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Transcription factor ANAC032 modulates JA/SA signalling in response to *Pseudomonas syringae* infection

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 March 2016

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. I am sorry for the slight delay in getting back to you. We have now reeived the full set of referee reports, pasted below, as well as referee cross-comments.

As you will see, the referees acknowledge that the findings are potentially interesting. However, referee 1 points out that the experiments need to be much better described, that statistics must be calculated and that known links between SA and JA signaling and known downstream defense mechanisms need to be integrated into the model. On a similar note, referee 3 remarks that it is unclear what the relevance of ANAC032 is in relation to several other transcription factors known to regulate SA-JA signaling. Both referess also note that the ChIP assay needs to be strengthened, and that the discussion should be more focused. On the other hand, that only one species of Pseudomonas is used in this study is not a concern.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board.

REFEREE REPORTS

Referee #1:

The authors have worked on analyzing the role of ANAC032 in the interaction of Arabidopsis with Pseudomonas syringae and show that there are opposing effects on isolated pre-selected parts of the SA/JA defense system. I am unable to fully assess the manuscript as the experimental and statistical details are largely missing. Further, while this is an interesting beginning, the model proposed does not fully integrate the known mechanistic links between SA and JA, nor does it contemplate other JA and SA regulated defenses that may be more important than the ones measured.

I have significant difficulty in interpreting any of the experiments as there is little to no description of the experimental design and/or replication. For the qRT-PCR all the authors state is that n = 3. Are these three technical replicates of the same sample, three leaves from the same plant, three plants in the same pot or plants across three independent experiments?

For the pathogen assays, there is no description of how the authors combined the data from the two separate experiments. A t-test does not allow for this.

In Figure 2E, how was the statistics done on this? Was there a False-discovery rate adjustment to the statistics? Additionally, the scale doesn't make sense as the maximal values are + and - 1, which means that there were no changes greater than two-fold?

There is no statistics in Figure 2D, this is a classical Chi-square test and easy to do.

In Figure 3E, the negative control had absolutely no signal? This is unusual for ChIP-qPCR as there is usually at least a background level. The authors should address this.

In Figure 6, the model is not accurate for the role of SA and JA in defense against Pst. JA induces the expression of key indolic compounds that are central to the defense response such as camalexin and the like and as such it is possible that these compounds are as important if not more important to the ANAC032 role than the stomatal opening. Similarly, there are other known connections between the SA and JA pathways that are not incorporated in the mechanistic model. Given this and other over-simplifications in the model, the current manuscript seems over-interpreted as the authors routinely give the impression that this model is the complete explanation.

A further over-simplification is that the authors have only utilized one species of Pseudomonas for their experiment yet they make claims that this is informative about how SA/JA works in all environments. The inputs are known to be highly conditional and as such, the current evidence only allows the authors to talk about SA/JA crosstalk in response to Pseudomonas syringae.

Referee #2:

Comments for the editor:
This is a solid and convincing study that identifies a novel regulator of plant defense signaling pathways.

Comments for the authors:
This study identifies ANAC032 as a critical regulator of SA and JA pathways. It convincingly shows that ANAC032 binds to the promoter of MYC2 and PDF1.2. Supported by expression data, this result suggests that ANAC032 inhibits the expression of JA-related genes. However, that ANAC032 inhibits MYC2 transcriptional activity by binding to its promoter is not experimentally demonstrated.
The authors aslo show that ANAC032 binds to the promoter of NIMIN1, which is a negative regulator of the SA pathway. Consequently, ANAC032 is shown to enhance expression of SA-regulated genes and positively control resistance to the biotroph pathogen Pseudomonas syringae. Finally, COR-mediated reopening of stomata is also negatively controlled by ANAC032.

This is a novel and interesting study that reveals a novel player in the tightly controlled SA and JA defense pathways. Experiments are convincing and conclusions are supported by the data.

Given the observation that ANAC032 expression is induced by MeJA treatment, there should be some discussion on the relevance of such finding with regard to herbivory. Since herbivores are known to trigger JA-Ile accumulation, ANAC032 induction would negatively impact defense against these attackers by inhibiting MYC2 transcriptional activity. Seems counterintuitive to me.

The Discussion could be shortened: there are unnecessary repeats of the Results section.

