Expression of the Human NAD(P)-Metabolizing Ectoenzyme CD38 Compromises Systemic Acquired Resistance in *Arabidopsis*

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Plant systemic acquired resistance (SAR) is a long-lasting, broad-spectrum immune response that is mounted after primary pathogen infection. Although SAR has been extensively researched, the molecular mechanisms underlying its activation have not been completely understood. We have previously shown that the electron carrier NAD(P) leaks into the plant extracellular compartment upon pathogen attack and that exogenous NAD(P) activates defense gene expression and disease resistance in local treated leaves, suggesting that extracellular NAD(P) [eNAD(P)] might function as a signal molecule activating plant immune responses. To further establish the function of eNAD(P) in plant immunity, we tested the effect of exogenous NAD(P) on resistance gene-mediated hypersensitive response (HR) and SAR. We found that exogenous NAD(P) completely suppresses HR-mediated cell death but does not affect HR-mediated disease resistance. Local application of exogenous NAD(P) is unable to induce SAR in distal tissues, indicating that eNAD(P) is not a sufficient signal for SAR activation. Using transgenic *Arabidopsis* plants expressing the human NAD(P)-metabolizing ectoenzyme CD38, we demonstrated that altering eNAD(P) concentration or signaling compromises biological induction of SAR. This result suggests that eNAD(P) may play a critical signaling role in activation of SAR.

Plants, like animals, are constantly subject to a wide variety of potentially dangerous microorganisms. To protect themselves from microbial invasion and colonization, plants have evolved a sophisticated immune system to detect the presence of microbes and to activate defense responses (Jones and Takemoto 2004). Plant cells can detect the presence of microbes by recognizing two general groups of pathogen-derived molecules. One group of such molecules is the so-called microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs), which are conserved molecules characteristic of a whole class of microbes. The other group is the effector molecules that are delivered into the plant cell by the pathogen's type III secretion system (He 1998; Nurnberger et al. 2004). Recognition of PAMPs by PAMP receptors activates the so-called PAMP-triggered immunity (PTI) (Gomez-Gomez et al. 1999; Ron and Avni 2004). Pathogens can often overcome the PTI by delivering effector molecules into the plant cell to interfere with

PTI. Plant cells, in turn, detect the presence of the effectors by resistance (R) proteins, inducing the so-called effector-triggered immunity (ETI) to restrict pathogen growth (Dangl and Jones 2001; Heath 2000; Martin et al. 2003). Both PTI and ETI contribute to the activation of a defense mechanism named systemic acquired resistance (SAR) in tissues distant from the primary infection site (Durrant and Dong 2004; Mishina and Zeier 2007; Ryals et al. 1996). Whereas it is generally accepted that SAR is activated by a blend of mobile signal molecules produced in the primarily infected tissues, the underlying molecular mechanisms are not entirely clear (Parker 2009; Vlot et al. 2008).

Pyridine nucleotide NAD(P) is a classic electron carrier that functions in metabolic reactions (Noctor et al. 2006) yet participates in both intracellular and extracellular signaling events (Berger et al. 2004). NAD(P) can be processed into the intracellular Ca^{2+} -mobilizing agents cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) that release Ca^{2+} from intracellular stores (Galione and Churchill 2000; Lee 2001). NAD also acts as an ADP-ribose donor and an acetyl group acceptor in protein ADP-ribosylation and deacetylation, respectively (Denu 2003). Upon environmental stress, cellular NAD(P) can be released into the extracellular compartment (ECC) by active exocytosis or diffusion through transmembrane transporters in living cells or passive leakage across membrane in dying cells (Billington et al. 2006b). In the ECC, NAD(P) is processed by ectoenzymes or perceived by cell surface receptors or channels, activating downstream signaling pathways. A number of NAD(P)-metabolizing enzymes, including CD38 and mono(ADP-ribosyl)transferases (ART), have been identified on the extracellular surface of the plasma membrane (Malavasi et al. 2006; Morabito et al. 2006; Partida-Sanchez et al. 2007; Zolkiewska 2005). CD38 is a multifunctional enzyme combining ADP-ribosyl cyclase, cADPR-hydrolase, and NAD-hydrolase activities, which utilizes NAD(P) as substrate to generate cADPR and NAADP (Ceni et al. 2003; Krebs et al. 2005; Partida-Sanchez et al. 2003). Both cADPR and NAADP can be transported across the plasma membrane to reach their intracellular targets and raise intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Billington et al. 2006a; De Flora et al. 2004). NAD^+ and NAADP can also function in the ECC to activate purinoceptors, resulting in increases in $[\text{Ca}^{2+}]_i$ (Heidemann et al. 2005; Singaravelu and Deitmer 2006). ART are glycosylphosphatidylinositol-anchored or secreted ectoenzymes that use NAD to ADP-ribosylate purinoceptor, CD38, integrin, and other raft-associated signaling proteins, resulting in inhibition of CD38 and activation of purinoceptors (Bannas et al. 2005; Han et al. 2000; Nemoto et al. 1996; Seman et al. 2003).

