Chloroplast clustering around the nucleus is a general response to pathogen perception in Nicotiana benthamiana

XUE DING1,2, TAMARA JIMENEZ-GONGORA1,2, BJÖRN KRENZ3 AND ROSA LOZANO-DURAN1,*

1Shanghai Center for Plant Stress Biology, CAS Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai, 201602, China
2University of the Chinese Academy of Sciences, Beijing, 100049, China
3Leibniz Institute DSMZ, 38124, Braunschweig, Germany

SUMMARY

It is increasingly clear that chloroplasts play a central role in plant stress responses. Upon activation of immune responses, chloroplasts are the source of multiple defensive signals, including reactive oxygen species (ROS). Intriguingly, it has been described that chloroplasts establish physical contact with the nucleus, through clustering around it and extending stromules, following activation of effector-triggered immunity (ETI). However, how prevalent this phenomenon is in plant–pathogen interactions, how its induction occurs, and what the underlying biological significance is are important questions that remain unanswered. Here, we describe that the chloroplast perinuclear clustering seems to be a general plant response upon perception of an invasion threat. Indeed, activation of pattern-triggered immunity, ETI, transient expression of the Rep protein from geminiviruses, or infection with viruses or bacteria all are capable of triggering this response in Nicotiana benthamiana. Interestingly, this response seems non-cell-autonomous, and exogenous treatment with H2O2 is sufficient to elicit this relocalization of chloroplasts, which appears to require accumulation of ROS. Taken together, our results indicate that chloroplasts cluster around the nucleus during plant–pathogen interactions, suggesting a fundamental role of this positioning in plant defence, and identify ROS as sufficient and possibly required for the onset of this response.

Keywords: chloroplasts, defence, ETI, geminivirus, nucleus, pathogen perception, PTI.

Plants have evolved a sophisticated multilayered immune system to defend themselves from potential pathogens. Upon perception of a pathogenic threat, signalling cascades leading to the onset of multiple immune responses are activated, which involves coordination between different subcellular compartments and organelles. The first layer of plant immunity is based on the recognition of conserved molecular patterns present in non-self by plasma membrane-localized pattern recognition receptors, and is therefore known as pattern-triggered immunity (PTI) (Bigeard et al., 2015; Macho and Zipfel, 2014). PTI is generally sufficient to fend off most pathogens; adapted pathogens, however, have evolved effector proteins that can suppress PTI and promote pathogenesis, leading to effector-triggered susceptibility. The susceptible interaction can be overcome by certain cultivars based on the presence of resistance (R) genes, which recognize effectors or their activities and initiate effector-triggered immunity (ETI) (Khan et al., 2016). PTI and ETI share signalling components and outputs, but the amplitude of ETI is higher and more sustained, often leading to the hypersensitive response (Chiang and Coaker, 2015). Recently, it has been proposed that pathogen molecules can be perceived as “invasion patterns”, and that plant immunity, comprising what has been previously artificially divided in PTI and ETI, acts as a global surveillance system (Cook et al., 2015).

Chloroplasts are endosymbiont-derived organelles present in plants, enabling photoautotrophy. In addition to their function as energy providers, an increasing body of evidence points at chloroplasts as playing an essential role in plant stress responses, serving as hubs in the integration of stimuli and source of downstream signalling molecules (Chan et al., 2016; de Souza et al., 2017; Zhu, 2016). Upon activation of immune responses, chloroplasts produce an array of pro-defence signals, including cytoplasmic calcium bursts, reactive oxygen species (ROS), and the phytohormones salicylic acid (SA) and jasmonic acid (JA) (Park et al., 2017; Sowden et al., 2017; de Torres Zabala et al., 2015). Consistent with a relevant role of chloroplast function in defence, effector proteins from different pathogens, including bacteria, viruses, fungi and oomycetes, have been shown to target this organelle (e.g. Fondong et al., 2007; Jelenska et al., 2007, 2010; Li et al., 2014; Liu et al., 2018; Park et al., 2017; Petre et al., 2016; Rodriguez-Herva et al., 2012; Rosas-Diaz et al., 2018; de Torres...
Zabala et al., 2015). Intriguingly, chloroplasts can establish contact with the nucleus, through clustering around it and extending tubular stroma-filled protrusions called stromules (Natesan et al., 2005), under different circumstances, including the activation of ETI triggered by the p50 protein from *Tobacco mosaic virus* (TMV) (Caplan et al., 2008, 2015; Kumar et al., 2018; Kwok and Hanson, 2004). Although the exact role of this physical interaction between chloroplasts and nucleus is at present unclear, it has been suggested that this connection could enable the transfer of molecules from plastids (e.g. NRIPI and H$_2$O$_2$) (Caplan et al., 2015). How prevalent the chloroplast perinuclear clustering is during different plant–pathogen interactions, how this response is induced and what the biological relevance of this physical association is in different contexts are currently open questions.

