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LETTER TO THE EDITOR

FLS2-Mediated Responses to Ax21-Derived Peptides: Response to the Mueller et al. Commentary[W]

In Danna et al. (2011), we reported the surprising result that sulfated synthetic peptide preparations, corresponding to derivatives of the *Xanthomonas oryzae* pv *oryzae* axY^{S}22 peptide, activate a variety of FLAGELLIN-SENSITIVE2 (FLS2)–dependent defense-related responses in *Arabidopsis thaliana* seedlings and plants. These included defense gene expression, an oxidative burst, and protection of seedlings from infection by *Pseudomonas syringae*. The Mueller et al. (2012) Commentary raises the important concern of potential contamination of synthetic peptide preparations with flg22 or flg22-like peptides. Although we had not considered the possibility that commercially synthesized peptides might be contaminated during synthesis, we were very cognizant of the fact that flg22 contamination in our peptide collection would explain our results. We therefore explicitly addressed the issue of contamination using mass spectrometry (liquid chromatography–electrospray ionization–mass spectrometry) analysis to determine the level of potential flg22 contamination in the most active peptide preparation. As explained in more detail below, our biological analysis suggested that contaminating flg22 would have had to be present at levels well over 10 nM to elicit a full protective response in seedlings, yet the liquid chromatography–mass spectrometry demonstrated that no flg22 was present at the 10 nM level of detection. Thus, we concluded that flg22 contamination was not a likely explanation for our results.

What, then, accounts for the experimental differences observed between our data and the data of Mueller et al. (2012)? As explained below, one explanation for the inability of Mueller et al. (2012) to reproduce our results is the clear experimental differences between the two studies. For example, Mueller et al. (2012) did not test the most active Ax21-derived peptide (called axY^{S}22-A1 or A1 in this report). The A1 peptide was originally synthesized as part of a structure/function analysis of Ax21 to determine which residues were essential for activation of rice (*Oryza sativa*) XA21-mediated immunity (Lee et al., 2009). A1 carries an Ala-to-Gly mutation in the N-terminal amino acid of the biologically active axY^{S}22 peptide and retains the sulfated Tyr at position 22 (Lee et al., 2009). The A1 peptide retains the ability to activate XA21-mediated immunity but does not occur in any of the *X. oryzae* pv *oryzae* sequenced genomes. Rather than testing the A1 peptide, Mueller et al. (2012) tested a variant of the peptide that is weakly active in our experimental system. They also used different methods to assess the plant responses than those used in the Danna et al. (2011) study. Finally, Mueller et al. (2012) do not provide evidence that the Ax21 synthetic peptides that they tested have activity in rice. These experimental differences may explain the failure of Mueller et al. (2012) to observe FLS2-dependent defense-related responses.

The Mueller et al. (2012) Commentary shows that at least one commercial preparation of INFLORESCENCE DEFICIENT IN ABSCISSION-like synthetic peptide was contaminated with traces of flg22. Although flg22 contamination potentially explains our data, we believe that the following issues need to be fully evaluated before reaching a final conclusion.

First, as reported by Danna et al. (2011), the peptides used by Mueller et al. (2012) had relatively little activity in our assays. Only the A1 peptide showed activity in our assays at a concentration of 10 μM. However, instead of testing the A1 peptide, Mueller et al. (2012) only tested the axY^{S}22 wild-type peptide and a version of axY^{S}22, called axY22A, in which the sulfotyrosine was replaced by an Ala. These peptides were 10 times less active than A1 in our assays. Is there any biological relevance to our finding that the A1 peptide elicited an immune response in *Arabidopsis*? It is not known whether the A1 peptide is synthesized by phytopathogenic bacteria. Although some bacterial species, such as *Lysinibacillus fusiformis* and *Cytophaga hutchinsonii*, synthesize proteins containing stretches of amino acids with relatively high homology to A1, including a Gly instead of an Ala at position #1 (see Supplemental Figure 1 online), the BLAST expectation value scores (5×10^{-4} and 7×10^{-4}, respectively) are relatively high, and it is not known whether these proteins are secreted and sulfated or have functions similar to the Ax21 protein in *Xanthomonas*.

Second, as noted above, the seedling protection and seedling oxidative burst assays used in our article require a relatively high level of flg22 to elicit a strong response, reducing the likelihood of flg22 contamination being responsible for the reported results. In response to concerns raised by Mueller et al. (2012), we repeated previous flg22 dose–response experiments to determine the minimum concentration of flg22 required to elicit a detectable level of protection from *P. syringae* infection in the *Arabidopsis* seedling protection assay. We focused our attention on this assay because we think it is the most physiologically relevant (i.e., a response to an immune elicitor that confers actual resistance to pathogen attack). Confirming previous data, this experiment showed that a significantly detectable response ($P \leq 0.001$) was obtained with concentrations of flg22 higher than 10 nM (see Supplemental Figure 2 online) and a full response, similar to that obtained with 1 μM flg22 as reported by Danna et al. (2011), was only obtained with a dose greater than 100 nM. Thus, flg22 contamination of the A1 peptide at a concentration greater than 100 nM would have been required to elicit the defense-related responses reported by Danna et al. (2011). However, contamination of the A1 peptide at this level appeared to be ruled out by liquid chromatography–

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electrospray ionization–mass spectrometry analysis, which showed that the maximum level of flg22 contamination, if any, was most likely less than 10 nM (see Supplemental Figure 6 in Danna et al., 2011). In additional experiments performed using matrix-assisted laser desorption/ionization–mass spectrometry while preparing this rebuttal, we found that one part flg22 could be detected when mixed with 1000 parts A1 (see Supplemental Figure 3 online). These latter data are important because, as discussed above, the difference in effective concentrations between flg22 (>100 nM) and the A1 peptide (~10 μM) was at most 100-fold (Danna et al., 2011). We are actively working to increase the sensitivity of our analytical mass spectrometry techniques to detect flg22 contamination at levels lower than 1 part in 1000.