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We have previously shown that, in *Arabidopsis*, exogenous application of NAD(P) activates both defense gene expression and disease resistance, and we hypothesized that extracellular NAD(P) [eNAD(P)] may be perceived by plant cells as a danger signal of pathogen attack, leading to the activation of immune responses (Zhang and Mou 2009). Although we reported that NAD(P) leaks into the ECC during pathogen infection, evidence supporting a role for endogenous eNAD(P) in plant immunity is still lacking. Here, in addition to testing the effect of exogenous NAD(P) on *R*-gene-mediated hypersensitive response (HR) and SAR, we expressed the human NAD(P)-metabolizing ectoenzyme CD38 in *Arabidopsis* to manipulate the levels of endogenous eNAD(P). We used CD38 because no plant NAD(P)-metabolizing ectoenzyme has been reported. Using the transgenic *Arabidopsis* plants expressing CD38, we provided evidence that eNAD(P) may participate in the activation of SAR.

RESULTS

Exogenous NAD(P) suppresses HR-mediated cell death but not HR-mediated disease resistance.

To test whether exogenous NAD(P) affects HR-mediated defense responses, we treated *Arabidopsis* plants with different concentrations of NAD⁺ followed by inoculation of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 carrying the *avrRpt2* gene (*Pst/avrRpt2*). Ion leakage was used to quantify HR-mediated cell death. Without NAD⁺ pretreatment, *Pst/avrRpt2* induced a significant ion leakage (Supplementary Fig. S1), whereas the negative control (10 mM MgCl₂ treatment) did not result in a strong ion leakage. Interestingly, NAD⁺ pretreatment inhibited *Pst/avrRpt2*-induced ion leakage in a concentration-dependent manner. We previously have shown that the amount of NAD(P) leaking into the ECC after *Pst/avrRpt2* infection is comparable with that after infiltration with 1 mM NAD(P) (Zhang and Mou 2009); therefore, we used this concentration in the experiment.

Pretreatment of *Arabidopsis* plants with 1 mM NAD⁺ or 1 mM NADP⁺ completely suppressed *Pst/avrRpt2*- and *Pst/avrRps4*-induced leaf collapse (Fig. 1A and B). These pretreatments also significantly suppressed *Pst/avrRpt2*- and *Pst/avrRps4*-induced ion leakage (Fig. 1C and D). Because HR-mediated cell death is generally associated with resistance (Coll et al. 2011; Mur et al. 2008), we tested whether NAD(P) also suppresses HR-mediated disease resistance. Wild-type Col-0 plants were treated with 1 mM NAD⁺ or 1 mM NADP⁺ followed by inoculation of *Pst/avrRpt2* or *Pst/avrRps4*. MgCl₂ pretreatment was included as a negative control. The fully susceptible mutants *rps2* (for *Pst/avrRpt2*) and *rps4* (for *Pst/avrRps4*) were also included as controls in the experiment. Consistent with the previous observations (Kunkel et al. 1993; Wirthmueller et al. 2007), *rps2* and *rps4* were highly susceptible to *Pst/avrRpt2* and *Pst/avrRps4*, respectively, and HR-mediated resistance significantly inhibited the growth of *Pst/avrRpt2* and *Pst/avrRps4* in the MgCl₂-pretreated plants (Fig. 1E and F). Surprisingly, although NAD(P)⁺ suppressed HR-mediated cell death, the growth of *Pst/avrRpt2* and *Pst/avrRps4* in NAD(P)⁺-pretreated plants was comparable with that in MgCl₂-pretreated plants (Fig. 1E and F). These results indicate that exogenous NAD(P) suppresses HR-mediated cell death but does not compromise HR-mediated resistance. To test whether exogenous NAD(P)-induced resistance depends on *R* genes, we treated *rps2* and *rps4* with 1 mM NAD⁺ followed by inoculation of *Pst/avrRpt2* and *Pst/avrRps4*, respectively. NAD⁺ pretreatment significantly inhibited the growth of both *Pst/avrRpt2* and *Pst/avrRps4* (Supplementary Fig. S2), though the effect of inhibition on *Pst/avrRps4* was stronger than that

on *Pst/avrRpt2*. These results suggest that exogenous NAD(P) mainly activates *R*-gene-independent disease resistance in *Arabidopsis*.

Local application of exogenous NAD(P) does not induce SAR.

After being released into the ECC, NAD(P) or its derivatives may function as a mobile signal to induce SAR in the distal tissues. To test this hypothesis, we infiltrated three lower leaves on each plant with different concentrations of NAD(P). Adenosine triphosphate (ATP) was included as a control in the experiment. After three days, the upper leaves were inoculated with the virulent bacterial pathogen *P. syringae* pv. *maculicola* ES4326. The in planta growth of ES4326 was determined 3 days later. A concentration of 5 mM NAD(H) induced a significant SAR (Fig. 2A), though much weaker than that induced by *Pst/avrRpt2*. However, when a physiologically relevant concentration (1 mM) was used (Zhang and Mou 2009), none of the five chemicals (NAD⁺, NADH, NADP⁺, NADPH, and ATP) induced a significant SAR. Therefore, under physiological conditions, eNAD(P) (or its derivatives) may not be a sufficient signal for activating SAR.

