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ORIGINAL PAPER

Hydrogen peroxide is not involved in HrpN from *Erwinia amylovora*-induced hypersensitive cell death in maize leaves

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Abstract Harpin elicits rapid and localized programmed cell death in plants, also known as the hypersensitive response (HR). Here we report that HrpN from *Erwinia amylovora* led to rapid cell death in maize leaves within 24 h and also induced the expression of systemic acquired resistance genes, such as *ZmPR1* and *ZmPR5*. Surprisingly, the results of DAB staining showed that there was no H_2O_2 accumulation in maize leaves during the HR process, and semi-quantitative RT-PCR revealed that there was also no difference in the expression of the *ZmRboh* genes. These results suggest that HrpN-induced cell death may be independent of H_2O_2 accumulation in maize leaves.

Keywords Cell death · HrpN · Hypersensitive response · Maize · H_2O_2 accumulation

Introduction

Plants deploy a variety of active defense responses when challenged by pathogens or elicitors. One of the active defense mechanisms is rapid cell death, also known as the hypersensitive response (HR) (Dangl et al. 1996; Greenberg and Yao 2004; Lamb and Dixon 1997), which contributes to pathogen limitation.

It is now apparent that one of the earliest events during plant HR is a rapid oxidative burst, caused by the release of reactive oxygen species (ROS) by elicited plant cells (Lamb and Dixon 1997; Levine et al. 1994). Pharmacological, molecular, and genetic studies strongly support the idea that the primary source of ROS is a superoxide (O_2^-) generating membrane-bound NADPH oxidase. Diphenylene iodonium (DPI), which is an inhibitor of NADPH oxidase, blocks the fungal elicitor-stimulated oxidative burst in plant cells (Desikan et al. 1996; Lamb and Dixon 1997).

Harpin protein HrpNEa coding by the *hrp* gene clusters secreted by *Erwinia amylovora* via its type III secretion system (T3SS) was one of the first bacterial elicitors characterized as inducing activation of MAPK cascades, cell death and systemic acquired resistance (SAR) (Desikan et al. 1999, 2001; Dong et al. 1999; Jang et al. 2006; Reboutier et al. 2007; Wei et al. 1992). Recently, Sinn et al. (2008) reported that the C-terminal half of HrpNEa plays a specific role in its secretion by the T3SS and in its virulence and avirulence activities while the N-terminal half of the protein was sufficient for cell-free elicitor activity. Transcription study showed that harpin may be involved in regulation of cell wall biogenesis, cellular communication in *Arabidopsis* (Livaja et al. 2008). Several studies have suggested the role of salicylic acid (SA) and ethylene-mediated signaling pathway in harpin-induced plant growth and defense response (Chuang et al. 2010; Dong et al. 1999; Liu et al. 2010; Peng et al. 2003; Samuel et al. 2005). Additionally, Methyl jasmonate treatment inhibited the harpin-induced cell death, H_2O_2 generation and phenylalanine ammonia-lyase (PAL) gen expression (Andi et al. 2001).

Harpin treatment also leads to H_2O_2 accumulation during HR in *Arabidopsis* and tobacco. It is now apparent that this oxidative burst is mediated by an NADPH oxidase-like enzyme in *Arabidopsis* and tobacco (Desikan et al. 1996; Zhang et al. 2009). In several previous studies, DPI

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treatment inhibited harpin-induced H_2O_2 accumulation (Ichinose et al. 2001; Xie and Chen 2000). It is also reported that harpin-induced H_2O_2 production is also from the chloroplasts and mitochondria, and H_2O_2 production by the mitochondrial electron transfer chain is less than chloroplastic H_2O_2 generation in photosynthetic tissues. However, several papers have previously reported that mitochondrial H_2O_2 has more important role rather than H_2O_2 generation from NADPH oxidases and H_2O_2 accumulation in the chloroplasts in harpin-induced cell death in *Arabidopsis* and tobacco (Garmier et al. 2007). Furthermore, alternative oxidase (AOX), a mitochondrial superoxide production oxidase, activity and transcripts level have been observed in plant during elicitor-induced cell death (Simons et al. 1999; Vidal et al. 2007). However, the relationships between H_2O_2 production and the cell death have never been established. A study with inhibitors has suggested that the accumulation of H_2O_2 is not necessary for harpin-induced cell death in tobacco suspension culture (Ichinose et al. 2001).

