Phosphoproteome Analysis of *Lotus japonicus* Roots Reveals Shared and Distinct Components of Symbiosis and Defense

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Plant roots form an intracellular symbiosis with nitrogen-fixing bacteria while maintaining the capacity for defending themselves against bacterial pathogens. To investigate the molecular relationship between these opposing cellular responses, we compared changes in the root phosphoproteome of the legume *Lotus japonicus* occurring within minutes after perception of nodulation factor (NF), a symbiotic signaling molecule, to those elicited by flagellin peptide (flg22), a conserved pathogen-associated peptide motif present in flagellar protein of a wide range of bacteria. Phosphoproteins were visualized by autoradiography of two-dimensional polyacrylamide gels after *in vivo* labeling with ³³P-orthophosphate. Comparisons of NF- and flg22-induced phosphoprotein patterns revealed signal-specific responses but also a surprisingly large overlap. Specificity of the responses was observed because the NF receptor kinases NFR1 and NFR5 were both required for NF- but not for flg22-mediated changes in the phosphoproteome. Moreover, NF did not stimulate an oxidative burst or activation of mitogen-activated protein kinases, two common markers for early defense responses that were induced by flg22. Inhibitor studies revealed that phosphorylation of at least some of the proteins in response to NF requires phospholipase D (PLD) whereas regulation of the flg22 phosphoproteome is PLD-independent. Although plant signal transduction during symbiosis and defense utilizes distinct components, phosphorylation of overlapping sets of proteins is achieved.

The symbiosis between roots of legumes and rhizobia is manifested by the development of a specialized plant organ, the root nodule. In exchange for ammonia, originating from fixation of atmospheric nitrogen by the bacteria, the plant supplies all other nutrients, including carbon sources. Although a

single infected root nodule cell can harbor hundreds of bacteria, they are not attacked by the plant's immune system. This remarkable feature of endosymbiosis has motivated research into the interplay between symbiotic and defense pathways in plants.

Symbiotic and defense responses can both be triggered by the same class of plant-specific receptors, the receptor-like kinases (RLK) (Shiu et al. 2004). Rhizobial lipochitooligosaccharides or nodulation factors (NF) induce symbiosis-related responses when applied to legume roots. In *Lotus japonicus*, two RLK, NFR1 and NFR5 (Madsen et al. 2003, 2011; Radutoiu et al. 2003) are likely to be directly involved in NF perception. Several of the symbiotic signal transduction components downstream of NF receptors have been defined by genetics, including a calcium and calmodulin-dependent protein kinase (Hayashi et al. 2010; Madsen et al. 2010). On the other hand, flg22, a peptide derived from a conserved region of the bacterial flagellum-protein, flagellin, activates plant defense responses (Boller and Felix 2009). In *Arabidopsis*, the perception of flagellin requires the FLS2 RLK, a direct receptor for the flg22 ligand (Chinchilla et al. 2006). In addition, mitogen-activated protein kinases (MAPK) have been implicated in the defense-related pathway downstream of flg22 perception (Bethke et al. 2009; Ichimura et al. 2006).

To analyze the interplay between symbiosis and defense, we needed a model system that responded to both symbiotic and defense-related stimuli. Unfortunately, the model plant *Arabidopsis* does not form any root symbiosis and is not suitable for this analysis. Therefore, we moved to the model legume *L. japonicus*. As a first step, we established that this plant also responds to the flg22 peptide. We then compared phosphorylation patterns of root proteins in response to NF and flg22 using phosphoproteomics. Phosphoproteome analysis by two-dimensional (2-D) gels allows the simultaneous display of proteins undergoing rapid changes in the rate of phosphorylation. The high reproducibility of 2-D gels allows direct comparisons of phosphoproteomes between different samples or treatments. This approach has been successful in identifying both soluble (Peck et al. 2001) and membrane-localized (Nühse et al. 2003) proteins of *Arabidopsis* that are rapidly phosphorylated in response to treatment with flg22. In the present work, we adapted the procedures developed for suspension cell cultures of *Arabidopsis* to detect changes in phosphorylation patterns in roots of *L. japonicus*. We observed a surprising level of conservation between NF- and flg22-induced phosphoproteomes, indicating that symbiotic and defense elicitors target a set of common proteins for phosphorylation.