Third, Mueller et al. (2012) did not use the same assays that we used to monitor the activity of Ax21-derived peptides. Mueller et al. (2012) used a cell tissue culture-based assay for monitoring flg22-elicited alkalinization of the medium. Although this tissue culture assay is very sensitive for monitoring flg22, the physiological and biological relevance of the assay is not known. Furthermore, there is no compelling reason to assume that the assay is also highly sensitive for monitoring the activity of Ax21-derived peptides. For example, if A1 perception requires a coreceptor that is not expressed in tissue culture cells, or if A1 fails to assume a proper three-dimensional conformation under the assay conditions employed, the tissue culture assay would lead to different results. Other unidentified lab-to-lab differences in experimental setup could also explain the negative results of Mueller et al. (2012). A related issue is that different FLS2-dependent flg22 assays require widely different levels of flg22 to observe flg22-mediated responses. For example, in the seedling assay, flg22 levels as low as 1 nM elicit robust expression of particular defense-related genes, whereas the protection assay requires more than 100 nM flg22 to elicit a full protective response. This appears to make biological sense because plants are constantly exposed to microbes in nature and it might be disadvantageous for plants to respond to microbes with a full energy-demanding protective response unless large numbers of microbes are detected. These data and reasoning suggest that there are unknown variables that influence the sensitivity of various flg22-mediated signaling events. Thus, the failure of the less active peptides used by Mueller et al. (2012) to elicit a response in their tissue culture assay cannot be used to logically conclude that the A1 peptide does not elicit a FLS2-dependent response in our seedling assays or that FLS2 is blind to all Ax21 peptide derivatives. A related issue is that it is not known what concentrations of Microbe-Associated Molecular Patterns are required to elicit a significant defense response under natural conditions.

Fourth, if flg22 contamination in our assays was responsible for the defense-related responses we observed, it would have had to be limited to the Ax21-derived peptide preparations, since elf26 peptide (in an *efr* mutant background) and flg22-Δ2 or flg22$^{A.tum}$ (*Agrobacterium tumefaciens*) peptides (in Columbia-0 wild-type plants) had no activity in the seedling protection assay at 100 μM (Danna et al., 2011; see Supplemental Figures 2 and 3 online). Moreover, three independent preparations of the A1 peptide from two different suppliers exhibited activity in the seedling protection assay.

In summary, the data reported by Danna et al. (2011) showed that synthetic *X. oryzae* pv *oryzae* Ax21-derived peptides elicited a variety of FLS2-dependent responses. Mass spectrometry analysis of A1 peptides and flg22 dose–response experiments indicate that these results are difficult to explain by flg22 contamination. Still, flg22 contamination is a possibility that we cannot categorically exclude, especially in light of the failure of Mueller et al. (2012) to detect any activity with the axY^{S}22 or axY22A peptides in their tissue culture assay. Indeed, the data presented by Mueller et al. (2012) are difficult to reconcile with our data unless one assumes that the response in cultured cells is specific to the A1 peptide, which was not tested by Mueller et al. (2012), or that tissue culture cells, in contrast with seedlings, simply cannot perceive Ax21-derived peptides. In this regard, although we stated in Danna et al. (2011) that it is possible that the A1 peptide is an FLS2 ligand, we also stated that it is possible that additional receptor-like kinases may partner with FLS2 to form heterodimers or other multimers that are required for A1 peptide recognition. We also stated that FLS2 might function as a coreceptor for another protein that is the actual receptor for A1. It is possible that these postulated A1 receptors are expressed in whole plants but not in tissue culture cells. To fully resolve whether Ax21-derived peptides can activate FLS2 signaling pathways, it will be necessary to order new batches of the flg22 and A1 peptides, carry out dose–response experiments in the seedling protection and cell culture alkalinization assays, and subject the peptides to careful analysis by mass spectrometry. Ideally, these experiments would be performed simultaneously with the same peptides in different laboratories.

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SUPPLEMENTAL DATA

The following materials are available in the online version of this article.

Supplemental Figure 1. Alignment of axY^S22-A1 and Two Naturally Occurring Proteins from *Lysinibacillus fusiformis* and *Cytophaga hutchinsonii*.

Supplemental Figure 2. flg22 Dose–Response Curve in the Seedling Protection Assay.

Supplemental Figure 3. Mass Spectrometry Analysis of Mixtures of flg22 and axY^S22-A1 Peptides.

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AUTHOR CONTRIBUTIONS

C.H.D., X.-C.Z., and A.K. performed the experiments described in the supplemental figures. C.H.D., A.F.B., P.C.R., and F.M.A. wrote the article.

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