To our knowledge, the previous reports of the effect of HrpN mainly focus on dicotyledons, such as *Arabidopsis* and tobacco, there is no study showing the effect of purified HrpN in monocotyledons. In this study, we aimed to investigate the effect of purified HrpN on cell death and H_2O_2 production in maize leaves. The present data indicates that HrpN induces HR and SAR genes, but does not lead to H_2O_2 accumulation in maize leaves.

Materials and methods

Plant material and treatments

Maize seedlings (*Zea mays* L. cv. Zhengdan 958) were cultured in Hoagland's solution (pH 6.0) under hydroponics greenhouse condition at 22/26°C (night/day) with a photosynthetic active radiation of 200 μmol m^{-2} s^{-1} and a photoperiod of 14/10 h (day/night) for 2 weeks at 3-leaf stage. Tobacco (*Nicotiana tabacum* cv. NC 89) plants were grown for 6 weeks in soil under controlled environmental condition at a photoperiod of 16/8 h (day/night) at a temperature of 20/25°C (night/day).

$Harpin_{Ea}$ protein was a gift from Dr Han-song Dong, and it was prepared and purified as described (Dong et al. 1999). The first fully expanded leaves of maize, and the third and fourth fully developed leaves of tobacco at 6-week-old were infiltrated with different concentrations of HrpN in TE buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA) using a 1-ml hypodermic syringe without a needle. The infiltrated zones were either observed for necrosis development or harvested using a scalpel and stored at −80°C for further analysis.

Detection of cell death in maize leaves using Evans blue staining

Cell death was detected in the leaves by staining with Evans blue according to the method described by Liu et al. (2008). Leaves were taken at the indicated times and soaked in 10 ml of 0.25% Evans blue. They were then washed briefly in 10 ml of water. Leaves were destained in boiling 96% ethanol for 10 min. Then the leaves were transferred to a 60% glycerol solution.

Histochemical detection of H_2O_2

The H_2O_2 was visually detected in the plant leaves using 3,3-diaminobenzidine (DAB) as a substrate. Briefly, the leaves were infiltrated with HrpN and then immersed overnight in a 1 mg ml^{-1} solution of DAB (pH 3.8). The leaves were then decolorized by boiling in ethanol (96%) for 10 min. After cooling, the leaves were extracted at room temperature with 60% glycerol and photographed.

Determination of H_2O_2 content in leaf extracts

The content of H_2O_2 was measured by monitoring the absorbance at 415 nm of the titanium peroxide complex following the method described by Jiang and Zhang (2001).

RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was isolated from leaves with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Totally, 3 μg of RNA was reverse transcribed into cDNA by a Revert Aid First Strand cDNA Synthesis Kit (200 units per reaction; Fermentas) at 42°C for 60 min followed by heat inactivation at 70°C for 10 min. PCR amplifications was for pre-denaturation at 94°C for 5 min, 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The cDNA was amplified by PCR using the following primers: *ZmRbohA*, forward GCTAAAAACGGCG TGGATA and reverse GGGCAGAAAGACTGAAAAGA GA; *ZmRbohB*, forward GGAAGGAGATGCTCGGTCAG and reverse TTGGTGGTCTGGTTCATTTAGG; *ZmPR1*, forward TGGGTGTCCGAGAAGCAGT and reverse CACAAATCGCCTGCATGGT; *ZmPR5*, forward ATCTC GGTCATCGACGGCT and reverse GCACACAAATCCA GCTACGT; and *ZmACTIN*, forward ATTCAGGTGATGG TGTGAGCCACAC and reverse GCCACCGATCCAGA CACTGTACTTCC. The PCR products were separated in a 1% agarose gel and stained with ethidium bromide. The

expression level of each sample was estimated based on the intensity of the band.

SA measurements

HrpN-infiltrated areas were pooled and 0.5 g of tissue was used for measuring the total SA, as described previously (Bowling et al. 1994).