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RESULTS

NF elicit reproducible changes in the phosphoproteome of roots from *L. japonicus*.

To examine changes in phosphorylation associated with NF signaling, roots from intact plants were incubated with ^{33}P orthophosphate to in vivo pulse-label phosphoproteins. Changes in the phosphoproteome were monitored by isolating the proteins, separating them on 2-D gels, and examining the phosphorimage of dried gels. To optimize the in vivo labeling of proteins, we germinated and grew seedlings in a minimal medium lacking phosphate (labeling medium [LM]). From preliminary analyses in which we examined different combinations of radioactive labeling times in coordination with NF elicitation, we concluded that co-treatment of ^{33}P orthophosphate and NF for 10 min gave the optimum contrast between radioactive incorporation into the responsive phosphoproteins and the constitutively phosphorylated proteins. Using these conditions, phosphoproteomes of *L. japonicus* roots were examined.

As a negative control for NF treatment, equivalent fractions from the reverse-phase chromatographic isolation as used for NF isolation were used from a *Mesorhizobium loti* mutant strain deficient for the production of NF. The phosphoproteome from the negative control treatment did not change compared with the untreated samples, indicating that no general nonspecific elicitor was present in these fractions.

Quantitation of radioactive incorporation.

Quantitation of changes in the phosphoproteome required standardization against constitutively phosphorylated proteins to control for potential variation in uptake of the radioactive orthophosphate in each experiment. Visual inspection of phosphorimaged gels from three biological experiments indicated numerous phosphoproteins that did not appear to change intensities. Densitometric quantitation of six of these spots in each experiment, including the two indicated by squares as controls in Figure 1, confirmed that the relative ratios of these control spots did not show any significant changes during any treatment

Fig. 1. Phosphoproteome comparison of *Lotus japonicus* roots. In vivo radiolabeled soluble proteins were extracted from roots 10 min after treatment and separated on 18-cm two-dimensional (2-D) gels (acidic side to the right). **A to D.** Labeled spots include constitutively phosphorylated control proteins (squares), proteins differentially phosphorylated in response to both nodulation factor (NF) and flg22, and phosphoproteins specifically responding to flg22, prefixed with F. Only proteins showing consistent changes in three independent experiments are marked. **A.** Phosphorimage of 2-D gel (IEF pH 4 to 7) of root phosphoproteome after control treatment. **B.** Phosphorimage of 2-D gel (IEF pH 4 to 7) of root phosphoproteome after NF treatment. **C.** Phosphorimage of 2-D gel (IEF pH 4 to 7) of root phosphoproteome after flg22 treatment. **D.** Analysis of protein co-migration on high-resolution 18-cm single isoelectric point (pI) unit 2-D gels. Phosphorimage of soluble proteins extracted from roots treated with NF (top), flg22 (middle), or a mixture of NF and flg22 (bottom). Numbering and labeling of spots are as in B and C. **E.** Densitometric analysis of phosphate incorporation into candidate spots after treatment with NF (dark bars) or flg22 (light bars). Values represent the fold change compared with control treatments. Results are average of three independent experiments $\pm$ standard deviation. Spot numbers correspond to labels in B and C. All values are $P < 0.05$, except for spots 6 and 12 (labeled with an *), for which $P < 0.1$ for the NF response.

conditions (average ratios in all three experiments = 1.03 ± 0.23). Therefore, these internal spots were used as reference values for relative quantitation for comparisons between gels.

Quantitation of differentially phosphorylated proteins was performed using two of these constitutively phosphorylated proteins (Fig. 1A and B, squares) as internal controls. A spot was considered to be reproducibly changing if the ratio of spot intensity in all three biological experiments showed a greater than 1.69-fold change (i.e., two confidence intervals from the 1.23-fold average of the controls). Spots consistently changing in intensity after treatment of roots with NF are shown in Figure 1A and B. The average fold change in ratio from all three experiments for NF-responsive phosphoproteins is shown in Figure 1E (dark bars). These data demonstrate that NF induced rapid and reproducible changes in the phosphoproteome from roots of *L. japonicus*.

Phosphoproteome comparisons of symbiosis and defense.

