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Wounding induces local resistance but systemic susceptibility to *Botrytis cinerea* in pepper plants

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ABSTRACT

Cotyledon wounding in pepper caused the early generation of hydrogen peroxide both locally (cotyledons) and systemically (upper true leaves). However, 72 h later there is a different wound response between local and systemic organs, as shown by resistance to the pathogenic fungus *Botrytis cinerea*, that increased locally and decreased systemically. Signaling by ethylene and jasmonic acid was assessed by using two inhibitors: 1-methylcyclopropene (MCP, inhibitor of ethylene receptors) and ibuprofen (inhibitor of jasmonate biosynthesis). MCP did not affect the modulation of resistance levels to Botrytis by wounding, ruling out the involvement of ethylene signaling. Ibuprofen did not inhibit wound-induced resistance at the local level, but inhibited wound-induced systemic susceptibility. Moreover, changes of biochemical and structural defenses in response to wounding were studied. Peroxidase activity and the expression of a peroxidase gene (*CAPO1*) increased locally as a response to wounding, but no changes were observed systemically. Lignin deposition was induced in wounded cotyledons, but was repressed in systemic leaves of wounded plants, whereas soluble phenolics did not change locally and decreased systemically. The expression of two other genes involved in plant defense (*CABPR1* and *CASC1*) was also differentially regulated locally and systemically, pointing to a generalized increase in plant defenses at the local level and a systemic decrease as a response to wounding. Wound-induced defenses at the local level coincided with resistance to the necrotroph fungus *B. cinerea*, whereas depleted defenses in systemic leaves of wounded plants correlated to induced susceptibility against this pathogen. It may be that the local response acts as a sink of energy resources to mount a defense against pathogens, whereas in systemic organs the resources for defense are lower.

Introduction

In nature, plants have to cope with changes in different environmental factors that cause stress. One of such factors is wounding, caused sometimes by the weather (*e.g.*, hail or heavy rain), but mainly by herbivore attack. After herbivore feeding, plant tissue is damaged and the resulting changes in the defensive status of the plant have been named the "plant wound response" (Heil, 2009). Some initial studies found that just mechanical damage is sometimes enough to elicit such a response, but other experiments revealed that the response to natural herbivory is more complex and induces changes that need the presence of specific insect-derived defence elicitors (Heil, 2009). The response to mechanical wounding has been well studied in solanaceous plants as tomato, where the plant hormones systemin and jasmonate are the key signals (Ryan, 2000; Wasternack et al., 2006). Besides systemin and jasmonates, other signaling molecules participate in wound response signaling, such as oligogalacturonides, abscisic acid, H_2O_2 and ethylene (Ryan, 2000; Savatin et al., 2014). The induction of both local and systemic expression of proteinase inhibitors is also a common feature of solanaceae in response to wounding. The presence of proteinase inhibitors in the plant protects the plant against herbivores. Besides proteinase inhibitor expression, nicotine accumulation (other direct defense against herbivores) is elicited in tobacco, a solanaceous plant (Kahl et al., 2000). Such direct defense is regulated by jasmonates and ethylene, whereas the indirect defense based on volatile terpenoids (mono and sesquiterpene emissions) is not regulated by ethylene (Kahl et al., 2000). After wounding, nicotine accumulates both locally and systemically in tobacco. Indeed, jasmonate signaling from the wounded leaves provokes the biosynthesis of nicotine in the roots and its translocation *via* xylem to the leaves (Mithöfer and Boland, 2012).

Abbreviations: DAB, 3,3-diaminobenzidine; MCP, 1-methylcyclopropene; PDA, potato dextrose agar; PVPP, polyvinylpolypyrrolidone.

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Besides defense against herbivores, the wound response also triggers defense mechanisms effective against pathogens (Bostock and Stermer, 1989). Mechanical wounding triggered an increase in resistance to pathogens such as the biotrophic fungus *Uromyces fabae* in *Vicia faba* or the necrotrophic fungus *Botrytis cinerea* in *Arabidopsis* (Chassot et al., 2008; Walters et al., 2006). In the solanaceae, wounding of tomato roots induced resistance in leaves against *B. cinerea* (a necrotroph, as stated above), and *Pseudomonas syringae* pv. *tomato*, a biotroph (Francia et al., 2007). Such induction of resistance is a logical consequence of wounding because some signals (jasmonate, ethylene, systemin) are both involved in plant defense against herbivores and pathogens. Indeed, wounding and systemin regulate resistance against the necrotroph *B. cinerea* in tomato, whereas ethylene was found to be a main key signal in such response (Díaz et al., 2002; Francia et al., 2007). Ethylene is an important plant hormone influencing resistance to *B. cinerea* in tomato, an effect independent of jasmonate signaling, but antagonistic to salicylic acid (Díaz et al., 2002). Salicylates, jasmonates and ethylene show a complex cross-talk to modulate plant immunity to pathogens and herbivores, besides other plant hormones, *e.g.*, abscisic acid (Pieterse et al., 2012). It is usually assumed that salicylates trigger defense against biotrophs and jasmonates/ethylene do the same against necrotrophs, based on studies carried out in *Arabidopsis* in most of the cases. In *Arabidopsis*, wounding induced the expression of genes related to jasmonate, ethylene and abscisic acid (Delessert et al., 2004). Wounding of *Arabidopsis* induced local resistance against *B. cinerea*, but in a transient way independent of jasmonate and ethylene signaling (Chassot et al., 2008).

