Line 129: should read„GAL4 DNA-binding domain" (instead of GAL4 DN-binding domain)

Reply: Corrected.

• Lines 252/253: authors suddenly speak about ´the CDF4GR transgenic plants´, but these plants were not introduced before. Shortly explain, what these are.

Reply: The explanation of the paragraph is as follows and in lines 246-248:

"By using the glucocorticoid-mediated transcriptional induction system in transgenic plants, we wanted to determine whether *NCED2* and *NCED3* were immediately downstream of *CDF4*."

• Line 450: authors state that JUB1 ´enhances various abiotic stresses by promoting DREB2A expression´. This statement is not correct; it has been reported that JUB1 enhances stress tolerance and the induction of DREB2A expression supports this.

Reply: Corrected.

• Line 598: authors ´The assay was conducted as previously described with slight modifications.´ However, a reference where this assay was described previously is missing. Please add.

Reply: Added.

Referee #3:

The revised manuscript EMBOR-2019-48967V2 by Xu et al has been improved significantly.

I have no further comment.

Reply: Thank you for your positive comments.

Cross-comments from referee 2:

I had a look at the manuscript again. Overall, it looks good. Regarding the comments raised by reviewer 1 I would comment as follows:

• I do not find the organization of the Introduction and Results confusing. For me, those parts are easily followable and also a non-expert of the field should be able to follow them.

Reply: Thanks!

• Regarding the EMSA experiments: competition assays are missing in all experiments. In the way the data are presented, authors cannot conclude that CDF4 binds to a specific cis-binding site. Authors should be requested to include data on competitive probes.

Reply: The competition assays were added. 5-, 20- or 50-fold excesses of unlabeled cold probes were used in the competition assay to all the EMSA experiments. These results further confirmed the interaction between CDF4 protein and the selected downstream target genes promoter regions. We have added the results in new Appendix figure S13.

• Figure S10 has been newly added to the manuscript. Unfortunately, however, a quantification of senescence is completely missing. Authors should provide chlorophyll level data and data on the expression of well-known senescence-associated genes (with a suitable number of replicates). Otherwise, the data provided in Figure S10 are not convincing.

Reply: The chlorophyll level data and the expression level of the senescence-associated gene *SAG12* in rosette leaves at various development stages were added. Three independent experiments were conducted. We have added the results in new Appendix figure S10.

• Figure 9 shows a model; and like all models, also this one has its limitations. At least, authors should explain (in the legend) what the solid and dashed lines indicate. It must also be explained what arrow- and T-ending lines, respectively, mean (although this might intuitively be clear to the trained reader). Unravelling all the details and intricacies of the model by lab experiments will require much more time, and this perhaps is not needed for the time being. However, a good model (with its legend) should highlight the open points to be investigated in the future. I think, authors should elaborate on this to some extent.

Reply: I quite agree with your point of view, and I have made modifications and supplements according to your requirements. We have added the following explanation paragraph in lines 835-837 of manuscript:

"In the legend, the solid and dotted lines represent the direct interaction obtained in this research and indirect interaction, respectively. And the arrow- and T-ending lines represent positive and negative regulatory pathways, respectively".

Minor point:

• Lines 280, 345, 397: "EMAS" must be replaced with "EMSA".

Reply: Corrected.

Cross-comments from referee 3:

Overall, I think the authors did a good job of addressing both the referee's comments and revising the manuscript. Among the referee #1's comments, I fully agree with two of them, which you perhaps consider before making a final decision.

Reply: Thanks, and we have carefully revised the paper according to your opinions.

Appendix Figure S13: It is necessary to perform a competitive binding assay to confirm the specificity of protein binding. EMSA without competitive assay is not conclusive.

Reply: Per your advice, the competition assays were added. 5-, 20- or 50-fold excesses of unlabeled cold probes were used in the competition assay to all the EMSA experiments. We have added the results in new Appendix figure S13.

Appendix Figure S10: senescence phenotypes must always be quantified, at least by measuring Chl content and the expression of a few senescence marker genes.

Reply: The chlorophyll level data and the expression level of the senescence-associated gene *SAG12* in rosette leaves at various development stages were added in the new Appendix figure S10. Three independent experiments were conducted.

Providing more detailed data for the feedback loop model (Figure 9) requires a fair amount of time and several additional experiments and I am not sure if it necessary for this manuscript.

Reply: I quite agree with you, and in order to illustrate our model more clearly. We have added the following explanation paragraph in lines 835-837 of manuscript:

“In the legend, the solid and dotted lines represent the direct interaction obtained in this research and indirect interaction, respectively. And the arrow- and T-ending lines represent positive and negative regulatory pathways, respectively”.

2nd Revision - Editorial Decision 30th Apr 2020

Dr. peipei Xu
sippe
Plant and environment interaction
fenglin road 300
Shanghai, xuhui district 200032
China

Dear Dr. Xu,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Esther

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Corresponding Author Name: Peipei Xu
Journal Submitted to: Embo Report
Manuscript Number: EMBOR-2019-48967

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We got sixteen independence transgenic lines for 35S::CDF4 plants；more than 3 independent lines for pER8::CDF4，pER8::CDF4-RNAi, pER8::amiCDF4 transgenic plants； two independent CS91480 and CS87649 mutants for CDF4; Five independence transgenic lines for 35S::CAT2 plants.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, also see the figure legend described.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, Student's t-test or ANOVA analysis with GraphPad8.0 was carried out, and differences were considered significant when $p<0.05$. The values represented as means ± standard deviation (SD).
Is there an estimate of variation within each group of data?	Yes

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	anti-HA polyclonal antibodies (Roche:118674231)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No data requred deposited in a public database
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20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No