To investigate the possible interplay between symbiosis and defense, we first needed to establish a system in *L. japonicus* that could serve as a defense model system. We therefore tested whether *L. japonicus* roots respond to flg22, a well-characterized elicitor of defense responses in *Arabidopsis*. We could observe a range of defense-related responses of *L. japonicus* to flg22 (discussed below). These results suggest that flg22 is also perceived by *L. japonicus* and leads to similar physiological responses as in *Arabidopsis*. Therefore, we proceeded to investigate the changes in the phosphoproteome in *L. japonicus* in response to flg22. Surprisingly, all 13 of the NF-induced phosphorylated proteins are contained within a subset of proteins differentially phosphorylated in response to treat-

ment with 100 nM flg22 (Fig. 1C), with an additional 6 spots specific for flg22 responses (Fig. 1C).

We investigated whether the similarities in complex phosphoproteomes arose from different proteins with similar electrophoretic characteristics that could not be resolved on the three isoelectric point (pI) unit gradients (pH 4 to 7) used in these experiments. Thus, we performed co-migration experiments using high-resolution 18-cm, single pI unit 2-D gels. Proteins were prepared for phosphoproteome analysis from roots treated with NF or flg22 and separated on 2-D gels either individually (representative gel regions) (Fig. 1D, top two panels) or after combining both fractions (Fig. 1D, bottom panel). The fact that all detectable phosphoproteins from NF- and flg22-treated samples perfectly co-migrated under high-resolution conditions provided strong evidence, if not proof, that identical differentially phosphorylated proteins were detected in both NF- and flg22-treated samples. Quantitative comparison of the NF- and flg22-regulated phosphoproteome showed that flg22 generally evoked stronger increases in radioactive incorporation, although some of the spots showed similar fold change (e.g., spots 11 and 13) (Fig. 1E).

Changes in the NF phosphoproteome require the receptor-like proteins NFR1 and NFR5.

The observed overlap of phosphorylation targets could be an artifact caused by contamination of the NF preparation with other defense elicitors (e.g., lipopolysaccharides). Alternatively, it could be a consequence of the structural relatedness between NF and chitin fragments, defense elicitors from fungal cell walls (Petutschnig et al. 2010). Both possibilities were eliminated by analysis of the root phosphoproteome of plant mutants carrying defective alleles of the NFR1 (*nfr1-1*) or NFR5 (*nfr5-2*). These LysM RLK are required for all symbiotic responses investigated but *nfr1* and *nfr5* mutants are still responsive to treatment with chitin fragments (Radutoiu et al. 2003), indicating that independent chitin receptors must be present. All NF-induced changes in the phosphoproteome seen in wild-type plants (Fig. 2A and B, representative regions) were abolished in the *nfr1* mutant (Fig. 2C) and *nfr5* mutant (data not shown). These results demonstrate that changes in the NF-responsive phosphoproteome were dependent on NFR1 and NFR5. Therefore, NF responses were not arising from cryptic defense elicitor activity in the NF preparation.

NF do not activate MAPK or an oxidative burst.

MAPK are well-characterized signaling components which, in *Arabidopsis*, are activated after treatment with flg22 (Asai et al. 2002; Nühse et al. 2000). Using in-gel kinase assays with myelin basic protein as a general substrate, we found that protein extracts from *L. japonicus* roots treated with flg22 for 10 min contained activated protein kinases with relative molecular masses of 44 and 46 kDa (Fig. 3A), consistent with the size expected for MAPK. These kinases were not activated in response to NF (Fig. 3A). Additional MAPK may be activated but not refolded properly in the in-gel assay. Therefore, we also performed immunoblot assays with monoclonal antibodies which specifically react with the di-phosphorylated form of activated MAPK (Fig. 3B). These experiments again showed activated MAPK of 44 and 46 kDa only in the flg22-treated samples. Thus, NF did not appear to activate MAPK. We were unable to determine whether the flg22-specific phosphoproteins shown in Figure 1C were downstream of MAPK because two different inhibitors of the upstream MAPK kinase, PD98059 and U0126, only blocked a small percentage of MAPK activity in *L. japonicus* roots (data not shown).

Induction of an oxidative burst has also been correlated with the response of plants to pathogens (Levine et al. 1994). In

Fig. 2. Phosphoproteome responses to nodulation factor (NF) in an *nfr1* mutant. Representative regions of phosphorimages of two-dimensional gels of root phosphoproteomes. **A**, Wild-type (WT) control; **B**, WT treated with NF; and **C**, mutant homozygous for the *nfr1-1* allele (Radutoiu et al. 2003) treated with NF. **D**, Densitometric analysis of spots of WT (dark bars) or *nfr1* mutant (light bars) treated with NF.