Here, we show that the clustering of chloroplasts around the nucleus seems to be a general response upon perception of a pathogenic threat. Indeed, activation of PTI, ETI, transient expression of the Rep protein from three different geminivirus species, infection by RNA viruses and infection by plant pathogenic bacteria are all capable of triggering this response in *Nicotiana benthamiana*. Moreover, exogenous treatment with H$_2$O$_2$ is sufficient to induce this targeted relocalization of chloroplasts around nuclei, which seems to require accumulation of ROS. In summary, our results show that chloroplasts actively cluster around the nucleus following activation of immune responses. This occurs upon perception of different kinds of invasion patterns or defence-related molecules, raising the idea that chloroplast–nuclear contacts may play a general role in plant defence.

While studying the subcellular localization of the replication-associated Rep protein from the geminivirus *Tomato yellow leaf curl virus* (TYLCV), we observed that transient expression of this protein (Rep, GFP-Rep, or Rep-GFP; Wang et al., 2017) in *N. benthamiana* triggered significant clustering of chloroplasts around the nuclei (Figs 1A–C and S1; Supplementary Table S1). Rep is the only protein required for replication of the circular single-stranded DNA genome of geminiviruses, but it is not a DNA polymerase itself; instead, it acts by reactivating the cell cycle in infected cells and recruiting the DNA replication machinery to the viral DNA (reviewed in Hanley-Bowdoin et al., 2013). Expression of a stroma marker (C4-GFP; Rosas-Diaz et al., 2018) allowed us to visualize stromules emitted by chloroplasts and potentially contacting the nuclei (Fig. 1B; Supplementary Videos S1 and S2). Transient expression of the other virus-encoded proteins did not induce chloroplast clustering around the nucleus, with the exception of C4 (Supplementary Fig. S2 and Table S2). Because in TYLCV the gene encoding C4 is contained within the Rep gene but in a different frame, we introduced a silent mutation in Rep that leads to an early stop codon in C4, with the aim of evaluating the potential contribution of C4 to the perinuclear chloroplast clustering induced by Rep (Supplementary Fig. S3). Expression of Rep but not C4 (Rep-C4mut) led to perinuclear chloroplast clustering to levels indistinguishable from those induced by expression of the wild-type Rep (Fig. 1D and Supplementary Table S1), suggesting that Rep is sufficient to induce this response. As expected, transient transformation of *N. benthamiana* leaves with a TYLCV infectious clone, which expresses Rep from the viral genome, triggered a similar chloroplast perinuclear clustering to that observed in tissues transformed with a cassette to express Rep from the 35S promoter (Figs 2 and S1; Supplementary Table S2).

Chloroplast clustering around the nucleus has been previously observed during ETI triggered by the p50 protein from TMV (Caplan et al., 2015; Kumar et al., 2018). Considering this, we wondered whether expression of Rep could result in the activation of immune responses in *N. benthamiana*. Interestingly, transient expression of Rep, GFP-Rep or Rep-GFP in *N. benthamiana* leaves promoted the expression of the defence-associated genes *NbCYP71D20* (Heese et al., 2007; Segonzac et al., 2011) and *NbACRE31* (Segonzac et al., 2011) (Supplementary Fig. S4), suggesting that Rep or its activity are recognized in this plant species, leading to the activation of defence responses.

Rep from different geminiviruses has conserved activities and protein domains (Hanley-Bowdoin et al., 2013). With the aim of ascertaining whether the effect in the chloroplast relocalization observed upon expression of Rep from TYLCV is specific to this virus or, on the contrary, is a general phenomenon following expression of geminiviral Rep proteins, we tested the position of chloroplasts in epidermal cells of *N. benthamiana* leaves transiently transformed with infectious clones of two other geminiviruses, *Beet curly top virus* (BCTV) and *Abutilon mosaic virus* (AbMV), or with constructs to express their corresponding Rep proteins. As shown in Fig. 2, Supplementary Fig. S1 and Supplementary Table S3, expression of either geminiviruses or their Rep proteins induced a perinuclear chloroplast clustering similar to that observed for TYLCV and its Rep (Fig. 1), indicating that this is a general effect of the geminivirus replication-associated protein.