CD38 is localized at the plasma membrane in transgenic *Arabidopsis*.

To establish a more direct relationship between eNAD(P) and plant immunity, we wished to express a protein that could alter the concentration of eNAD(P) or its signaling in *Arabidopsis* plants. Due to the lack of any known NAD(P)-metabolizing ectoenzyme in plants, we chose the well-characterized human protein CD38, an integral membrane protein with its enzyme-active site on the extracellular surface of the plasma membrane (Deaglio and Malavasi 2006). In human cells, CD38 metabolizes eNAD(P) to the secondary messengers cADPR and NAADP, which activate transmembrane signaling (Billington et al. 2006a; De Flora et al. 2004; Partida-Sanchez et al. 2003). Although the function of extracellular NAADP in plant immunity has not been defined, cADPR has been shown to induce phenylalanine ammonia lyase and pathogenesis-related (*PR*)I expression in tobacco leaf disks (Durner et al. 1998). We expected that expression of CD38 in plants would modify eNAD(P)-activated immune responses.

In human cells, CD38 is localized in the plasma membrane (Malavasi et al. 2008). To test whether CD38 is correctly localized in transgenic plants, we used the green fluorescence protein (GFP) as a reporter. The *GFP* coding region was fused to the 3' end of the *CD38* cDNA, and the expression of the *CD38-GFP* fusion gene is under the control of the constitutive *Cauliflower mosaic virus* 35S promoter (Odell et al. 1985). To generate a construct expressing an inactive form of CD38, we introduced a point mutation into the 35S::CD38-GFP construct to change the glutamate residue at the active site, Glu-226, to glycine (named hereafter E226G) (Munshi et al. 2000). Both constructs (35S::CD38-GFP and 35S::E226G-GFP) were transformed into wild-type Col-0 plants. Transgenic lines were screened by protein gel-blot analysis using a monoclonal anti-GFP antibody, and those lines expressing CD38-GFP or E226G-GFP were used for further subcellular localization study.

The subcellular localization of CD38-GFP was examined by confocal microscopy. Plants expressing GFP alone were included as a control in the experiment. GFP was localized in the cytosol, whereas both CD38-GFP and E226G-GFP were localized predominantly in an area corresponding to the plasma membrane (Fig. 3). In order to visualize the plasma membrane better, the *Arabidopsis* root cells were plasmolyzed. After plasmolysis, both CD38-GFP and E226G-GFP appeared to be

Fig. 1. Effect of exogenous NAD(P) on hypersensitive-response-mediated cell death and resistance. **A**, Wild-type Col-0 leaves pretreated with 10 mM MgCl₂, 1 mM NAD⁺, or 1 mM NADP⁺ followed by inoculation with the avirulent bacterial pathogen *Pst/avrRpt2*. **B**, Wild-type Col-0 leaves pretreated with 10 mM MgCl₂, 1 mM NAD⁺, or 1 mM NADP⁺ followed by inoculation with the avirulent bacterial pathogen *Pst/avrRps4*. **C**, Ion leakage from wild-type Col-0 leaves treated with 10 mM MgCl₂ or *Pst/avrRpt2*, or pretreated with 1 mM NAD⁺ or 1 mM NADP⁺ followed by inoculation with *Pst/avrRpt2*. **D**, Ion leakage from wild-type Col-0 leaves treated with 10 mM MgCl₂ or *Pst/avrRps4*, or pretreated with 1 mM NAD⁺ or 1 mM NADP⁺ followed by inoculation with *Pst/avrRps4*. **E**, Growth of *Pst/avrRpt2* in *rps2* and wild-type Col-0 plants pretreated with 10 mM MgCl₂, 1 mM NAD⁺, or 1 mM NADP⁺. **F**, Growth of *Pst/avrRps4* in *rps4* and wild-type Col-0 plants pretreated with 10 mM MgCl₂, 1 mM NAD⁺, or 1 mM NADP⁺. Error bars represent standard deviation. Experiments were repeated three times with similar results.

localized in the plasma membrane and not in the cell wall (Fig. 3). To confirm the plasma membrane localization of CD38-GFP, we produced protoplasts by digesting the cell wall of the root cells expressing CD38-GFP. Whereas GFP alone was localized in the cytosol of the cell, CD38-GFP and E226G-GFP were strictly localized to the plasma membrane of the cell in a nonhomogeneous pattern (Fig. 3). To further confirm that CD38-GFP is localized in the plasma membrane, we separated total membranes from soluble fractions by membrane fractionation. CD38-GFP and E226G-GFP were present in the membrane fraction but not in the soluble supernatant (Supplementary Fig. S3A). We also measured total NAD(P) levels in Col-0, 35S::CD38-GFP, and 35S::E226G-GFP plants using both denatured (heated at 95°C for 5 min) and undenatured samples. We reasoned that, if CD38-GFP is localized in the plasma membrane and functions as an NAD(P)-metabolizing ectoenzyme, it should change the total NAD(P) levels in undenatured samples but not in denatured samples. Total NAD(P) levels were similar in the denatured samples from all genotypes, whereas NADP levels were significantly lower and NAD levels were also lower in the undenatured samples from the 35S::CD38-GFP plants than in those from the wild-type Col-0 and 35S::E226G-GFP plants. Taken together, these results clearly demonstrate that CD38 is localized in the plasma membrane when expressed in plant cells.