Results

HrpN induces cell death in maize leaves

Maize leaves were infiltrated with 25 μg ml^{-1} HrpN, a concentration sufficient to cause cell death when infiltrated into tobacco leaves (Fig. 1a). As shown in Fig. 2a, cell death of the infiltrated zone appeared about 24 h after HrpN application, followed by complete drying of the infiltrated zone at 72 h post-infiltration (pi). This rapid dehydration event is believed to play an important role in plant resistance (Dangl et al. 1996; Ren et al. 2002).

To determine whether HrpN-induced cell death in maize leaves, we used Evans blue staining to detect cell death. Cell death was first observed at 12 h pi of maize leaves, leading to complete necrosis at 48 h pi (Fig. 2b). Infiltration of the leaves with buffer alone did not produce any cell death symptoms (Fig. 2a, b). Furthermore, concentrations of HrpN greater than 25 μg ml^{-1}, such as 100, 250 and 500 μg ml^{-1}, did not result in a significant increase in the cell death of maize leaves (data not shown).

HrpN induces SAR gene expression

In addition to inducing cell death in *Arabidopsis* and tobacco, harpin induces expression of PR genes (Peng et al. 2003), which are normally induced during the development of SAR. Therefore, we assayed the expression of *ZmPR1* and *ZmPR5* in maize leaves treated with HrpN. The results showed that these two genes had similar expression profiles after HrpN treatment (Fig. 3a). As shown in Fig. 3a, the expression of *ZmPR1* and *ZmPR5* was very low within 4 h, and significant increased at 8 h, which was maximized at 12 h and remained high for 48 h after HrpN infiltration.

Salicylic acid plays a central role in plant defense against pathogens, most prominently as an essential agent in the establishment of SAR. Previous results suggested that induction of SAR by harpin in challenged plant cells requires the participation of SA (Peng et al. 2003; Samuel et al. 2005). Therefore, we measured the total SA accumulation in maize leaves following infiltration with a HrpN concentration (25 μg ml^{-1}) that led to cell death over a period of 24–72 h. The leaves after infiltration displayed a high level of SA accumulation over a period of 24 h (Fig. 3b), and the level of SA is also increased after 8 h, which is sufficient to induce the expression of *ZmPR1* and *ZmPR5*. These results clearly suggest that HrpN induces SAR genes and SA accumulation in maize leaves.