We next wondered whether the Rep-induced clustering of chloroplasts around the nucleus is a cell-autonomous effect. To answer this question, we transformed isolated cells in a *N. benthamiana* leaf with a construct to express Rep-GFP through biolistics and followed chloroplast behaviour in nearby non-transformed cells. As shown in Supplementary Fig. S5, the clustering of chloroplasts around the nucleus could also be observed in non-transformed cells following expression of Rep-GFP, but not of free GFP, suggesting that this effect is indeed non-cell-autonomous.

So far, chloroplast assembly around the nucleus has been described to occur upon expression of viral proteins, namely p50 from TMV and Rep from geminiviruses. Nevertheless, how widespread this response is across different plant–pathogen interactions is still not clear, it has been suggested that this connection could enable the transfer of molecules from plastids (e.g. NRIPI and H$_2$O$_2$) (Caplan et al., 2015). How prevalent the chloroplast perinuclear clustering is during different plant–pathogen interactions, how this response is induced and what the biological relevance of this physical association is in different contexts are currently open questions.
interactions is unknown. To shed some light on this matter, we checked chloroplast behaviour during local leaf infection with the RNA viruses TMV and Tobacco rattle virus (TRV), the plant pathogenic bacterium Pseudomonas syringae pv. tomato DC3000, which activates ETI in N. benthamiana, or the P. syringae pv. tomato DC3000 ΔhopQ1-1 mutant, which causes disease. Infection by all these different pathogens induced the perinuclear positioning of chloroplasts (Fig. 3 and Supplementary Table S4), suggesting that this may be a common response following pathogen perception by the plant. In order to further explore this idea, we tested chloroplast behaviour upon activation of PTI by exogenous treatment with the epitope peptide of bacterial flagellin, flg22 (Gómez-Gómez et al., 1999), or activation of ETI by overexpressing the R protein RPS2 (Yu et al., 1993). As shown in Fig. 4A,B and Supplementary Table S5, triggering either PTI or ETI results in the clustering of chloroplasts around the nucleus. In the case of flg22 treatment, which allows for a temporal dissection of the response, this cellular change occurs within 1 hour following application of the peptide (Supplementary Video S3). Taken together, these results suggest that chloroplasts

Fig. 1 Expression of geminivirus Rep triggers clustering of chloroplasts around the nucleus in pavement cells of Nicotiana benthamiana leaves. (A) Subcellular localization of Rep-GFP, GFP-Rep (described in Wang et al., 2017) and free GFP in transiently transformed epidermal pavement cells of N. benthamiana leaves. AF, autofluorescence. Arrowheads indicate nuclei shown in the right column (close-up). White scale bar, 25 μm; orange scale bar, 5 μm. (B) 3D image of chloroplasts clustering around a nucleus upon transient expression of Rep-RFP and the chloroplast stroma marker C4-GFP. Scale bar, 5 μm. (C) Percentage of nuclei with four or more chloroplasts around in leaves expressing Rep-GFP, GFP-Rep or free GFP. Three biological replicates were performed with similar results. Raw data and statistical analyses of all replicates are presented in Supplementary Table S1. (D) Percentage of nuclei with four or more chloroplasts around in leaves expressing Rep-GFP, Rep-C4mut or free GFP. Three biological replicates were performed with similar results. Raw data and statistical analyses of all replicates are presented in Supplementary Table S1.
Fig. 2  Clustering of chloroplasts around the nucleus occurs in response to different geminiviruses. (A) Clustering of chloroplasts around the nucleus upon transient expression of TYLCV (see Wang et al., 2017), BCTV (Briddon et al., 1989) and AbMV (Kleinow et al., 2009) in pavement cells of Nicotiana benthamiana leaves. Free GFP is co-expressed in all cases to facilitate visualization of nuclei. AF, autofluorescence. Arrowheads indicate nuclei shown in the right column (close-up). White scale bar, 25 μm; orange scale bar, 5 μm. (B) Subcellular localization of BCTV Rep-GFP (expressed from the pGWB5 vector, described in Nakagawa et al., 2007b) and AbMV Rep-GFP (expressed from the pGWB505 vector, described in Nakagawa et al., 2007a) in pavement cells of N. benthamiana leaves. AF, autofluorescence. Arrowheads indicate nuclei shown in the right column (close-up). White scale bar, 25 μm; orange scale bar, 5 μm. (C) Percentage of nuclei with four or more chloroplasts around in leaves expressing TYLCV, BCTV, AbMV, BCTV Rep-GFP, AbMV Rep-GFP or free GFP. Three biological replicates were performed with similar results. Raw data and statistical analyses of all replicates are presented in Supplementary Table S3.
rearrangement around the nucleus is most likely a general phenomenon upon perception of invasion patterns, since it can be similarly observed following activation of PTI or ETI.