MInor points:
L182 NIMIN1 expression is reduced in 35S::ANAC032 and not enhanced.
L222: Fig2E should read Fig3E
L275: Fig3D should read Fig5E

Referee #3:

The ms. of Allu et al. presents some convincing data that the transcription factor ANAC032 directly represses the transcription of three quite different genes to repress some JA-dependent responses and to activate certain SA-dependent responses. It therefore proposes a central function of ANAC032 in plant immune responses and is of interest to a range of plant and microbial researchers.

General comments
1- The term 'crosstalk' (or cross-communication) is confusing. It should be removed or replaced if necessary with the term 'interplay' for at least 2 reasons. 1st, 'crosstalk' was coined in electronics as any phenomenon by which a signal transmitted IN one circuit (read IN a signaling pathway) creates an undesired effect IN another circuit. Such 'crosstalk' in biological systems is therefore generally suppressed, and mechanistic examples of direct circuit or pathway interactions, as opposed to feedbacks, are rare in cells (Mundy et al. 2006 TIPS 11, 63-4). In contrast, 'interplay' implies a reciprocal relationship, action or influence, which is what most authors hint at when they say 'crosstalk'. 2nd, the following statement (p6, line 185) exemplifies vague, indeed mistaken uses of 'crosstalk': 'Activation of SA-responsive defence genes and concomitant suppression of JA-responsive genes suggest that ANAC032 modulates the crosstalk between SA- and JA-dependent defence signalling in the response to Pst'. If ANAC032 directly regulates the transcription of NIMIN1, MYC2 and PDF1.2, where does the crosstalk mechanistically occur IN their signaling pathways? In fact it probably doesn't, which makes ANAC032 all the more interesting.

2- The ms would benefit from editing throughout and the discussion should be more focused and shorter. The authors may find two standard guides useful for this and future manuscripts:
http://www.amazon.com/The-Elements-Style-William-Strunk/dp/1557427283;
http://www.orwell.ru/library/essays/politics/english/e_polit/.
For starters, the abstract could begin: Plant immune responses to pathogens, including host transcriptional reprograming, require partially antagonistic signaling pathways dependent on the phytohormones salicylic (SA) and jasmonic (JA) acids. However, upstream factors modulating the interplay of these pathways are not well characterized.

3- The clinching ChIP experiment, building upon the binding site selection, appears somewhat convincing but a) it is unclear from the supplemental data or methods which promoter regions/sequences were amplified, and b) the negative control promoter is that of MPK9, a putative positive regulator of ABA signaling in guard cells (Jammes 2009 PNAS 106, 20520), which seems like a potentially biased choice. In addition, ChIP in 35S over-expression lines, rather than in lines complemented with the native promoter, are always iffy unless supported by transient expression assays with a target promoter reporter (MYC, NIMIN and/or PDF::GUS with internal 35S::LUC transfection control) and overexpression of the factor (35S::ANAC032-GST vs. 35S::GST control) in the anac032-1 knockout background.

Specific comments
- Discussion should be shorter and more focused.
- It should be made clearer why ANAC032 is important as one of several known TF regulators of SA-JA signaling.

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- ANAC032, as well as other NACs, have previously been shown to be JA- and Et-inducible, and this work should therefore be cited: Nakano et al. (2006) J Plant Res. 119, 407-13.
- Is the proposed function of ANAC032 in immunity related to its apparent function in ABA-dependent leaf senescence (Takasaki et al. 2015 Plant J. 84, 1114)?
- How were the 123 defense/stress-related genes selected, and were any other relevant genes omitted by this selection?
- Does the anac032-1 knockout have any discernable mutant phenotypes vs. wild type when grown under control conditions?
- What effect did 35S::ANAC032 have on PDF1.2 repression in Fig. 4C?

1st Revision - authors' response 14 July 2016

Response to Editor:

Thanks a lot for giving us the opportunity to submit a revised version of our manuscript (EMBOR-2016-42197V1). We greatly appreciate the thorough assessment of our article and believe that the constructive criticism made by all the three reviewers has led to a significant improvement of our presentation. We responded to each of their specific comments and recommendations. This includes:

1- More detailed description of experiments (e.g. number of replications and statistical tests) are now provided in the Figure legends and Materials and Methods.

