Brain, Behavior, and Immunity xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Extracellular self-DNA as a damage-associated molecular pattern (DAMP) that triggers self-specific immunity induction in plants

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ARTICLE INFO

Article history:
Received 3 July 2017
Received in revised form 4 October 2017
Accepted 13 October 2017
Available online xxxx

Keywords:
Damaged-self recognition
DAMP
Defence signalling
eDNA
MAPK signalling
ROS

ABSTRACT

Mammals sense self or non-self extracellular or extranuclear DNA fragments (hereinafter collectively termed eDNA) as indicators of injury or infection and respond with immunity. We hypothesised that eDNA acts as a damage-associated molecular pattern (DAMP) also in plants and that it contributes to self versus non-self discrimination. Treating plants and suspension-cultured cells of common bean (*Phaseolus vulgaris*) with fragmented self eDNA (obtained from other plants of the same species) induced early, immunity-related signalling responses such as H_2O_2 generation and MAPK activation, decreased the infection by a bacterial pathogen (*Pseudomonas syringae*) and increased an indirect defence to herbivores (extrafloral nectar secretion). By contrast, non-self DNA (obtained from lima bean, *Phaseolus lunatus*, and *Acacia farnesiana*) had significantly lower or no detectable effects. Only fragments below a size of 700 bp were active, and treating the eDNA preparation DNAse abolished its inducing effects, whereas treatment with RNAse or proteinase had no detectable effect. These findings indicate that DNA fragments, rather than small RNAs, single nucleotides or proteins, accounted for the observed effects. We suggest that eDNA functions a DAMP in plants and that plants discriminate self from non-self at a species-specific level. The immune systems of plants and mammals share multiple central elements, but further work will be required to understand the mechanisms and the selective benefits of an immunity response that is triggered by eDNA in a species-specific manner.

1. Introduction

Multicellular organisms suffer different types of cellular damage that may, or may not, include infectious processes. Janeway's classical model states that the immune system evolved to distinguish the infectious non-self from the non-infectious self (Janeway et al., 2001). However, in most environments, injury to the outer layers of an organism (the skin or gut epithelia in the case of mammals, the epidermis of leaves and roots in the case of plants) inevitably leads to infection. Moreover, responses such as wound sealing and tissue repair are also required in non-infected injured tissues and, in most cases, they are independent of the exact nature of the harming agent. Thus, multicellular organisms require an endogenous signalling pathway that enables them to perceive injury and mount adequate local and systemic responses (Heil and Land 2014). The danger model holds that the onset of a successful immune response depends on the detection of 'danger' or 'damage'-associated molecular patterns (DAMPs): endogenous indicators of injury (Land et al., 1994; Matzinger 2002, 1994). During injury, tissue disruption and the resulting de-compartmentalization of cells lead to the release of intra-cellular molecules into the extracellular space and to the fragmentation of macromolecules (Heil and Land, 2014). All these molecules potentially can be perceived by the surrounding, intact cells as DAMPs that trigger 'damaged-self recognition': an induction of immunity in damaged organisms that is independent of exogenous molecules such as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) (Heil, 2009; Heil and Land, 2014).

In mammals, well-studied DAMPs include high-mobility group box proteins (HMGBs), extracellular ATP, or extracellular and cytosolic DNA fragments (Garg et al., 2015; Vénéreau et al., 2015). For the sake of simplicity, hereinafter we employ the term 'eDNA' collectively for extracellular and extranuclear (i.e., cytosolic) DNA. Whereas eDNA molecules of nuclear and mitochondrial origin are considered DAMPs (Toussaint et al., 2017), bacterial and viral DNA molecules are considered MAMPs or PAMPs (Altfeld and Gale, 2015; Dempsey and Bowie, 2015; Jounai et al., 2013; Kaczmarek et al., 2013; Tang et al., 2012; Wang et al., 2016; Wu and Chen, 2014). However, it remains matter of discussion whether mitochondrial DNA is perceived as DAMP or rather as