L. japonicus roots, flg22 treatment resulted in the accumulation of reactive oxygen species (ROS) but we failed to detect any accumulation after application of NF (Fig. 3C).

A phospholipase D inhibitor, *n*-butanol, alters phosphoproteome in response to NF but not flg22.

One of the earliest responses to plant-defense elicitors and to NF is an accumulation of the lipid-signaling molecule, phosphatidic acid (PA). PA can be synthesized upon phospholipase C (PLC) stimulation in the case of flg22 or chitotetraose elicitors (van der Luit et al. 2000) or by co-activating PLC and phospholipase D (PLD) in the case of NF (den Hartog et al. 2001, 2003). Thus, inhibition of PLD by *n*-butanol may differentially inhibit components of the NF versus flg22 phosphoproteomes. Indeed, *n*-butanol blocked radioactive incorporation into 9 of the 16 common phosphoproteins (Fig. 3D, spot 13) in response to NF whereas other spots were not affected (Fig. 3D, spot 11). No phosphoproteins responsive to flg22 were affected (data not shown). A summary of the NF-responsive spots inhibited by PLD is presented in Figure 4. Thus, even though both NF and flg22 stimulate the phosphorylation of identical target proteins, only a subset of the NF-induced responses were inhibited by *n*-butanol.

Fig. 3. Separation of nodulation factor (NF) and flg22 responses. **A** and **B**, Mitogen-activated protein kinase (MAPK) activation by flg22 but not by NF **A**, In-gel kinase assays with proteins extracted 10 min after elicitation with either NF or flg22. **B**, Immunoblots activated MAPK were detected with a monoclonal antibody against the characteristic pTEpY motif. Proteins from NF- or flg22-treated roots were extracted at the time points indicated, resolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis, and blotted to nylon filters for detection. Ponceau S staining of the blot is shown as control for equal protein loading. **C**, Induction of an oxidative burst in *Lotus japonicus* roots in a medium with luminol and peroxidase after treatment with either NF or flg22. **D**, Inhibition of phospholipase D (PLD) blocks a subset of the phosphoproteome response to NF. *L. japonicus* seedlings were treated with and without NF in the presence or absence of 0.5% *n*-butanol. Representative regions of phosphorimages of two-dimensional gels are shown.

DISCUSSION

In planta phosphoproteome analysis.

Plants have evolved specific types of receptors, RLK, which are committed to survey the cellular environment for developmental cues. The kinase domains of RLK are closely related and form a monophyletic group (Shiu et al. 2004) and it is an interesting question how signaling specificity downstream of such receptors is mediated. As a first step in detecting molecular specificity downstream of RLK, we choose to investigate two opposing developments, symbiosis and defense, which both can be triggered by RLK. The RLK NFR1 and NFR5 mediate the perception of bacterially secreted NF for symbiotic interactions (Madsen et al. 2003; Radutoiu et al. 2003) while others, such as FLS2 (Chinchilla et al. 2006), recognize pathogen-associated molecular patterns such as flagellin and activate defense reactions. To examine RLK-induced changes in protein phosphorylation, we developed a sensitive and reproducible method to label and display phosphoproteins of *L. japonicus* roots. In the current study, we applied this method to compare responses to symbiosis-related (NF) and defense-related (flg22) signaling molecules. However, the method should be directly applicable to the study of signaling pathways in response to a range of nutritional, hormonal, abiotic, and biotic stimuli in *L. japonicus* roots and be adaptable to other plant species.