2- In Figure 6 (model), we added other known connections between the SA and JA pathways including WRKY TFs (WRKY70, 50, 51 …) and their effect on SA- and JA-dependent defense signaling, the negative regulation of PDF1.2A by TGAs (TGA2, 5, 6)/GRX480, and the negative effect of ANAC055, 019 and 072 on SA biosynthesis.

3- We have considerably shortened and at the same time re-structured the Discussion so that it is more focused now (additions are shown in BLUE). We also tried to emphasize the importance of ANAC032 next to several other known TF regulators of SA-JA signaling (to address the comment raised by reviewer 3).

4- We performed new ChIP experiments using lines expressing ANAC032-GFP fusion protein from the native *ANAC032* native promoter in *anac032-1* plants. Furthermore, we changed the negative control primers to *ACTIN7* (AT5G09810), as used in many papers (e.g. Ng et al., Plant Cell, 2011). Figure 3C-E has been replaced with the new ChIP data. Primer sequences for *ACTIN7* are added to Table EV1.

Few additional points:
5- In the previous version, we missed to include the information about the total number of *UGT* genes (15 genes) tested by qRT-PCR in *ANAC032* transgenic and WT upon *Pst* infection. We now provided this information and added their primer sequences in Table EV1.

6- Figure EV2E is newly added (*ANAC032prom-ANAC032-GFP* plant lines showing expression of *ANAC032* compared to wild-type (WT) plants upon treatment with *Pst* for 6 h).

Referee #1:

The authors have worked on analyzing the role of ANAC032 in the interaction of Arabidopsis with Pseudomonas syringae and show that there are opposing effects on isolated pre-selected parts of the SA/JA defense system. I am unable to fully assess the manuscript as the experimental and statistical details are largely missing. Further, while this is an interesting beginning, the model proposed does not fully integrate the known mechanistic links between SA and JA, nor does it contemplate other JA and SA regulated defenses that may be more important than the ones measured.

I have significant difficulty in interpreting any of the experiments as there is little to no description of the experimental design and/or replication.

RESPONSE: We now provide details of the experimental procedures in the revised manuscript.

-For the qRT-PCR all the authors state is that n = 3. Are these three technical replicates of the same sample, three leaves from the same plant, three plants in the same pot or plants across three independent experiments?

RESPONSE: For all qRT-PCR experiments (expression analysis and ChIP), three independent biological repeats were performed. More detailed information is now added to the Figure legends and Material and Methods part of the revised manuscript.

For the pathogen assays, there is no description of how the authors combined the data from the two separate experiments. A t-test does not allow for this.

RESPONSE: For pathogen assays, two independent experiments were performed, each with three biological replications per experiment, providing 6 measurements in total. In each replicate, three plants grown in individual pots were pooled for analysis. We now added this information to the legends of Figures 2B and EV3C.

In Figure 2E, how was the statistics done on this? Was there a False-discovery rate adjustment to the statistics? Additionally, the scale doesn't make sense as the maximal values are + and -1, which means that there were no changes greater than two-fold?

RESPONSE: As it was indicated in the Figure legend 2E, the heat map shows fold change (log2 basis). We now added this information to the Figure 2E. We also added asterisks indicating significant differences from WT plants (Student's t test, $p \leq 0.05$) and the corresponding description to the legend of Figure 2E.

There is no statistics in Figure 2D, this is a classical Chi-square test and easy to do.

RESPONSE: The reviewer is right. We performed a Chi-square test for the data shown in Figure 2D, EV3B and EV5B and added the corresponding information to the figures and their legends.

In Figure 3E, the negative control had absolutely no signal? This is unusual for ChIP-qPCR as there is usually at least a background level. The authors should address this.

RESPONSE: We performed new ChIP experiment using lines expressing ANAC032-GFP fusion protein under the control of the native ANAC032 promoter in anac032-1 plants. Furthermore, following the comment of Reviewer 3, we changed the negative control primers to ACTIN 7 (AT5G09810). Please see Figure 3C-E of our revised manuscript for the new data.

In Figure 6, the model is not accurate for the role of SA and JA in defense against Pst. JA induces the expression of key indolic compounds that are central to the defense response such as camalexin and the like and as such it is possible that these compounds are as important if not more important to the ANAC032 role than the stomatal opening. Similarly, there are other known connections between the SA and JA pathways that are not incorporated in the mechanistic model. Given this and other over-simplifications in the model, the current manuscript seems over-interpreted as the authors routinely give the impression that this model is the complete explanation.