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https://doi.org/10.1016/j.bbi.2017.10.010
0889-1591/

a MAMP when it appears outside of cells (Zhang et al., 2010). This situation is paralleled by fructans, plant storage polysaccharides that have been suggested to act as DAMPs when they appear in the apoplast, but that might also be of bacterial or fungal origin and then represent MAMPs (Versluys et al., 2017). Nevertheless, mammalian cells sense DAMPs as well as MAMPs via a range of receptor-dependent and -independent pathways that involve, among others, toll-like receptors (TLRs), purinergic receptors, DNA-dependent activator of IFN-regulatory factors (DAI), interferon regulatory factor (IRF), or the NACHT, LRR and PYD domains-containing protein 3 (NLPR3) inflammasome (Di Virgilio et al., 2017; Lupfer and Anand, 2016) Magna and Pisetsky, 2016; Schlee and Hartmann, 2016; Takahashi et al., 2017; Takaoka et al., 2007). In fact, mammalian immune cells sense eDNA independently of whether it has been released from dying host cells or produced, e.g., by retroviral reverse transcriptase (Altfeld and Gale, 2015; Gallucci and Maffei, 2017; Kato et al., 2017). The activation of these sensors triggers immunity-related responses like mitogen-activated protein kinase (MAPK) signalling, the formation of reactive oxygen species (ROS), the synthesis of interferons (IFNs) and multiple other signalling processes that lead to inflammation, the maturation of dendritic cells to antigen-presenting cells and, ultimately, to active innate and adaptive immune response (Land, 2015).

Research into the mechanisms that enable the mammalian immune system to discriminate "self from non-self" in the sensing of nucleic acids has mainly focused on the differentiation of host (self) versus viral or microbial (non-self) eDNA (Schlee and Hartmann, 2016). For plants, by contrast, recent studies revealed a surprising level of specificity at which DAMPs of different taxonomic origin trigger immunity. For example, treating intact leaves of common bean (*Phaseolus vulgaris*) with leaf homogenate – which arguably contains a complex blend of DAMPs – induced various immunity-related responses, but only when using homogenate prepared from conspecific leaves (Duran-Flores and Heil, 2014). Even the application of homogenate from the closely related lima bean (*Phaseolus lunatus*) led to a significantly reduced response (Duran-Flores and Heil, 2014). However, it remains unknown which ones of all the molecules that are released from damaged tissue account for this surprising specificity in the plant immune response.

Based on the central role of eDNA in the mammalian immune system and recent anecdotal evidence for an equivalent function in plants (summarized in Gallucci and Maffei, 2017; Gust et al., 2017),we hypothesized that eDNA is a particularly promising candidate of a DAMP that could contribute to the species-specificity in plant damaged-self recognition; mainly for the following reasons. First, delocalized self nucleic acids — such as extranuclear DNA or extracellular RNA — are well-known DAMPs in mammals, "because they are reliable indicators of cellular damage" (Desmet and Ishii, 2012). Upon its recognition, eDNA triggers the generation of ROS, downstream MAPK signalling cascades, the release of cytokines, inflammation and other immunity-related responses (Altfeld and Gale, 2015; Anders and Schaefer, 2014; Dempsey and Bowie, 2015; Heil and Land, 2014; Jounai et al., 2013; Kaczmarek et al., 2013; Patel et al., 2011; Tang et al., 2012; Wang et al., 2016). Second, eDNA has been suggested to act in plant immunity (Duran-Flores and Heil, 2015; Gallucci and Maffei, 2017; Gust et al., 2017; Hawes et al., 2011) because it was reported as an indicator of bacterial infection in *Arabidopsis thaliana* (Yakushiji et al., 2009), as an inducer of immunity to fungal infections in pea roots (*Pisum sativum*) (Wen et al., 2009) and, most recently, as a trigger of Ca^{2+} signalling and membrane depolarization in lima bean and maize (*Zea mays*) (Barbero et al., 2016). Third, the effects of eDNA can depend on the taxonomic distance between the source and the receiver: the application of non-self eDNA from lima bean or an insect did not result in membrane depolarization in maize (Barbero et al., 2016) and the inhibitory effect of eDNA on the growth of organisms in different phyla (Mazzoleni et al., 2015a,b, Mazzoleni et al., 2014) showed taxonomic specificity: eDNA of *Lepidium sativum* inhibited the root growth of Arabidopsis in a dosage-dependent manner, but 'self eDNA' prepared from Arabidopsis had a much stronger effect (Mazzoleni et al., 2015a). Based on the above-mentioned reports, we reasoned that self eDNA might contribute to the taxonomic specificity in plant damaged-self recognition (Duran-Flores and Heil, 2015).