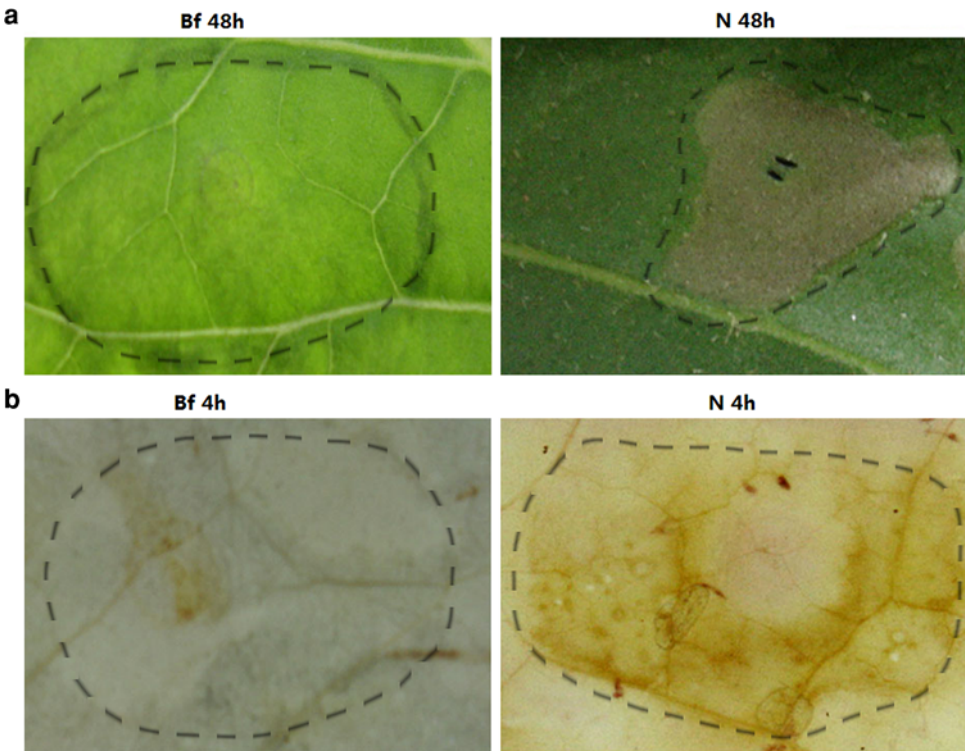

Fig. 1 Induction of cell death and H_2O_2 by HrpN in tobacco. **a** Leaves were infiltrated with buffer and HrpN (25 μg ml^{-1}), and the infiltrated areas are shown at 48 h after buffer infiltration (*Bf*) and HrpN infiltration (*N*). **b** Histochemical detection of H_2O_2 production with DAB staining at 4 h after buffer infiltration (*Bf*) and HrpN infiltration (*N*). Infiltrated leaf areas are indicated by *circles*