RESPONSE: Following the comment of the reviewer, we now added other known connections between the SA and JA pathways including WRKY TFs (WRKY70, 50 and 51) and their effect on SA- and JA-dependent defense signaling, the negative regulation of PDF1.2A by TGAs (TGA2, 5, 6)/GRX480 and the negative effect of ANAC055, 019 and 072 on SA biosynthesis in our model (revised Figure 6).

With respect to the camalexin pathway: PAD3 (CYP71B15), the key enzyme that converts indole-3-acetaldoxime (IAOx) to camalexin, was among the 123 defense-/stress-related genes (Table EV1) whose expression was checked in 6-weekold ANAC032 transgenic and WT plants after Pst infection by qRT-PCR. Expression of PAD3 was not altered by ANAC032. We now checked expression of further camalexin biosynthesis genes (CYP71A12, CYP71A13) and their upstream TFs (MYB51, MYB122, MYB34) in 6-week-old ANAC032 transgenic and WT plants after Pst infection (at 6 hpi) by qRT-PCR. There is no difference in their expression levels compared to WT indicating that

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ANAC032 is not involved in the transcriptional regulation of camalexin biosynthesis genes. Therefore, we do not include it in our model (Figure 6).

In addition, camalexin is thought to be more important for the resistance to necrotrophic fungal pathogens, such as Alternaria brassicicola and Botrytis cinerea, while it does not contribute substantially to resistance to the hemibiotrophic bacterium P. syringae (Glazebrook and Ausubel, 1994). In addition, camalexin biosynthesis is highly complex. JA has both positive and negative effects on camalexin biosynthesis. Although JA induces the expression of key indolic compounds, it has been shown that in Arabidopsis roots, coronatine, which is a JA-Ile analog, negatively regulates camalexin biosynthesis via the MYC2 TF (Millet et al., 2010). SA has been also found to be necessary for the accumulation of camalexin (Zhao and Last, 1996; Zhou et al., 1998).

A further over-simplification is that the authors have only utilized one species of Pseudomonas for their experiment yet they make claims that this is informative about how SA/JA works in all environments. The inputs are known to be highly conditional and as such, the current evidence only allows the authors to talk about SA/JA crosstalk in response to Pseudomonas syringae.

RESPONSE: We did not claim that ANAC032 regulates crosstalk/interplay between SA and JA in all environments. As also indicated in the title of our manuscript, our current data confirm an involvement of ANAC032 in the regulation of the SA/JA interplay in the response to Pseudomonas syringae infection. However, to be more precise and following the reviewer's comment, we now added the term ''in response to Pseudomonas syringae'' whenever we speak about an involvement of ANAC032 in the SA/JA interplay.

Referee #2:

Comments for the editor: This is a solid and convincing study that identifies a novel regulator of plant defense signaling pathways.

Comments for the authors: This study identifies ANAC032 as a critical regulator of SA and JA pathways. It convincingly shows that ANAC032 binds to the promoter of MYC2 and PDF1.2. Supported by expression data, this result suggests that ANAC032 inhibits the expression of JA-related genes. However, that ANAC032 inhibits MYC2 transcriptional activity by binding to its promoter is not experimentally demonstrated. The authors aslo show that ANAC032 binds to the promoter of NIMIN1, which is a negative regulator of the SA pathway. Consequently, ANAC032 is shown to enhance expression of SA-regulated genes and positively control resistance to the biotroph pathogen Pseudomonas syringae. Finally, COR-mediated reopening of stomata is also negatively controlled by ANAC032.

This is a novel and interesting study that reveals a novel player in the tightly controlled SA and JA defense pathways. Experiments are convincing and conclusions are supported by the data.
Given the observation that ANAC032 expression is induced by MeJA treatment, there should be some discussion on the relevance of such finding with regard to herbivory. Since herbivores are known to trigger JA-Ile accumulation, ANAC032 induction would negatively impact defense against these attackers by inhibiting MYC2 transcriptional activity. Seems counterintuitive to me.