Expression of CD38 suppresses exogenous NAD(P)-induced PR gene expression and disease resistance.

After showing that both CD38-GFP and E226G-GFP are localized in the plasma membrane in transgenic *Arabidopsis*, we were able to test whether CD38 functions in plants using the transgenic plants. We also generated 35S::CD38 transgenic plants in case fusion of CD38 with GFP affects CD38 activity. We expected that transgenic *Arabidopsis* expressing the active form of CD38 would exhibit altered responses to eNAD(P) no matter whether plants and animals employ similar mechanisms to perceive eNAD(P).

We first tested the induction of *PR1* gene expression by different concentrations of exogenous NAD⁺ in the transgenic plants. Consistent with our previous observation (Zhang and Mou 2009), *PR1* was induced by NAD⁺ in a concentration-dependent manner (Fig. 4A). Intriguingly, induction of *PR1* gene expression by exogenous NAD⁺ at a concentration of 1 mM or lower was completely blocked in both the 35S::CD38 and 35S::CD38-GFP plants, whereas induction of *PR1* expression in the 35S::E226G-GFP plants by exogenous NAD⁺ was comparable with that in the wild-type Col-0 plants. We noticed that the induction of *PR1* gene expression by exogenous NAD⁺ was significantly decreased in a total of 29 independent 35S::CD38 transgenic lines, though the induction of *PR1* was not tightly correlated with the expression levels of CD38 (Supplementary Fig. S4). These results indicate that CD38 blocks eNAD(P) signaling for *PR* gene induction and that the enzyme-active site of CD38 is required for suppressing exogenous NAD⁺-induced *PR1* gene expression.

We then tested NAD⁺-induced disease resistance in the transgenic plants. For this purpose, lower concentrations (0.2 and 0.4 mM) of exogenous NAD⁺ were used because these concentrations are sufficient to induce strong disease resistance in wild-type Col-0 plants (Fig. 4B) (Zhang and Mou 2009). Induction of disease resistance in both the 35S::CD38 and 35S::CD38-GFP plants was significantly suppressed but not affected in the 35S::E226G-GFP plants, suggesting that CD38 suppresses NAD⁺-induced disease resistance and that the enzyme-active site of CD38 is required for suppressing exogenous NAD⁺-induced disease resistance.

Expression of CD38 depletes extracellular NAD(P).

Expression of the human ectoenzyme CD38 in plants suppresses exogenous NAD⁺-induced immune responses, suggesting that CD38 may metabolize the exogenously applied NAD⁺. To test whether CD38 metabolizes the endogenous eNAD(P) accumulated during pathogen infection, we measured NAD(P) concentrations in the extracellular washing fluid (EWF) collected from pathogen-infected leaves of the wild-type Col-0 and the transgenic plants. Consistent with our previous observation (Zhang and Mou 2009), NAD(P) leaked into the ECC after the *Pst/avrRpt2* infection (Fig. 5). Interestingly, the concentrations of NADP in the EWF collected from the *Pst/avrRpt2*-infected 35S::CD38 or 35S::CD38-GFP leaves were significantly lower than those from the wild-type Col-0 or 35S::E226G-GFP leaves (Fig. 5A), whereas the concentrations of NAD in the EWF from the *Pst/avrRpt2*-infected 35S::CD38 or 35S::CD38-GFP leaves were only slightly lower than those from the wild-type Col-0 or 35S::E226G-

Fig. 2. Induction of systemic acquired resistance by local application of exogenous NAD(P). **A**, Growth of *Pseudomonas syringae* pv. *maculicola* ES4326 in the systemic leaves of wild-type Col-0 plants with lower leaves treated with 10 mM MgCl₂, *Pst/avrRpt2*, 5 mM NAD⁺, 5 mM NADH, 5 mM NADP⁺, 5 mM NADPH, and 5 mM adenosine triphosphate (ATP). **B**, Growth of *P. syringae* pv. *maculicola* ES4326 in the systemic leaves of wild-type Col-0 plants with lower leaves treated with 10 mM MgCl₂, *Pst/avrRpt2*, 1 mM NAD⁺, 1 mM NADH, 1 mM NADP⁺, 1 mM NADPH, and 1 mM ATP. Error bars represent standard deviation, and different letters above the bars indicate significant differences ($P < 0.05$, t test). Experiments were repeated three times with similar results.

