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Letter

Prevention of necrosis caused by transient expression in *Nicotiana benthamiana* by application of ascorbic acid

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instruction of Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Kenji Miura (miura.kenji.ga@u.tsukuba.ac.jp).

Dear Editor,

The overexpression of recombinant proteins can sometimes result in the accumulation of unfolded or misfolded proteins, triggering leaf necrosis, and/or dehydration and subsequent degradation of recombinant proteins. To enhance the accumulation of active proteins, a fundamental and a simple treatment to overcome leaf necrosis is required. In this study, treatment of *Nicotiana benthamiana* plants with high concentrations (100 mM or more) of sodium ascorbate (AsA) via foliar spraying after agroinfiltration suppressed the necrosis of leaves expressing human Cul1 (hCul1) and F-box protein, Fbxw7. The suppression of necrosis accordingly enhanced the accumulation of recombinant proteins by three-fold or more. Treatment with 200 mM AsA enhanced the formation and accumulation of PMab-2, an anti-RAP (rat Aggrus/podoplanin [PDPN]) tag antibody with antigen-binding activity. Thus, spray application of high concentrations of AsA is simple and easily applicable in several kinds of facilities to effectively suppress leaf necrosis, leading to an increase in the accumulation of functional recombinant proteins.

Compared with traditional cell culture-based systems, whole plant-based systems for the production of recombinant proteins have advantages, including cost-effectiveness and production scalability (Buyel et al., 2017). Transient gene expression using a deconstructed viral vector is a promising technique for rapidly producing high amounts of recombinant proteins (Desai et al., 2010; Ma et al., 2013). However, some recombinant proteins, such as hepatitis B surface antigen and human growth hormone, causes necrosis and/or dehydration of *N. benthamiana* leaves (Gils et al., 2005; Huang et al., 2008) and there has been no way to prevent necrosis. To increase the yield of these recombinant proteins, a fundamental solution is required.

We constructed pBYR2HS-hCul1 and pBYR2HS-human F-box protein (hFbxw7; Supplemental Figure S1) and transfected those to *N. benthamiana* leaves by agroinfiltration. Leaves expressing hCul1 and hFbxw7 exhibited necrosis (Figure 1A; 0 mM). As necrosis appeared, expression of binding immunoglobulin protein (BiP) and H_2O_2 was induced (Supplemental Figure S2), suggesting that endoplasmic reticulum (ER) stress-triggered accumulation