RESPONSE: JA-dependent defense signaling pathways are often key in plant responses to herbivores. ANAC032 expression is induced by JA, but in turn it represses the expression of MYC2, a key TF regulating the expression of JA-responsive genes. Thus, ANAC032 might function as a negative regulator of plant responses to herbivore attack, forming a negative feedback loop in herbivore defense/JA signaling. However, as phytohormone signaling pathways are highly complex and plant responses to pathogens with different life styles are divergent, it is currently not possible to predict the actual role of ANAC032 in the response of plants to insect/herbivore attacks; unravelling this is an interesting aspect for future studies. We now added a short statement on this to the Discussion (lines 362-366).

The Discussion could be shortened: there are unnecessary repeats of the Results section.
RESPONSE: We considerably shortened the discussion.

MInor points:

L182 NIMIN1 expression is reduced in 35S::ANAC032 and not enhanced.
RESPONSE: Thanks to the reviewer for pointing this out. We corrected it.

L222: Fig2E should read Fig3E
RESPONSE: corrected.

L275: Fig3D should read Fig5E
RESPONSE: corrected.

Referee #3:
The ms. of Allu et al. presents some convincing data that the transcription factor ANAC032 directly represses the transcription of three quite different genes to repress some JA-dependent responses and to activate certain SA-dependent responses. It therefore proposes a central function of ANAC032 in plant immune responses and is of interest to a range of plant and microbial researchers.

General comments
1-The term 'crosstalk' (or cross-communication) is confusing. It should be removed or replaced if necessary with the term 'interplay' for at least 2 reasons. 1st, 'crosstalk' was coined in electronics as any phenomenon by which a signal transmitted IN one circuit (read IN a signaling pathway) creates an undesired effect IN another circuit. Such 'crosstalk' in biological systems is therefore generally suppressed, and mechanistic examples of direct circuit or pathway interactions, as opposed to feedbacks, are rare in cells (Mundy et al. 2006 TIPS 11, 63-4). In contrast, 'interplay' implies a reciprocal relationship, action or influence, which is what most authors hint at when they say 'crosstalk'. 2nd, the following statement (p6, line 185) exemplifies vague, indeed mistaken uses of 'crosstalk': 'Activation of SA-responsive defence genes and concomitant suppression of JA-responsive genes suggest that ANAC032 modulates the crosstalk between SA-and JA-dependent defence signaling in the response to Pst'. If ANAC032 directly regulates the transcription of NIMIN1, MYC2 and PDF1.2, where does the crosstalk mechanistically occur IN their signaling pathways? In fact it probably doesn't, which makes ANAC032 all the more interesting.

RESPONSE: We very much thank the reviewer for pointing this out to us. We have now replaced 'crosstalk' (or cross-communication) with interplay.
2-The ms would benefit from editing throughout and the discussion should be more focused and shorter. The authors may find two standard guides useful for this and future manuscripts:
http://www.amazon.com/The-Elements-Style-William-Strunk/dp/1557427283;
http://www.orwell.ru/library/essays/politics/english/e_polit/.

RESPONSE: We edited the manuscript throughout and considerably shortened the discussion

For starters, the abstract could begin: Plant immune responses to pathogens, including host transcriptional reprograming, require partially antagonistic signaling pathways dependent on the phytohormones salicylic (SA) and jasmonic (JA) acids. However, upstream factors modulating the interplay of these pathways are not well characterized.

RESPONSE: We thank the reviewer for the comment. We included the suggested sentences in the Abstract.

3-The clinching ChIP experiment, building upon the binding site selection, appears somewhat convincing but a) it is unclear from the supplemental data or methods which promoter regions/sequences were amplified, and b) the negative control promoter is that of MPK9, a putative positive regulator of ABA signaling in guard cells (Jammes 2009 PNAS 106, 20520), which seems like a potentially biased choice. In addition, ChIP in 35S over-expression lines, rather than in lines complemented with the native promoter, are always iffy unless supported by transient expression assays with a target promoter reporter (MYC, NIMIN and/or PDF::GUS with internal 35S::LUC transfection control) and overexpression of the factor (35S::ANAC032-GST vs. 35S::GST control) in the anac032-1 knockout background.