GFP leaves (Fig. 5B). Although CD38 appears to have a stronger effect on eNADP than on eNAD, these results indicate that CD38 does, indeed, metabolize the NAD(P) released into the ECC during pathogen infection.

Expression of CD38 compromises biological induction of SAR.

Although eNAD(P) is not a sufficient mobile signal molecule for SAR activation, it may participate in mounting SAR through interaction with other signal molecules. To test this hypothesis, we investigated the biological induction of SAR in 35S::CD38, 35S::CD38-GFP, 35S::E226G-GFP, and wild-type Col-0 plants. Three lower leaves on each plant were infected with the avirulent bacterial pathogen *Pst/avrRpt2*. The upper uninfected leaves were either collected after 2 days for *PR* gene analysis or inoculated after 3 days with the virulent bacterial pathogen *P. syringae* pv. *maculicola* ES4326, and the in planta growth of ES4326 was determined 3 days later. The induction of *PR1*, *PR2*, and *PR5* in the upper uninfected leaves of the 35S::CD38 and 35S::CD38-GFP plants was decreased compared with that in the 35S::E226G-GFP and Col-0 plants (Fig. 6A). Whereas the *Pst/avrRpt2*-induced SAR resistance to ES4326 was strong and comparable in the 35S::E226G-GFP and Col-0 plants, the induction of SAR resistance by *Pst/avrRpt2* was significantly inhibited in both the 35S::CD38 and 35S::CD38-GFP plants (Fig. 6B). These results indicate that eNAD(P) is positively involved in the biological induction of SAR. Because CD38 is an NAD(P)-metabolizing ectoenzyme which converts extracellular NAD and NADP to cADPR and NAADP, respectively, SAR induction in 35S::CD38 and 35S::CD38-GFP plants might be inhibited by cADPR or NAADP. To test this possibility, we treated lower leaves with cADPR or NAADP prior to *Pst/avrRpt2* inoculation for SAR induction. cADPR and NAADP did not significantly affect *Pst/avrRpt2*-induced SAR resistance (Supplementary Fig. S5), indicating that CD38 may inhibit biological induction of SAR through reduction of eNAD(P) levels.

DISCUSSION

SAR is a secondary immune response activated in the distal parts of a plant by mobile signal molecules generated in the primarily infected tissues. SAR confers long-lasting resistance to subsequent infections by a broad spectrum of pathogens (Durrant and Dong 2004). It is generally believed that multiple mobile signal molecules coexist but the nature and action mode of the signal molecules remain controversial (Attaran et al. 2009; Chaturvedi et al. 2008; Parker 2009). Evidence thus far has indicated that reactive oxygen species, methyl-salicylic acid, jasmonic acid, azelaic acid, and glycerol-3-phosphate (G3P) are likely mobile signal molecules activating SAR (Alvarez et al. 1998; Chanda et al. 2011; Jung et al. 2009; Park et al. 2007; Truman et al. 2007). Our data suggest that eNAD(P) may play an important signaling role in the activation of SAR.

NAD(P) is usually found inside the cell acting as an electron carrier in metabolic reactions or participating in intracellular signaling events unrelated to metabolism (Berger et al. 2004). However, it is known that certain animal cells actively release NAD(P) into the ECC upon stimulation or passively leak out NAD(P) during cell death and inflammation (Bruzzone et al. 2001; Contreras et al. 2003; Smyth et al. 2004). In the ECC, NAD(P) are perceived or processed on the adjacent cell plasma membrane via distinct mechanisms. NAD and NADP can be metabolized by the ectoenzyme CD38 to cADPR and NAADP, respectively, which, in turn, move across the plasma membrane and trigger intracellular signaling events (Billington et al. 2006a; De Flora et al. 2004). NAD(P) can also act di-

rectly on plasma membrane receptors and NAD can be utilized to ADP-ribosylate plasma membrane receptors (Bannas et al. 2005; Han et al. 2000; Judkins et al. 2006; Moreschi et al. 2006; Nemoto et al. 1996; Seman et al. 2003). To test whether similar mechanisms are evolved in plants, we generated *Arabidopsis* transgenic plants expressing the human CD38 protein that metabolizes NAD and NADP to cADPR and NAADP, respectively. As in human cells, CD38 is localized in the plasma membrane in the transgenic plants (Fig. 3), which is in agreement with its ectoenzyme character (Malavasi et al. 2006; Morabito et al. 2006). Interestingly, we found that expression of CD38 inhibited exogenous NAD(P)-induced immune responses and that the enzyme-active site of CD38 is required for the inhibition (Fig. 4), suggesting that the human ectoenzyme CD38 is active when expressed in *Arabidopsis*. Consistently, we detected less NAD and significantly less NADP in the EWF collected from pathogen-infected leaves of the transgenic plants expressing the active form of CD38 than in the EWF from wild-type or transgenic plants expressing the inactive form of CD38 (Fig. 5). These results suggest that, although the human ectoenzyme CD38 can process eNAD(P) in the plant ECC, the resulting secondary messengers cADPR and NAADP may not be able to activate immune responses. Thus, plants and animals may use different mechanisms to process eNAD(P) for immunity activation, though it is possible that, in plants, CD38-generated cADPR and NAADP trigger some signaling pathways other than immune response. Whether, in plants, eNAD(P) could act directly on plasma membrane receptors requires further investigation.