In the present study, we aimed at investigating whether eDNA can cause the same species-specific responses in bean as they had been observed after the application of leaf homogenates. We used *P. vulgaris* as the receiver species and applied fragmented self-eDNA, prepared from different individuals but the same cultivar as the receiver, as well as non-self eDNA, which was prepared from *P. lunatus* and *Acacia farnesiana* (*A. farnesiana* is a member of the Fabaceae family but does not belong to the same subfamily as bean). We quantified the generation of ROS and the activation of MAPKs as two early, general responses to stress and the secretion of extrafloral nectar (EFN) and the infection by a bacterial phytopathogen as two indicators of the phenotypic components of the plant immune system. The secretion of EFN is a widespread, inducible plant response to herbivory. EFN attracts ants, predators, parasitoids and other natural enemies of the herbivores to the plant, thereby serving as a means of 'natural biological control' (see Heil, 2015 for a recent overview). Putative effects of RNA or proteins on the observed responses were excluded using nucleases and proteinases, respectively. Based on our results, we suggest that eDNA is likely to represent a DAMP that contributes to the specificity in plant damaged-self recognition.

2. Material and methods

2.1. Biological material

For all experiments in plants, four-week-old common bean plants were used as receivers (*Phaseolus vulgaris*, Negro San Luis variety; seeds were obtained from the national germplasm collection at INIFAP, Celaya, GTO, México). The plants were grown under greenhouse conditions and natural light (average day-time temperature, 28 °C; night-time temperature, 20 °C), watered on Mondays, Wednesdays and Fridays, and fertilized weekly with a commercial fertilizer (Ferviafol 20-30-10®, Agroquímicos Rivas S. A. de C.V., Celaya, GTO, México). Lima bean (*Phaseolus lunatus*) seeds were collected from a wild population 5-km west of Puerto Escondido, in the state of Oaxaca in Southern Mexico (~15°55′ N and 097°09′ W), and cultivated under greenhouse conditions. Before cultivation, the seeds were surface-sterilized with 70% ethanol for 1 min and with a 20% hypochlorite solution for 10 min and then washed five times with sterile water. Wild *Acacia farnesiana was* collected from the area around CINVESTAV - Irapuato, in the state of Guanajuato in Central Mexico (~20°72′ N and 101°33′W). The bacterial phytopathogen (rifampicin-resistant Pseudomonas syringae pv. *syringae* strain 61) was provided by Dr. Choong-Min Ryu (KRIBB, Daejeon, South Korea).

2.2. Suspension cells

Surface-sterilized common bean seeds were germinated under sterile conditions in solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with a pH of 5.8 and 3% sucrose. After seven days, the apical meristem or root was cut 3 mm from the tip. These tips were transferred to solid MS medium with a pH of 5.8 that was enriched with 0.5 mg L^{-1} of indoleacetic acid (IAA) and 5

Please cite this article in press as: Duran-Flores, D., Heil, M. Extracellular self-DNA as a damage-associated molecular pattern (DAMP) that triggers self-specific immunity induction in plants. Brain Behav. Immun. (2017), https://doi.org/10.1016/j.bbi.2017.10.010