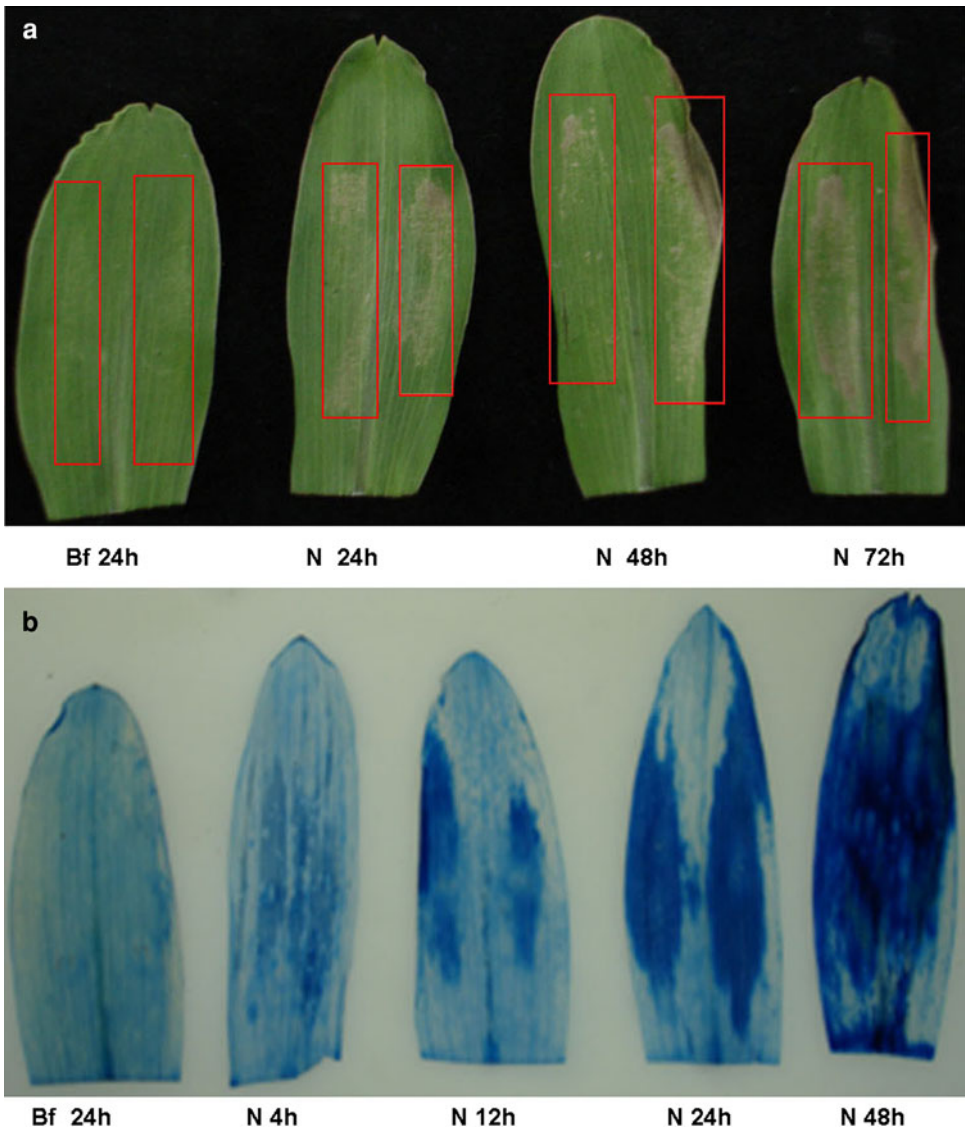

Fig. 2 HrpN-induced cell death in maize. **a** Leaves were infiltrated with HrpN (25 μg ml^{-1}), and the infiltrated areas are shown at 24 h after buffer infiltration (*Bf*) and 24, 48, and 72 h after HrpN infiltration (*N*). Infiltrated leaf areas are indicated by *red boxes*. **b** Leaves were stained with Evans blue at 24 h after buffer infiltration (*Bf*) and 4, 12, 24, and 48 h after HrpN treatments. Cell death is indicated by a *blue color* (color figure online)

HrpN does not induce H_2O_2 accumulation in maize leaves

Previous studies have shown that harpin-induced cell death was accompanied by rapid generation of hydrogen peroxide (H_2O_2), one of the ROS (Desikan et al. 1996; Zhang et al. 2009). Harpin-induced H_2O_2 production in leaf extracts was examined using histochemistry with DAB staining and spectrophotometry; DAB reacts with H_2O_2 in the presence of peroxidases to produce a detectable brown polymerization product. Treatment with HrpN-induced significant H_2O_2 accumulation at 4 h pi of tobacco leaves (Fig. 1b). Surprisingly, a time-course analysis of DAB staining showed that treatment with HrpN did not lead to H_2O_2 accumulation in the infiltrated zones over a period of 12 h but that the color mainly appeared in the major veins of the leaves (Fig. 4a). Spectrophotometry was used to quantify the content of H_2O_2 in the maize leaves. HrpN did not lead to H_2O_2 accumulation in maize leaves within 12 h after HrpN treatment (Fig. 4b).

Genetic evidence shows that H_2O_2 generated by NADPH oxidase plays important roles in elicitor-induced HR and that harpin-induced H_2O_2 accumulation is inhibited by DPI, an inhibitor of the NADPH oxidase (Desikan et al. 1996). Therefore, we analyzed the expression of *ZmRbohA* and *ZmRbohB* after HrpN treatment using semi-quantitative RT-PCR. *ZmRbohA* was constitutively expressed at a very low level, whereas *ZmRbohB* was not induced within 12 h in the presence of HrpN (Fig. 4c). These results suggest that HrpN does not induce H_2O_2 accumulation in maize leaves during cell death.