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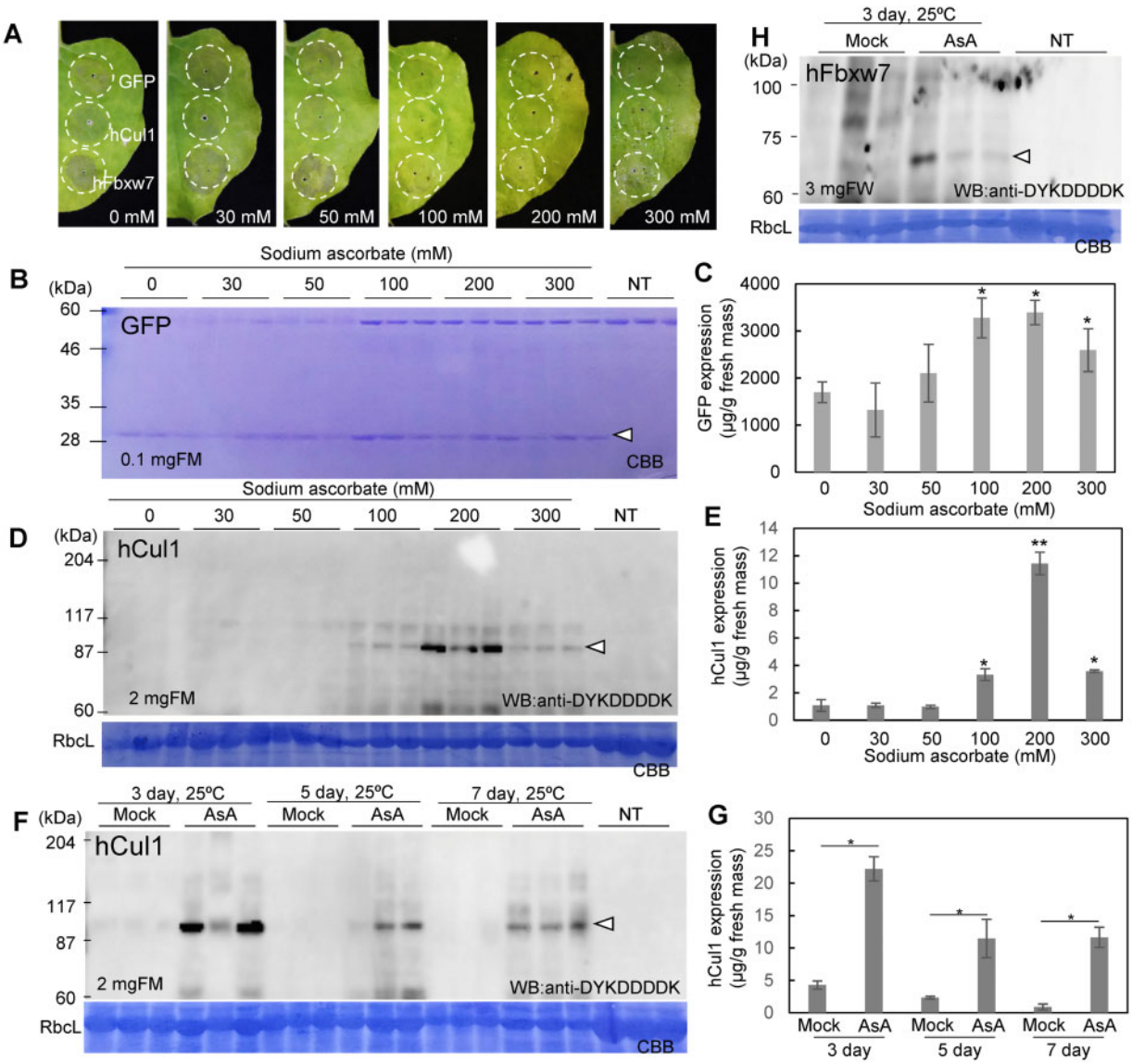

Figure 1 Suppression of necrotic effects by foliar spray application of high concentration of AsA. A, Syringe agroinfiltration was performed to express GFP, hCul1, and hFbxw7 in the leaves of *N. benthamiana*, followed by foliar spray application of AsA at the indicated concentration at 2-d intervals. The plants were incubated at 20°C for 7 d. Soluble proteins from the indicated fresh mass (FM) of these leaves were separated by SDS-PAGE. GFP (B) or hCul1 (D) were detected by Coomassie Brilliant Blue (CBB) staining or anti-DYKDDDDK antibody. NT indicates nontransfected plants. The amount of GFP (C) or hCul1 (E) was determined from band intensities. *N. benthamiana* plants agroinfiltrated with different vectors were incubated at 25°C following the foliar spray application of 200 mM AsA, and soluble proteins were extracted from leaves at 3, 5, or 7 d after agroinfiltration. hCul1 (F) or hFbxw7 (H) were detected with anti-DYKDDDDK antibody. The amount of hCul1 (G) were determined from band intensities. Data represent the means ± SD (n = 3–4) and significance was determined using unpaired Student's t tests (*$P < 0.05$) (C, E, G).

of H_2O_2 (Ozgur et al., 2014). Treatment with ER stress inhibitors had no appreciable improvement in plants (Supplemental Figure S3).