RESPONSE: We agree with the reviewer. We performed new ChIP experiments using lines expressing ANAC032-GFP fusion protein from the native ANAC032 native promoter in anac032-1

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plants. Furthermore, we changed the negative control primers to ACTIN7 (AT5G09810), as used in other studies (e.g. Ng et al., Plant Cell, 2011). Please see Figure 3C-E of our revised manuscript for the new data.

-Discussion should be shorter and more focused.
RESPONSE: We considerably shortened the discussion and restructured it to make it more focused.

-It should be made clearer why ANAC032 is important as one of several known TF regulators of SAJA signaling.

RESPONSE: A key finding of our research is that ANAC032 enhances salicylic acid (SA) but decreases jasmonic acid (JA) signalling thereby shifting the plant´s response activity towards biotrophic over necrotrophic invaders. ANAC032 exerts its function by direct transcriptional regulation of core pathogen response genes and key elements in JA signaling (MYC2 and PDF1.2A) and SA signalling (NIMIN1). This we now included in lines 307-310 of the Discussion. Several other TFs including e.g. WRKY70, WRKY50, and WRKY 51 have been identified as regulators acting in the SA-JA interplay (Mao et al., 2007; Gao et al., 2011), however, details on their regulatory networks and their direct target genes are currently not available. This information we now added to the Discussion (lines 378-382).

ANAC032 is the first TF identified to directly suppress the transcription of NIMIN1, a negative regulator of the NPR1-TGA-PR1 module. This information is given in lines 319-322 of the Discussion.

We now shortened the discussion and tried to more highlight the importance of ANAC032 as a regulator of the interplay between SA-and JA-dependent defense signalling.

-ANAC032, as well as other NACs, have previously been shown to be JA-and Et-inducible, and this work should therefore be cited: Nakano et al. (2006) J Plant Res. 119, 407-13.
RESPONSE: We now cite Nakano et al. (2006) in line 119 of the revised manuscript; reference number 40. We changed all other reference numbers accordingly.

-Is the proposed function of ANAC032 in immunity related to its apparent function in ABA-dependent leaf senescence (Takasaki et al. 2015 Plant J. 84, 1114)?

RESPONSE: Based on results shown in Takasaki et al. (2015), ANAC032 does not play a prominent role in mediating ABA signaling during senescence. As shown in Figure 2 of the paper, the anac032 single mutant did not alter ABA-induced senescence phenotype compared to WT. Furthermore, as shown in Figure 2C of the paper, the septuple SNAC-A mutant plants (lacking 7 NACs including ANAC032) showed the same delay in senescence as the sextuple mutant (containing functional ANAC032), again indicating that ANAC032 is not involved in ABA-stimulated senescence. This observation correlates with our data indicating that ANAC032 is not a limiting factor in mediating ABA-dependent signaling in response to Pst.

-How were the 123 defense/stress-related genes selected, and were any other relevant genes omitted by this selection?

RESPONSE: The genes included in this study were extracted through literature mining comprising several of the previous studies carried on understanding the plant defense response against the invading pathogens, in particular Pseudomonas syringae (Beckers and Spoel, 2006; Robert-Seilaniantz et al., 2011; Sendon et al., 2011; Gimenez-Ibanez et al., 2013; Van der Does et al., 2013). We aimed to have a comprehensive list of genes that were known to be involved in the defense network. We tried our best to include the genes that have been shown to be more relevant for the defense pathways acting in response to Pst. We added a statement about this and several references to the manuscript text (lines 157-160).

-Does the anac032-1 knockout have any discernable mutant phenotypes vs. wild type when grown under control conditions?
RESPONSE: Under the conditions used in the current study, no significant morphological differences were noticed between WT and anac032-1 knockout plants.

-What effect did 35S::ANAC032 have on PDF1.2 repression in Fig. 4C?
RESPONSE: Upon SA treatment, expression of PDF1.2 was slightly (but not significantly) more reduced in 35S:ANAC032 than in WT seedlings. We now included the data for expression of PDF1.2A in 35S:ANAC032 seedlings in the Figure 4C and added a sentence to the text (lines 262-264).

2nd Editorial Decision 27 July 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the comments from the referees that were asked to assess it, and both in principle support the publicaiton of your work now.

Referee 1 has a few more comments that all need to be addressed. Please carefully check and remove all overinterpretations.