Treatment of roots with NF resulted in defined and reproducible changes in protein phosphorylation within 10 min. At least three genes required for nodulation encode kinases, which indicated that protein phosphorylation is a likely event following NF perception (Endre et al. 2002; Gleason et al. 2006; Madsen et al. 2003; Radutoiu et al. 2003; Stracke et al. 2002; Tirichine et al. 2006). Here, we provide direct evidence that such phosphorylation responses downstream of NFR1 and NFR5 indeed occur. The 16 phosphoproteins influenced by NF treatment represent new molecular markers for the genetic dissection of symbiotic signaling. Previously, the only early responses that could separate genetic mutants included membrane depolarization, ion fluxes, and accumulation of PA, which all occur within the first 1 to 2 min, and calcium spiking, which starts approximately 10 min after NF treatment. Thus, the

Fig. 4. Model summarizing phosphoprotein responses to nodulation factor (NF) and flg22. Perception of NF by NFR1 and NFR5 leads to phosphorylation of proteins (corresponding spot numbers 1 to 13), of which a subset is sensitive to inhibition by *n*-butanol, indicating a requirement for phospholipase D (PLD) (4, 5, 6, 7, 8, 10, 13). Recognition of flg22 by a hypothetical *Lotus* FLS2 also leads to responses of all NF-responsive phosphoproteins but via a PLD-independent mechanism, as indicated by resistance to *n*-butanol treatment. In addition, flg22 results in phosphorylation of additional proteins (spot numbers F1-F6), as well as activation of mitogen-activated protein kinases.

analysis of the phosphoproteome in different mutant backgrounds will facilitate a more refined dissection of the early molecular response pathway during symbiosis. Identification of differentially phosphorylated proteins is an obvious next step in the analysis. Although it appears relatively straightforward to elute individual proteins from 2D gels and identify them by mass spectrometry, this alone would not be sufficient in the present case. The confirmation that protein identities obtained from mass spectrometry correspond to the differentially phosphorylated proteins of interest requires significant additional experimentation, such as immunodepletion experiments with antibodies against the candidate protein or analysis of plant lines expressing no or modified versions of the candidate protein. Therefore, both of these approaches exceeded the scope of the present study.

Overlapping phosphorylation patterns via distinct signaling components.

Perhaps the most surprising finding was that NF-responsive phosphoproteins are also phosphorylated in response to the bacterial defense elicitor, flg22. These results are consistent with previous microarray studies that found transcriptional regulation of several genes shared between plant-microbe and plant-pathogen interactions (Mitra et al. 2004). Thus, the similarity in phosphoproteomes between NF and flg22 reflect a high level of conservation between defense and symbiotic response pathways. Because flg22 responses still occur in *nfr1* and *nfr5* mutant plants, this convergence of pathways appears to be downstream of specific RLK (Fig. 4). However, the fact that NF did not elicit changes in the phosphoproteome in *nfr1* or *nfr5* mutants and did not activate two well-characterized defense responses, activation of MAPK and formation of ROS, clearly demonstrates that perception of NF and signaling downstream are specific and distinct from pathogen-associated molecular pattern-triggered defense signaling. Interestingly, a functional comparison of the *Arabidopsis* chitin receptor kinase CERK1 and the *L. japonicus* NF receptor NFR1 revealed peptide motifs within the kinase domain that were responsible for symbiosis-specific downstream signaling (Nakagawa et al. 2011). An important question that remains to be resolved is whether these pathways both occur in the same cell types.

Interestingly, even though the two elicitors lead to phosphorylation of the same proteins, we found that inhibition of PLD with *n*-butanol blocked phosphorylation of approximately half the proteins in response to NF but not flg22. One interpretation of this difference is that different kinases phosphorylate the same substrates. NF activates both PLC and PLD (den Hartog et al. 2001, 2003) whereas flg22 only activates PLC (van der Luit et al. 2000). Although both phospholipases produce PA, a potential signaling molecule, the PA produced is not equivalent. PA from PLC comes from phosphorylation of diacylglycerol (DAG) by DAG kinase whereas PA from PLD comes directly from the structural lipids (Arisz et al. 2009). Therefore, PA will have different fatty acid compositions that may activate unique kinases. Further specificity may arise from differential localization of PLC and PLD activity within the cell and from differential effects on the MAPK pathway (Testerink et al. 2007).

In other systems, activation kinetics and the duration of the response are known to coordinate signaling events leading to vastly different biological responses. For instance, the commitment to differentiation or apoptosis in mammalian thymocytes is thought to be regulated by differences in the kinetics and amplitude of kinase activation, possibly by activating different sets of transcription factors (Werlen et al. 2003). In a similar fashion, biologically different outcomes may arise from the strength of signals generated by different RLK in symbiosis (NFR1 and NFR5) as opposed to defense (FLS2).