We have shown that exogenous NAD(P) induces salicylic acid (SA)- and NPR1-dependent *PR* gene expression and disease resistance in local treated leaves (Zhang and Mou 2009), indicating that exogenous NAD(P) induces signaling pathways similar to those activated during SAR. Previous studies showed that preexisting SAR suppresses HR-mediated cell death (Devadas and Raina 2002; Rate and Greenberg 2001). Pretreatment of plants with exogenous NAD(P) induces SAR signaling pathways and, thus, also suppresses HR-mediated cell death (Fig. 1A–D). NAD(P) released into the ECC from the dying cells during HR does not have a strong effect on the HR-mediated cell death, because eNAD(P) accumulates after cell death occurs. However, eNAD(P) accumulated during HR might contribute to preventing the spread of HR-mediated cell death to the adjacent healthy cells. Because exogenous NAD(P)-induced disease resistance to two avirulent bacterial pathogens is largely independent of their corresponding *R* genes, it will be interesting to identify the signaling components downstream of eNAD(P).

The transgenic *Arabidopsis* plants expressing the human ectoenzyme CD38 allowed us to assess the function of eNAD(P) in defense gene induction, HR, basal resistance, and SAR. We found that SA- and *Pst/avrRpt2*-induced *PR1* expression, *Pst/avrRpt2*-induced HR (macroscopic leaf collapse), and basal resistance to *Pst/avrRpt2* and *P. syringae* pv. *maculicola* ES4326 were not altered in the CD38 transgenic plants (Supplementary Fig. S6), suggesting that depleting eNAD(P) by CD38 in the transgenic plants may not influence defense responses in the primarily infected tissues. We are unable to conclude that eNAD(P) does not play a role in the defense of primarily infected tissues, because CD38 cannot completely remove eNAD(P) from the ECC. Surprisingly, we found that biological induction of SAR was significantly inhibited in the transgenic plants expressing the active form of CD38 but not in the transgenic plants expressing the inactive form of CD38 (Fig. 6). This result indicates that depleting eNAD(P) accumulated in the primarily infected tissues prevents SAR induction. However, local application of a

physiological concentration of NAD(P) did not induce SAR in systemic leaves (Fig. 2B), suggesting that eNAD(P) alone is not sufficient for activation of SAR. This is reminiscent of the signal molecule SA, which activates SAR signaling path-

ways in local treated leaves but does not induce SAR in systemic leaves. Whereas SA accumulation is required for both basal resistance and SAR, removal of eNAD(P) by expression of the human ectoenzyme CD38 only affects SAR in-

Fig. 3. Subcellular localization of green fluorescent protein (GFP), CD38-GFP, and E226G-GFP. GFP images, propidium iodide (PI) staining images, and overlays are shown in the top, second, and third row, respectively. GFP images of plasmolyzed cells and protoplasts are shown in the fourth and the bottom row, respectively.

duction, suggesting that eNAD(P) accumulated after primary infection may play a more important role in the activation of SAR, a secondary immune response. It is possible that eNAD(P) functions together with other signal molecules, or that a transporter is required for translocation of eNAD(P) or its derivatives to distal tissues. The latter scenario has recently been observed for the likely SAR mobile signal molecule G3P, which requires the lipid transfer protein DIR1 for activating SAR (Chanda et al. 2011).

The nucleotide ATP has been shown to function in plant ECC as a modulator of cell death and pathogen defense (Chivasa et al. 2005, 2009). Extracellular ATP (eATP) and eNAD(P) appear to play opposite functions in regulating plant immunity. Whereas eATP negatively regulates both cell death and defense response (Chivasa et al. 2005, 2009), eNAD(P) seems to suppress HR-mediated cell death but activate defense response (Fig. 1). Whereas exogenous application of ATP inhibits SA biosynthesis, *PR* gene expression, and disease resistance (Chivasa et al. 2009), exogenous NAD(P) induces all these immune responses (Zhang and Mou 2009). The antagonistic relationship between eATP and eNAD(P) in regulating plant immunity deserves more detailed investigation.

Research in animal cells has established eNAD(P) as a signal molecule in activating intracellular signaling events, including immune responses (Billington et al. 2006b). Our data indicate that eNAD(P) may also function as a signal molecule in plant immune responses (Zhang and Mou 2009). Although it is not clear whether plants employ mechanisms similar to those found in animal cells to process and perceive eNAD(P), results from characterization of *Arabidopsis* transgenic plants expressing the human ectoenzyme CD38 suggested otherwise. Thus far, no proteins similar to animal NAD(P)-utilizing ectoenzymes and cell surface receptors have been identified in

plants (Sanchez et al. 2004; Zolkiewska 2005). Future studies will need to combine genetic and biochemical approaches to uncovering the identities of plant eNAD(P) receptors and downstream components of the eNAD(P) signaling pathway (Zhang and Mou 2008). It is also desired to establish connections between the eNAD(P) signaling pathway and other well-characterized defense signaling pathways in plants.