At the moment it is unclear whether n stands for the number of independently performed experiments or technical replicates performed in parallel; this needs to be clarified in the figure legends. N should be used for the number of independently performed experiments, and I suggest to use this term instead to avoid confusion. Error bars can only be shown if at least 3 independent experiments were performed. In principle no data should be shown for a single experiment only. Fig 2B and EV3C cannot show error bars or calculate p-values if only 2 independent experiments were performed. Please repeat the experiement one more time or show all data points of both experiments along with their mean and remove the error bars and p-value.

REFEREE REPORTS

Referee #1:

The authors have improved the description of materials and methods. This however raises a few relatively minor questions.

Is it journal policy to allow experiments to only be done once? For example, Figure 1 shows only single experiments with three internal bio-replicates.

In Figure 3 D/E, why did the authors not do statistical analysis? The authors nicely did independent biological replication on the CHiP. I understand that the CHiP community hasn't enforced the use of statistics but this is essentially like qPCR data and can be analyzed similarly.

I still have difficulty with the legend in Figure 2E, I agree with the authors that this is in a log2 but the axis on the legend says that the maximal change is +1 log 2 which is only a 2 x change and the minimal is a -1 log 2. I find it hard to believe that the maximal change is +1 log2 in a pathogen experiment but this is what the figure legend is explicitly stating. While this might seem a minor point, it will be an issue in the future when people read the paper and ascertain what they do and do not believe. Especially as ANAC032 showed +1 log 2 changes in Figure 1.

I still feel that the authors are over-interpreting in parts. For example, the title for Figure 6 ends with "upon pathogen attack" but as the authors point out, this model would not work for necrotrophic pathogens as there is no camalexin. As such, I would urge the authors to better caveat with the specific Pseudomonas syringae DC3000 rather than slipping into the imprecise and potentially incorrect usage of the general term "pathogen". This may seem an over-reach but even if the authors went to a maculicula strain of Pst they would then have to bring in the sax loci and associated plant defense compounds that are regulated by myc2 which do not play a role in resistance against DC3000 (Fan et al., 2011). So there is already existing literature saying that using the general "pathogen" is incorrect and inaccurate and even "Pseudomonas" is incorrect.

Fan, J., Crooks, C., Creissen, G., Hill, L., Fairhurst, S., Doerner, P., and Lamb, C. (2011).

Pseudomonas sax Genes Overcome Aliphatic Isothiocyanate-Mediated Non-Host Resistance in Arabidopsis. Science 331, 1185-1188.

Referee #3:

I have examined the revised ms and think that the authors have met most of the important issues raised by the reviewers.

2nd Revision - authors' response 10 August 2016

Response to Editor:

I am herewith submitting the revised version of our manuscript (EMBOR-2016-42197V2). We addressed all the comments given by you and referee 1.

1- We removed all over-interpretations. This includes changing the title of Figure 6 from "upon pathogen attack" to "in response to *Pst* DC3000", and the removal of the last sentence in the chapter "Model for ANAC032 action".

2- With respect to number of experiments, we tried to make it clear in the figure legends and used "n" to describe the number of independent experiments.

3- In Figures 2B and EV3C, We changed the graph so that we removed the error bars and show the data points of the two individual experiments along with their mean.

4- With respect to Figures EV2C,D,E, here we performed single experiments to identify transgenic lines. Technically, this experiment cannot be repeated with different plants or experiments as all transgenic lines at this stage (T0) are individuals and genetically different from each other. To this end, leaves of individual T0 plants are harvested for gene expression analysis. We now added this information to the figure legend. For all other experiments, plants of subsequent generations (derived from the original transformants) were used, where biological replications are then possible and were done (phenotyping, gene expression, ChIP,…). Importantly, the expression level of the transgene is always determined in biological replications in all the lines used for further analysis.

5- We added scale bars to microscopy images.

Response to Referee #1:

The authors have improved the description of materials and methods. This however raises a few relatively minor questions. Is it journal policy to allow experiments to only be done once? For example, Figure 1 shows only single experiments with three internal bio-replicates.

RESPONSE: Figure 1 shows three independent experiments. This is now described in the legend.

-In Figure 3 D/E, why did the authors not do statistical analysis? The authors nicely did independent biological replication on the CHiP. I understand that the CHiP community hasn't enforced the use of statistics but this is essentially like qPCR data and can be analyzed similarly.