Another explanation exists for generating different biological outcomes (i.e., symbiosis or defense) from utilization of the same response components. Even though the same protein may be phosphorylated during two different responses, phosphorylation does not have to occur on the same residues. Phosphorylation of a protein alters its pI and causes its position to shift to a more acidic region of a 2-D gel. The amount of the shift is generally not dependent on the particular residue phosphorylated; therefore, the perfect co-migration we observed between NF- and flg22-phosphorylated proteins only demonstrates that these are the same proteins, not that they are phosphorylated on the same residues. Numerous cases have been documented in which protein phosphorylation on different residues results in very different biological outcomes (e.g., mammalian Raf kinase) (Marshall 1995) either by differentially altering protein activity or by leading to formation of new multiprotein complexes. Addressing these possibilities will require not only identifying the individual components but also determining the precise residues phosphorylated during each response.

MATERIALS AND METHODS

In planta labeling and treatments.

NF were isolated from supernatants of *M. loti* R7A (Sullivan et al. 2002) by reverse-phase chromatography (Firmin et al. 1993), and quality control was performed by SELDI-MS (Ciphergen, Fremont, CA, U.S.A.). Supernatant preparations from an NF-deficient *M. loti* strain R7AC2 (nodC::Tn5 mutant) (J. T. Sullivan and C. W. Ronson, *personal communication*) were used as controls. *L. japonicus* L. ecotype Gifu seed were scarified by immersion in concentrated H₂SO₄ for 10 min and washed with sterile water. Sterilized seed were transferred to LM (0.9 mM CaCl₂, 0.5 mM MgSO₄, 20 mM ferric citrate, 0.8 mM MnCl₂, 0.6 mM CuSO₄, 0.7 mM ZnCl₂, 1.6 mM H₃BO₃, 0.54 mM Na₂MoO₄, and 5 mM MES) containing aminoethoxyvinylglycine (0.1 mg/liter) and 0.6% agar, pH 6.8. Five days after germination, 10 seedlings were transferred to 12-well microtiter plates containing LM, pH 5.5. Treatments with the elicitors (100 nM flg22 or 100 nM NF) were carried out in the presence of radioactive ³²P orthophosphate (1,850 kBq/ml) for 10 min. PLD inhibition studies were performed by adding *n*-butanol to the medium to 0.5% and incubating for 15 min before labeling as described above.

Protein methods.

After treatments, roots were rapidly dissected from seedlings and proteins were extracted from roots in 2-D homogenization buffer as described previously (Peck et al. 2001). Acetone protein pellets were resuspended in 2-D gel loading buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% immobilized pH gradient (IPG) buffer, and 2% dithiothreitol). Isoelectric focusing of 400 µg of protein on 18-cm IPG strips (pH 4 to 7) was performed using an IPGPhor first-dimension electrophoresis unit (GE Healthcare, Amersham, U.K.) using a program of 1 h at 500 V, 1 h at 1,000 V, 10-h gradient to 4,500 V, and 12 h at 4,500 V. Co-migration experiments were performed as above except that 18-cm single pI unit gels spanning pH 4.5 to 5.5 and pH 5.0 to 6.0 were used for the first dimension. The second dimension and silver staining were as previously described (Peck et al. 2001).

Densitometric analysis of phosphate incorporation.

Volumetric quantitation of candidate spots was performed using an AIDA Image Analyser (version 3.0; Raytek Scientific, Sheffield, U.K.). The readings were normalized to internal controls (two per gel) and represented as a fold change com-

pared with control gels. The data used for the analysis represent the average of three independent experiments.

In-gel kinase assays, immunoblots, and ROS assays.

Proteins were isolated from treated roots in Laemmli buffer supplemented with 10 nM calyculin and 100 μ M sodium orthovanadate. In-gel kinase assays with 50 μ g of protein and myelin basic protein as substrate were performed as previously described (Nühse et al. 2003). Immunoblots were performed using polyvinylidene difluoride and monoclonal anti-MAPK, activated (pTEpY) (Sigma-Aldrich, St. Louis) according to the manufacturer's instructions. ROS production was measured with a luminol-based assay imaged with Photech high-resolution photon counting system (HRPCS 218, camera model 6045-2/2149-1; Photech, <http://www.photech.com>) as described previously (Nühse et al. 2007).

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