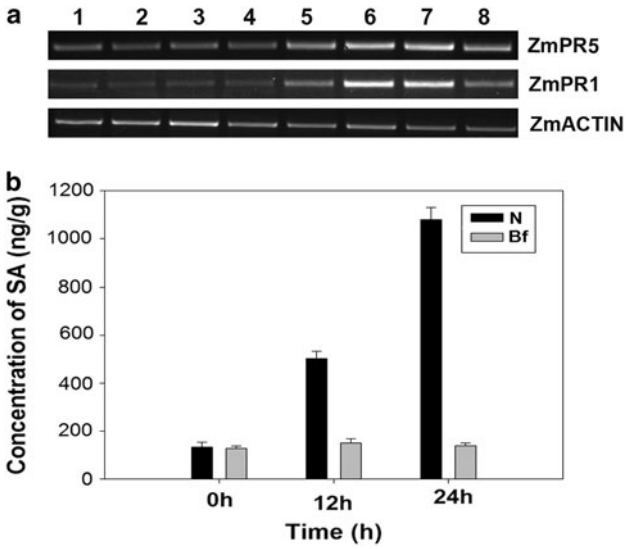

Fig. 3 Accumulation of PR mRNAs and SA in leaf areas infiltrated with HrpN. **a** *ZmPR1* and *ZmPR5* expression determined by RT-PCR. *Lane 1* buffer 24 h; *lane 2* HrpN 1 h; *lane 3* HrpN 2 h; *lane 4* HrpN 4 h; *lane 5* HrpN 8 h; *lane 6* HrpN 12 h; *lane 7* HrpN 24 h; *lane 8* HrpN 48 h. **b** Leaves were infiltrated with buffer (*Bf*) and HrpN (*N*) for various times and the infiltrated areas were harvested and analyzed for SA accumulation by HPLC. **a** Experiments were repeated at least five times with similar results. **b** The values are the means of at least three independent experiments

Discussion

The aim of this study was to determine the disease resistance induced by *E. amylovora* HrpN in maize. In previous work, harpin was shown to induce cell death and SAR genes in *Arabidopsis* and tobacco (Peng et al. 2003), and our data confirm that HrpN does induce HR in tobacco (Fig. 1a). The results of the present study showed that HrpN not only induced HR, but also induced the expression of *ZmPR1* and *ZmPR5* in maize leaves (Figs. 2, 3a), suggesting that HrpN can lead to induced HR and SAR both in dicotyledons and monocotyledons.

The role of SA in the induction of cell death has been clearly established by both gain- and loss-of function data (Raffaele et al. 2006; Samuel et al. 2005). Harpin-induced cell death does not occur in *NahG* transgenic *Arabidopsis* and tobacco, which do not accumulate SA (Peng et al. 2003; Samuel et al. 2005). Our results showed that SA accumulation preceded cell death following HrpN infiltration (Fig. 3b), partially supporting the requirement for SA in harpin-induced cell death in maize.

Plant cells challenged with HR-inducing pathogens or elicitors display two peaks of H_2O_2 generation. The first rapid but weak transient burst of H_2O_2 production is not