RESPONSE: We performed statistical analysis (Student´s t-test) for Figure 3D/E and added corresponding information to the figure and its legend.

-I still have difficulty with the legend in Figure 2E, I agree with the authors that this is in a log2 but the axis on the legend says that the maximal change is +1 log 2 which is only a 2 x change and the minimal is a -1 log 2. I find it hard to believe that the maximal change is +1 log2 in a pathogen experiment but this is what the figure legend is explicitly stating. While this might seem a minor point, it will be an issue in the future when people read the paper and ascertain what they do and do not believe. Especially as ANAC032 showed +1 log 2 changes in Figure 1.

RESPONSE: The axis on the legend does not explain that the maximum change is +1 log 2. It only indicates what the strongest colors in the heat map represent. For example the strongest red color in the heat map represents +1 log 2 fold change and in this case all the differences equal or above +1 log 2 changes are shown in dark red color. However for better presentation, we changed the scale to +1.5 log 2.

-I still feel that the authors are over-interpreting in parts. For example, the title for Figure 6 ends with "upon pathogen attack" but as the authors point out, this model would not work for necrotrophic pathogens as there is no camalexin. As such, I would urge the authors to better caveat with the specific Pseudomonas syringae DC3000 rather than slipping into the imprecise and potentially incorrect usage of the general term "pathogen". This may seem an over-reach but even if the authors went to a maculicula strain of Pst they would then have to bring in the sax loci and associated plant defense compounds that are regulated by myc2 which do not play a role in resistance against DC3000 (Fan et al., 2011). So there is already existing literature saying that using the general "pathogen" is incorrect and inaccurate and even "Pseudomonas" is incorrect.

Fan, J., Crooks, C., Creissen, G., Hill, L., Fairhurst, S., Doerner, P., and Lamb, C. (2011). Pseudomonas sax Genes Overcome Aliphatic Isothiocyanate-Mediated Non-Host Resistance in Arabidopsis. Science 331, 1185-1188.

RESPONSE: We changed title of Figure 6, and replaced "upon pathogen attack" to "in response to Pst DC3000".

We also removed the last sentence in the chapter "Model for ANAC032 action".

3rd Editorial Decision 17 August 2016

Thank you for the submission of your revised manuscript to our editorial offices. You have sufficiently addressed the remaining points of the referee and the editorial requests. However, before we can proceed with the formal acceptance of your manuscript, some further minor revisions are necessary.

I suggest changing the title to: Transcription factor ANAC032 modulates JA/SA signalling in response to Pseudomonas syringae infection.

We cannot publish a figure or data already published in another journal. Otherwise we would need to formally ask for permission to the publisher. This refers to Figure EV1. It also seems it is not really necessary to show this figure again. You could easily mention these data in the text supported by the relevant reference (if needed also mentioning the relevant figure panel(s) from that reference). We therefore ask you to remove Figure EV1 and mention this data in the text as indicated above.

3rd Revision - authors' response 23 August 2016

Following your instructions we have now updated our submission in the following form:
- We changed the title of our manuscript to: Transcription factor ANAC032 modulates JA/SA signalling in response to *Pseudomonas syringae* infection.
- Figure EV1: the graphs shown in this figure are made from data that we had extracted from Genevestigator (https://genevestigator.com/gv/) and they are not shown as graphs in any other journal. However, we agree with you that it is not necessary to show this figure in our manuscript. Therefore, we now removed Figure EV1 and only refer to the relevant reference. We renamed the remaining EV figures accordingly.

4th Editorial Decision 23 August 2016

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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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Salma Balazadeh
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42197

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	As descrbed in Materials and Methods, for mature plants, rosettes leaves of at least three plants grown in individual pots were harvested and pooled as one replicate. Experiments were perfromed in at least 3 independent replications.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	For plant teratments with hormones, as described in Materials and Methods, three independent biological replicates were performed for hormone treatments and gene expression analyses. Each replicate consisted of at least 20 seedlings selected randomly.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	YES
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	YES
Is there an estimate of variation within each group of data?	N/A
Is the variance similar between the groups that are being statistically compared?	N/A

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).	N/A
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	
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G- Dual use research of concern

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