Non-cell-autonomous responses require cell-to-cell communication. Because we detect this phenomenon upon activation of defence, we reasoned that molecules produced during this response and potentially acting as systemic signals may relay the information to ultimately trigger this change. ROS, which during PTI and ETI are generated in the apoplast by the NADPH oxidase RBOHD (Nuhse et al., 2007; Torres et al., 2002; Zhang et al., 2007), have been shown to mediate systemic signalling in both abiotic and biotic stress responses (Gilroy et al., 2016), and hence are good candidates. Supporting this potential role of ROS as trigger, we found that exogenous application of H$_2$O$_2$ is sufficient to cause the perinuclear clustering of chloroplasts (Fig. 4C and Supplementary Table S5).

To date, the molecular cues activating the chloroplast perinuclear clustering during defence responses remain elusive. Since application of the ROS H$_2$O$_2$ is sufficient for this phenomenon to occur, we set out to test whether accumulation of ROS is also required. For this, we treated *N. benthamiana* leaves with the NADPH-oxidase inhibitor diphenyleneiodonium (DPI), as well as with the ROS scavengers dimethylthiourea (DMTU) and Tiron, while activating PTI by application of flg22 peptide. Strikingly,
all three chemicals decreased the number of chloroplasts around nuclei upon flg22 treatment (Fig. 4D and Supplementary Table S5), suggesting that ROS are not only sufficient, but also required for chloroplasts to establish contact with the nucleus following activation of PTI.

Chloroplasts are emerging as environmental sensors and central signalling hubs in plant biology. These organelles communicate with the nucleus through the so-called retrograde signalling pathway, which transmits developmental and environmental information to regulate the expression of thousands of nuclear genes (Chan et al., 2016). During plant–pathogen interactions, chloroplasts act as a source of signalling and/or defensive compounds, including ROS, SA, JA and cytoplasmic calcium bursts, and are required for transcriptional reprogramming and for the plant to mount a full defence response (Nomura et al., 2012; Park et al., 2017; Pogorelko et al., 2016; Sowden et al., 2017; de Torres Zabala et al., 2015). It has been previously shown that, upon activation of ETI through expression of the p50 protein from TMV, chloroplasts tend to cluster around the nucleus and emit stromules (Caplan et al., 2015; Kumar et al., 2018); interestingly, infection by the geminivirus AbMV also results in the induction of stromules (Krenz et al., 2010, 2012). Here, we show that chloroplast perinuclear clustering is a general response to the perception of a biotic threat, occurring both during PTI and ETI, and in response to bacteria and viruses. The biggest question, however, is still unanswered, since the biological significance of the relocalization of this organelle remains elusive.

Caplan et al. (2015) suggested that molecules such as proteins or ROS might be transferred from chloroplasts to the nucleus, which would be enabled by the prior establishment of contact; a
trafficking role of stromules has also been proposed by Krenz et al. (2012). Even in the absence of direct passage of molecules, it could be envisioned that fast activation of retrograde signalling through signalling cascades would likely benefit from physical proximity, which would maximize efficiency of communication. These enticing ideas, nevertheless, will require further support by experimental evidence.

Intriguingly, chloroplasts surrounding nuclei have been previously observed in contexts other than defence (Kwok and Hanson, 2004). Defining how general this phenomenon is, and what the specific triggers are, could greatly contribute to further our understanding of the chloroplast–nucleus interaction.

In pavement epidermal cells of N. benthamiana leaves, chloroplasts are generally found towards the cell periphery, pushed outwards by the large central vacuole. Upon activation of defence responses, however, chloroplasts rapidly rearrange and surround the nucleus. Three obvious questions emerge from this observation: (1) What are the signals perceived by chloroplasts to initiate their movement?; (2) How do chloroplasts move inside the cell directionally towards the nucleus?; and (3) How do chloroplasts stay in close proximity to the nucleus once they reach this position? Some insight to the first question has been obtained in this work: exogenous treatments with ROS are sufficient to promote chloroplast perinuclear clustering, and accumulation of ROS seems to be required for this change to occur. These results suggest the idea that ROS might be somehow perceived by the plastids, directly or indirectly, and elicit their migration towards the nucleus. On the other hand, recent work by Kumar et al. (2018) has shed light onto questions two and three, proving that chloroplasts move associated to microtubules, but anchor themselves to the nucleus through actin filaments. It is to be expected that these latest discoveries will pave the way for future studies, which will hopefully help unravel the functional relevance of this intriguing phenomenon.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Additional confocal images from the experiments shown in Figs 1 and 2.