In plants, high levels of reactive oxygen species (ROS) can serve as a trigger for programmed cell death (van Breusegem and Dat, 2006). To remove ROS, a high concentration of AsA was applied to *N. benthamiana* plants expressing green fluorescent protein (GFP), hCul1, and hFbxw7. Leaves expressing hCul1 and hFbxw7 treated with 50 mM or less concentration of AsA via foliar spraying exhibited necrotic symptoms. In contrast, leaves treated with AsA at a concentration 100 mM or higher appeared healthy (Figure 1A). Consistently, accumulation of H_2O_2 was significantly reduced in leaves treated with 200 mM AsA (Supplemental Figure S2B).

To establish whether the alleviation of necrosis by AsA also resulted in an enhancement in the protein expression, soluble proteins were extracted from leaves and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) . Peak GFP expression (~4 mg/g fresh mass [FM]) occurs 3 d postagroinfiltration (dpa), and thereafter declines (Yamamoto et al., 2018). At 7 dpa, only ~2 mg/g FM of GFP protein accumulated in the leaves, whereas when AsA was applied at concentrations of at least

100 mM, ~3 mg/g FM of GFP expression in these leaves (Figure 1, B and C). Similarly, in leaves expressing hCul1, no clear bands (< 1 μg/g FM) were observed when 50 mM or less AsA was treated, but notable protein expression (11.4 μg/g FM when applied at 200 mM AsA) was observed after treatment with AsA at concentrations of 100 mM or higher (Figure 1, D and E), indicating high concentrations of AsA have the effect of inhibiting protein degradation. Leaves were incubated at 25°C for 3, 5, and 7 d and 200 mM AsA was applied. A marked increase in the expression of hCul1 and hFbxw7 was observed in response to treatment with AsA, with peak hCul1 expression being attained at 3 dpa (Figure 1, F–H). These results indicate that the application of a high concentration of AsA can mitigate the necrosis and markedly enhances protein expression.

Approximately 0.3 mg/g FM of PMab-2, an antibody against the RAP epitope, which is in rat PDPN (Fujii et al., 2017), was accumulated in *N. benthamiana* without AsA treatment (Miura et al., 2020). In this study, 200 mM AsA was applied to *N. benthamiana* expressing the PMab-2 heavy chain (HC) and light chain (LC). The PMab-2 HC and LC were detected using anti-mouse IgG (H) and anti-mouse IgG (L) antibodies, respectively (Figure 2, A and B). The full tetrameric assembly of PMab-2 (2 HCs and 2 LCs) was detected by immunoblot analysis using native-PAGE (Figure 2C). Application of AsA increased the accumulation of PMab-2 HC and LC and enhanced the formation of tetrameric PMab-2. Without AsA application, the formation of antibodies was not stable and the peak of HC and LC production may be shifted. PMab-2 was purified from *N. benthamiana*. Fluorescence-activated cell sorting analysis indicated that AsA-treated plant-derived PMab-2 recognized Chinese hamster ovary (CHO)-K1 cell-expressed rat PDPN as did hybridoma-derived PMab-2 or plant-derived PMab-2. No substantial changes were observed in the CHO-K1 cells treated with PMab-2 (Supplemental Figure S4). Immunoblot analysis revealed that rat PDPN was similarly detected by PMab-2 derived from hybridoma cells and plant cells with or without AsA treatment (Figure 2D). These results indicate that the application of AsA did not affect the function of a recombinant antibody. No apparent phenotypic difference was observed when a high concentration of AsA was applied.

In this study, ER retention signal was fused with genes of interest. Some proteins require late posttranslational modification, such as the formation of complex glycan. These modifications are typically performed downstream of the ER along secretory pathways (Faye et al., 2005). Thus, the production of modified proteins is likely to be enhanced by spray application of AsA.