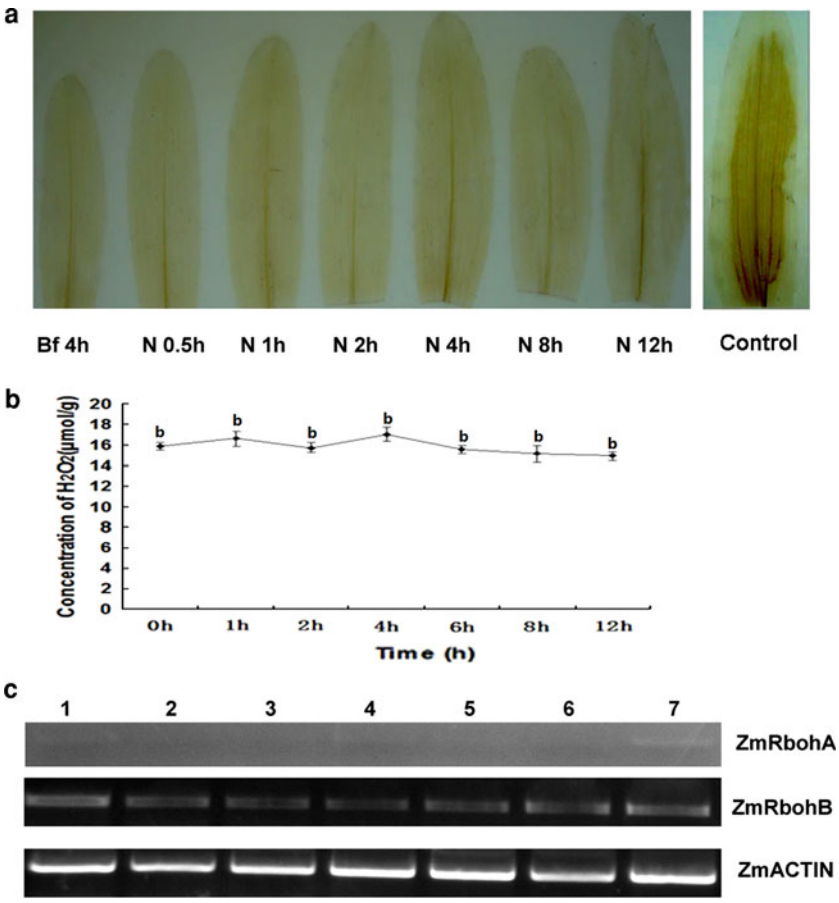

Fig. 4 H_2O_2 generation after HrpN elicitation. **a** Histochemical detection of H_2O_2 production with DAB staining. **b** H_2O_2 was assayed by spectrophotometry as described in "Materials and methods". **c** Expression of *ZmRbohA* and *ZmRbohB* in leaf areas infiltrated with HrpN as analyzed by RT-PCR. *Lane 1* buffer 2 h; *lane 2* HrpN 1 h; *lane 3* HrpN 2 h; *lane 4* HrpN 4 h; *lane 5* HrpN 6 h; *lane 6* HrpN 8 h; *lane 7* HrpN 12 h. **a**, **c** Experiments were repeated at least five times with similar results. **b** The values are the means of at least three independent experiments. Means denoted by the *same letter* did not differ significantly at $P < 0.05$ according to Duncan's multiple range test. *Bf* buffer; *N* HrpN; *Control* 4 h after 100 mM H_2O_2 infiltration

specific to HR. The second increase of H_2O_2, which occurs between 3 and 6 h after inoculation, precedes the onset of HR cell death and is specific for HR-inducing pathogens or elicitors (Lamb and Dixon 1997). Harpin treatment leads to H_2O_2 accumulation in tobacco and *Arabidopsis*, and our data also shows that treatment with 25 μg ml^{-1} HrpN for 4 h leads to H_2O_2 accumulation in infiltrated areas of tobacco (Fig. 1b). Intriguingly, a time-course analysis showed that treatment with HrpN did not lead to H_2O_2 accumulation in maize leaves or increased expression of *ZmRbohA* and *ZmRbohB* (Fig. 4). In studies by Xie and Chen (2000) and Ichinose et al. (2001), DPI, an inhibitor of the oxidative burst, failed to inhibit harpin-induced hypersensitive cell death. Several papers have previously reported that the rapid alternation of mitochondrial function and low levels of ATP play very important roles in harpin-induced hypersensitive cell death and that H_2O_2 is not the direct cause for harpin-induced inhibition of ATP (Garmier et al. 2007; Krause and Durner 2004; Vidal et al. 2007; Xie and Chen 2000). It is not clear whether low levels of ATP serve as a signal for the induction of HR cell death. However, these results indicated that generation of H_2O_2 after harpin treatment is not required for cell death induced by harpin or other elicitors. In addition, Delledonne et al. (1998) also reported that H_2O_2 alone does not induce cell death in soybean cells, which is in agreement with our studies in maize (our unpublished data). Moreover, harpin and exogenous H_2O_2 have different effects on defense gene expression in *Arabidopsis* suspension cultures, as harpin led to increased H_2O_2 generation and expression of *PAL*, glutathione *S*-transferase (*GST*) and anthranilate synthase (*ASA1*) mRNA, and H_2O_2 only led to increased *PAL* and *GST* mRNA (Desikan et al. 1998). These results suggest that both H_2O_2-dependent and H_2O_2-independent pathways are involved in HR cell death signaling in plants. Our results indicated that HrpN induces an H_2O_2-independent rather than an H_2O_2-dependent pathway in HR cell death in maize leaves.

Furthermore, in a previous study, active MAPKKs induce cell death and ROS production in tobacco and *Arabidopsis* (Ren et al. 2002). More recently, it has been reported that $OsMKK4^{DD}$-induced cell death without extracellular ROS production in rice cells, while $OsMKK4^{DD}$-induced cell death and ROS production in *Nicotiana benthamiana* (Kishi-Kaboshi et al. 2010). This is consistent with our observation that HrpN induces cell death and H_2O_2 production in tobacco, while HrpN induces cell death without extracellular H_2O_2 production in maize leaves. However, further studies needed to investigate the mechanism of the different process of HrpN-induced cell death in dicotyledons and monocotyledons. Taken together, HrpN-induced cell death in maize leaves may be not the result of the generation of H_2O_2.

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