Fig. 5. Depletion of extracellular NAD(P) by CD38. **A**, NADP levels in extracellular washing fluids collected from wild-type Col-0, *35S::CD38*, *35S::CD38-GFP*, and *35S::E226G-GFP* leaves infected with *Pst/avrRpt2*. **B**, NAD levels in extracellular washing fluids collected from wild-type Col-0, *35S::CD38*, *35S::CD38-GFP*, and *35S::E226G-GFP* leaves infected with *Pst/avrRpt2*. Error bars represent standard deviation, and different letters above the bars indicate significant differences ($P < 0.05$, t test). Experiments were repeated three times with similar results.

Fig. 4. Suppression of exogenous NAD(P)-induced immune responses by CD38. **A**, Induction of *PR1* gene expression by different concentrations of exogenous NAD⁺ in wild-type Col-0, *35S::CD38*, *35S::CD38-GFP*, and *35S::E226G-GFP* plants. The experiment was repeated with similar results. **B**, Growth of *Pseudomonas syringae* pv. *maculicola* ES4326 in Col-0, *35S::CD38*, *35S::CD38-GFP*, and *35S::E226G-GFP* plants with or without NAD⁺ (0.2 or 0.4 mM) pretreatment. Error bars represent standard deviation, and different letters above the bars indicate significant differences ($P < 0.05$, t test). The experiment was repeated three times with similar results.

MATERIALS AND METHODS

Plant materials and growth conditions.

The wild type used was the Columbia ecotype (Col-0). *Arabidopsis* seed were sown on autoclaved soil (Metro-Mix 200; Grace-Sierra, Malpitas, CA, U.S.A.) and vernalized at 4°C for 3 days. Plants were germinated and grown at 23 to 25°C under a regime of 16 h of light and 8 h of darkness.

Plasmid construction and plant transformation.

A pair of primers—*Xho* I-*CD38F1* (5'-CCGCTCGAGATG GCGCAACTGCGAGTTTCAG-3') and *Nco* I-*CD38R* (5'-CATG CCATGGCGATCTCAGATGTGCAAGATG-3')—was used to amplify the coding region of *CD38* from the cDNA clone of *CD38* (provided by A. Yen, Cornell University). The polymerase chain reaction (PCR) products were digested with *Xho*I and *Nco*I and then ligated into the corresponding sites of the vector pRTL2-mGFP, resulting in the plasmid pRTL2-*CD38*-GFP. Then, the 35S::*CD38*-GFP cassette was recovered using *Hind*III and subcloned into *Hind*III-digested and calf-intestinal alkaline phosphatase-treated pCB302 to generate the plasmid pCB302-35S::*CD38*-GFP. Site-directed mutagenesis of the amino acid residue (E226) at the active site of *CD38* was performed in the pCB302-35S::*CD38*-GFP construct using a PCR-based Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The primers used for the site-directed mutagenesis were *CD38MuF* (5'-CACTTTTGGGAG TGTGGGAGTCCATAATTGCAAC-3') and *CD38MuR* (5'-

GTTGCAAATTATGGACTCCCACACTCCCCAAAAGTG-3'). The presence of the expected mutation in the resulting construct was verified by DNA sequencing. A pair of primers—*Sal* I-*CD38F* (5'-ACGCGTCGACATGGCCAACTGCGAGTT CAG-3') and *Sac* I-*CD38R* (5'-CGAGCTCTCAGATCTCAGA TGTGCAAGATG-3')—was used to amplify the coding region of *CD38* from the cDNA clone of *CD38*. The PCR products were digested with *Sal*I and *Sac*I and then ligated into the corresponding sites of the vector pBI1.4T, resulting in the plasmid pBI1.4T-35S::*CD38*. The plasmids were introduced into *Agrobacterium* GV3101(pMP90) by electroporation, and plant transformation was performed following the floral dip method (Bent 2000).

Chemical treatment and pathogen infection.

NADPH-Na, NADP-Na, NADH-Na, NAD-Na, and ATP-Na were dissolved in water, and the pH of the resulting solutions were adjusted to approximately 6.0 with 0.1 M NaOH. The solution was infiltrated into *Arabidopsis* leaves with a 1-ml syringe. For *PR* gene expression, the infiltrated leaves were collected for RNA extraction 24 h after the treatment. For disease resistance test, the infiltrated leaves were inoculated with bacterial pathogens 5 h after the treatment. Infection of plants with *P. syringae* pv. *maculicola* ES4326 was performed as described previously (Zhang et al. 2007). Briefly, *Arabidopsis* leaves were infiltrated with NADPH, NADP⁺, NADH, NAD⁺, ATP, or MgCl₂. After 5 h, the infiltrated leaves were inoculated with the bacterial pathogen ES4326 (optical density at 600 nm [OD₆₀₀] = 0.001). Eight leaves were collected 2 or 3 days post-inoculation to examine the growth of the pathogen. For cADPR and NAADP treatment, *Arabidopsis* leaves were infiltrated with cADPR and NAADP dissolved in 10 μM sodium phosphate buffer (pH 7.0). After 5 h, the infiltrated leaves were inoculated with the bacterial pathogen *Pst/avrRpt2* (OD₆₀₀ = 0.002) for SAR induction.