As stated above, the response of tomato to mechanical wounding is well characterized (Ryan, 2000) and leads to resistance against some pathogens (Francia et al., 2007), but there are less studies with other solanaceous species. Pepper (*Capsicum annuum*) is another solanaceous plant that also shows a response to wounding involving systemin and proteinase inhibitors (Moura and Ryan, 2001). Wounding of pepper also results in a local increase in lignin, shikimate dehydrogenase and peroxidase activity, probably responsible in part of wound healing (Díaz and Merino, 1998). Such a local lignin accumulation prevents water-loss and represents a barrier against pathogen infection, but a possible systemic effect of wounding on lignin accumulation has not been studied so far in pepper. In *Arabidopsis*, the expression of lignin biosynthesis genes is triggered after wounding, but only at the local site of the wound, not systemically, as it occurs with other genes related to defense against pathogens (*e.g.*, a PR1-like gene) (Delessert et al., 2004). In wounded pepper, plant defenses against pathogens were not tested so far neither locally (where the wound is inflicted) nor systemically (in other part of the plant). The study of both local and systemic effects is interesting because could reflect differences in trend (induction/repression or resistance/susceptibility).

In the present study, we tested the effect of wounding on resistance to *B. cinerea* both locally and systemically, as well as changes in plant defense compounds (hydrogen peroxide production, peroxidase activity, chitinase activity, total soluble phenolics and lignin) and the expression of three defense genes. We also assayed resistance to *B. cinerea* in plants previously treated with inhibitors of plant hormone action. The results point to an induced resistance response at the local level, but, surprisingly, to a systemic induced susceptibility response.

Materials and methods

Plant material, wounding treatment and inhibitors treatment

Before sowing, seeds of *Capsicum annuum* L. cv. Padrón (obtained in our greenhouse facilities) were disinfected in 1% (v/v) commercial bleach for 30 min. Then, seeds were washed with running water and soaked overnight in distilled water before being sown in vermiculite. Plants were grown in a growth chamber at 25 °C and a photoperiod of 16 h light and 8 h darkness, and 70% relative humidity. Three weeks after sowing, plants were transferred to a mixture of potting soil and perlite (3:1) and were grown in a growth chamber in the same conditions. Two weeks later plants were treated, keeping convenient control groups. Cotyledons and leaves were at fully expanded but non-senescing stage.

In wounding treatments, pepper plants were wounded by puncturing each cotyledon with a needle at 100 evenly distributed locations (modified from Díaz and Merino, 1998). Both cotyledons of the plant were punctured. Cotyledons were further analysed to test local response to wounding as described below for biochemical and molecular parameters as well as *Botrytis cinerea* resistance (Fig. 1A). The first pair of true leaves were also analysed to check the systemic response (Fig. 1A). In the *B. cinerea* inoculation experiments, plants were inoculated 72 h after wounding as described below.

In experiments with 1-methylcyclopropene (MCP, an inhibitor of ethylene perception, kindly provided by Rohm and Haas), pepper plants were treated with the inhibitor in a sealed container (Díaz et al., 2005) at a final concentration of 0.2 $\mu L L^{-1}$. A control group of plants was kept in a container with no chemical added. Containers were opened after 24 h of treatment and, following aeration, plants were then wounded as indicated above and 72 h later were inoculated as described below.

In experiments with ibuprofen (an inhibitor of jasmonic acid biosynthesis, purchased from Sigma-Aldrich, I1892), pepper plants were transferred to perlite three weeks after sowing, and watered with Hoagland nutrient solution. Two days before wounding treatment, the nutrient solution was supplemented with the inhibitor at a final concentration of 1 mM (concentration based in Oikawa et al., 2001). Plants were wounded as indicated above and 72 h later were inoculated as described below.

Inoculation with Botrytis cinerea

The *B. cinerea* isolate B0510 was kindly provided by Dr. Jan van Kan (Wageningen University), and grown in tomato-PDA (Díaz et al., 2002). The inoculum was prepared by flooding the dishes with sterile distilled water and filtering the suspension. The concentration was adjusted to 10^6 conidia mL^{-1} as described by Díaz et al. (2002). Inoculation with *B. cinerea* was carried out by putting 2 drops (3 μL) of the inoculum on each cotyledon or leaf. After inoculation, the plants were placed into a wet chamber at room temperature to preserve high humidity conditions. The diameters of the lesions caused by *B. cinerea* were measured 48 and 72 h postinoculation, and symptomatic area was calculated.

In vivo hydrogen peroxide staining

Hydrogen peroxide was visually detected in the cotyledons and the leaves by using 3,3-diaminobenzidine (DAB) as staining agent as described by Orozco-Cardenas and Ryan (1999) with a few modifications. Briefly, plants were excised at the base of stems with a razor blade and fed through the cut stems with a 1 mg mL^{-1} solution of DAB, pH 3.8, for 6 hr under light at 25 °C. Cotyledons were wounded as indicated above. After wounding, the plants were continually supplied with DAB solution for 4 h. Then, cotyledons and leaves were boiled in ethanol (96%, v/v) for 10 min to decolorize them except for the deep brown polymerization product produced by the reaction of DAB with hydrogen peroxide. Then, cotyledons and leaves were cooled, extracted at room temperature with fresh ethanol for 24 h and photographed.