Overall, our research demonstrated the application of high concentrations of AsA suppresses necrosis caused by the expression of foreign recombinant proteins in *N. benthamiana*, increasing in protein accumulation and AsA did not affect the antigen-binding activity of the recombinant antibody. The application of AsA by a foliar spray is simple, thus, it is easily applicable to several kinds of facilities.

Supplemental data

The following supplemental materials are available.

Supplemental Figure S1. Schematic representation of the T-DNA regions of the plasmids pBYR2HS-hCul1 and pBYR2HS-hFbxw7.

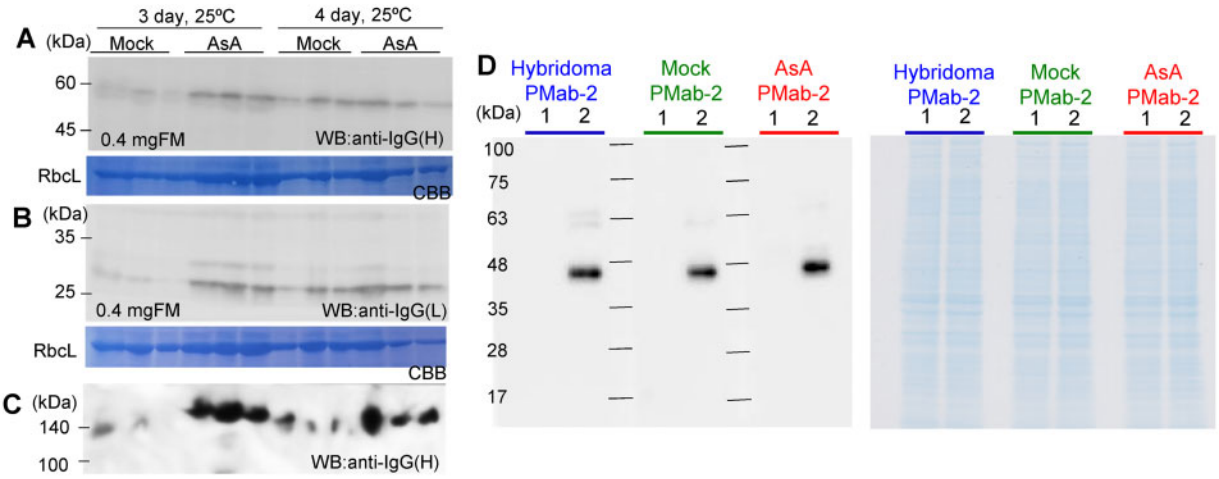

Figure 2 Application of AsA increases the production of an antibody that retains binding activity. *N. benthamiana* leaves were infiltrated with a mixed preparation of Agrobacterium harboring pBYR2HS-PMab2H and pBYR2HS-PMab2L, after which, the leaves were treated with water (Mock) or 200 mM AsA via foliar spray application. At 3 or 4 d postinfiltration, leaves were harvested. Total protein extracts were separated and were transferred to PVDF membranes. The membranes were incubated with anti-mouse IgG(H) or anti-mouse IgG(L) to detect the HC (A) and LC (B) of PMab-2, respectively. C, Native PAGE was used to detect the tetrameric assembly of PMab-2 (two HCs and two LCs). D, Immunoblot analysis with PMab-2 to detect rat PDPN. Soluble proteins from CHO-K1 cells (1) or CHO-K1 expressing rat PDPN (2) were separated on SDS-PAGE. Rat PDPN was detected using PMab-2 derived from hybridoma cells (hybridoma) or *N. benthamiana* leaves treated without (Mock) or with 200 mM AsA. Total proteins were visualized by CBB staining.

Supplemental Figure S2. BiP expression and H_2O_2 accumulation were induced by hCul or hFbxw7.

Supplemental Figure S3. Necrosis was observed in leaves treated with ER stress inhibitors.

Supplemental Figure S4. AsA-treated plant-derived antibody retained its binding activity.

Supplemental Methods. The methodology used in this study.

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Conflict of interest statement. None declared.

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