Ion leakage measurement.

For each treatment or genotype, 20 leaf disks (approximately 7 mm in diameter) were removed 10 min after pathogen infiltration and washed twice with distilled water for a total of 50 min; then, sets of five leaf disks each were placed in 6 ml of fresh water in a test tube. Conductance of the water was measured over time with an Orion 3 Star portable conductivity meter (Thermo Electron Corporation, Beverly, MA, U.S.A.).

RNA and protein analysis.

RNA extraction and RNA gel blot analysis were carried out as described by Cao and associates (1997). Reverse-transcription and quantitative PCR were performed as previously described (Defraia et al. 2010). Protein gel-blot analysis was performed as previously described (Mou et al. 2000).

Membrane fractionation.

Total membrane proteins were obtained by homogenization of 5 g of leaf tissue in extraction buffer (250 mM Tris-HCl [pH 8.5], 290 mM sucrose, 25 mM EDTA, and 5 mM β-mercaptoethanol; and protease inhibitors *N*-tosyl-L-phenylalanine chloromethyl ketone at 50 μg ml⁻¹, 1-chloro-3-tosylamido-7-amino-L-2-heptanone at 50 μg ml⁻¹, and 0.6 mM phenylmethylsulfonyl fluoride) with a mortar and pestle on ice. The homogenate was filtered through cheesecloth and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant (crude lysate) was then centrifuged at 100,000 × g for 2 h at 4°C. The supernatant of this spin was the soluble fraction and the pellet was resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis buffer to form the membrane fraction and subject to protein gel immunoblot analysis.

Fig. 6. Suppression of biological induction of systemic acquired resistance (SAR) by *CD38*. **A**, Expression of *PR1*, *PR2*, and *PR5* in the systemic leaves of wild-type Col-0, 35S::*CD38*, 35S::*CD38*-GFP, and 35S::*E226G*-GFP plants with lower leaves infected with *Pst/avrRpt2* (optical density at 600 nm [OD₆₀₀] = 0.002). **B**, Growth of *Pseudomonas syringae* pv. *maculicola* ES4326 in the systemic leaves of wild-type Col-0, 35S::*CD38*, 35S::*CD38*-GFP, and 35S::*E226G*-GFP plants with lower leaves either infected with *Pst/avrRpt2* (OD₆₀₀ = 0.002) (+SAR) or not infected (-SAR). Error bars represent standard deviation, and different letters above the bars indicate significant differences ($P < 0.05$, *t* test). The experiment was repeated three times with similar results.

Collection of EWF and pyridine nucleotide assay.

EWF was collected as described by Ogawa and associates (1996). Briefly, leaves were inoculated with the bacterial pathogen *Pst/avrRpt2* (OD₆₀₀ = 0.02). When the leaves started to become water soaked (approximately 14 h after the inoculation), they were detached and rinsed three times with water, then dipped in water and subjected to vacuum infiltration. After infiltration, water on the surface of the leaves was carefully removed with paper tissues. The infiltrated water was then collected by centrifugation of the leaves at 500 × g for 5 min, and designated as the EWF. The EWF was immediately subjected to the pyridine nucleotide assay. For each treatment, 108 leaves (approximately 2 g) were used. Pyridine nucleotide assay was performed as previously described (Zhang and Mou 2009). For total NAD and NADP assay, 100 mg of tissue was ground into fine powder in liquid nitrogen. For undenatured samples, 1 ml of extraction buffer (10 mM Tris-HCl, pH 7.0) was added to the powder and the mixture was incubated at room temperature for 5 min. For denatured samples, 1 ml of extraction buffer preheated to 95°C was added to the powder and the mixture was incubated at 95°C for 5 min. After brief vortexing and centrifugation at 16,000 × g for 5 min at 4°C, the supernatants were immediately subjected to the pyridine nucleotide assay.

Microscopy.

Confocal microscopy was performed as described previously (Mou et al. 2003). Protoplasts were produced as described (Zhang and Mou 2009). Propidium iodide staining and plasmolysis were performed as described by Libault and associates (2010). Briefly, 5-day-old *Arabidopsis* seedling roots were stained in a propidium iodide water solution (5 µg ml⁻¹) for 1 min, rinsed with distilled water, and visualized using an excitation wavelength of 543 nm and a 565- to 615-nm emission filter. For plasmolysis, roots were incubated for 10 min in 1 M NaCl solution prior to observation.

Statistical methods.

All statistical analyses were performed with the data analysis tools (*t* test: two samples assuming unequal variances) in Microsoft Excel of Microsoft Office 2004 for Macintosh.

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