Fig. S2 Transient expression of Rep or C4 from *Tomato yellow leaf curl virus* (TYLCV) induces chloroplast clustering around the nucleus. Percentage of nuclei with four or more chloroplasts around in *Nicotiana benthamiana* leaves transiently expressing Rep-GFP, C2-GFP, C3-GFP, C4-GFP, V2-GFP, CP-GFP or free GFP.
(constructs are described in Wang et al., 2017). Two biological replicates were performed with similar results. Raw data and statistical analyses of all replicates are presented in Supplementary Table 2.

**Fig. S3** Schematic representation of the mutation introduced in Rep to generate a C4 null mutant. The Rep-C4 mutant was generated by using primers Rep-C4mut-Fw: 5′-ACATCTCCATGTGCTTAATCAATCGAAGGC-3′ and Rep-C4mut-Rv: 3′-TGTAGAGGTACGCATTAGGTAAAGCTTCCG-5′; the resulting mutant expresses the wild-type Rep but has an early stop codon replacing the leucine in position 3 of the C4 protein.

**Fig. S4** Transient expression of Rep in *Nicotiana benthamiana* activates the expression of defence-related genes. (A) Relative expression of the defence-associated gene *NbCYP71D20* in *N. benthamiana* leaves upon transient expression of Rep-GFP, GFP-Rep and Rep compared with free GFP, or after a 1-hour treatment with flg22 (as control; 100 nM) compared with mock, as measured by qRT-PCR. Three biological replicates were performed, with three technical replicates each; the mean of three biological replicates is shown. (B) Relative expression of the defence-associated gene *NbACRE31* in *N. benthamiana* leaves upon transient expression of Rep-GFP, GFP-Rep, and Rep compared with free GFP, or after a 1-hour treatment with flg22 (as control; 100 mM) compared with mock, as measured by qRT-PCR. Three biological replicates were performed, with three technical replicates each; the mean of three biological replicates is shown.

**Fig. S5** The Rep-mediated promotion of the perinuclear clustering of chloroplasts is non-cell autonomous. (A) Pictures show single cells expressing GFP or Rep-GFP after bombardment (performed as in Ueki et al., 2010). The arrowhead indicates the nucleus of a cell expressing Rep-GFP. Squares mark regions of interest (ROI) 1 and 2 (see B). Hoescht staining for nuclear visualization was used at 4 μg/mL. (B) Close-ups of ROI1 and ROI2 (from A).

**Table S1** Raw data of the chloroplast counts presented in Fig. 1C and D. *P* value was calculated using a Student’s *t*-test to compare the average number of chloroplasts around each nuclei in samples compared to the corresponding GFP control. In each replicate, two leaves of one plant were used per construct.

**Table S2** Raw data of the chloroplast counts presented in Supplementary Fig. 2. *P* value was calculated using a Student’s *t*-test to compare the average number of chloroplasts around each nuclei in samples compared to the corresponding GFP control. In each replicate, two leaves of one plant were used per construct.

**Table S3** Raw data of the chloroplast counts presented in Fig. 2C. *P* value was calculated using a Student’s *t*-test to compare the average number of chloroplasts around each nuclei in samples compared to the corresponding GFP control. In each replicate, two leaves of one plant were used per construct.

**Table S4** Raw data of the chloroplast counts presented in Fig. 3. *P* value was calculated using a Student’s *t*-test to compare the average number of chloroplasts around each nuclei in samples compared to the corresponding GFP/mock control. In each replicate, two leaves of one plant were used per construct.

**Table S5** Raw data of the chloroplast counts presented in Fig. 4. *P* value was calculated using a Student’s *t*-test to compare the average number of chloroplasts around each nuclei in samples compared to the corresponding mock control. In each replicate, two leaves of one plant were used per construct.

**Video S1** 3D images of GFP-labelled chloroplasts around the nucleus of a cell expressing Rep-RFP.

**Video S2** 3D images of GFP-labelled chloroplasts around the nucleus of a cell expressing Rep-RFP.

**Video S3** Chloroplasts clustering around the nucleus in a GFP-expressing cell treated with flg22 (1 hour).