Methyl jasmonate, benzothiadiazole and oxalic acid induce resistance and defense gene expression against *Sclerotinia sclerotiorum* in oilseed rape

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Abstract

To determine effects of methyl jasmonate, benzothiadiazole or oxalic acid on induced resistance of oilseed rape to S. *sclerotiorum*, disease resistances and changes of mycelium growth were investigated. Chemical-pretreated plants exhibited enhanced local and systemic resistance, with a significant reduction of disease severity and inhibition of mycelium growth in leaves inoculated with *S. sclerotiorum*, in comparison with control plants pretreated with water. Furthermore, pretreatment with methyl jasmonate, benzothiadiazole or oxalic acid increased the level of H_2O_2 in inoculated plants as showed by strong red brown after staining with 3,3-diaminobenzidine. To further understand the mechanism of disease resistance, expression of seven defense-related genes in chemical treated plants or inoculated plants was studied. The results showed that treatment with methyl jasmonate, benzothiadiazole or oxalic acid or inoculation with S. *sclerotiorum* elevated expression levels of most of these defense-related genes, but the expression level of *NPR1* was not obviously affected. Inoculation with *S. sclerotiorum*, as well as treatment with oxalic acid, induced up-regulated expression of APX, PDF, PR-1. These suggest that the mechanism of defence to *S. sclerotiorum* in oilseed rape may involved activation of different signalling pathways.

Key words: chemical activators, induced resistance, H_2O_2, defense-related genes

Introduction

Plants have evolved a number of mechanisms to defend themselves against environmental stresses. In many cases, localized infection by pathogens or treatment by signal molecules can induce a broad spectrum of resistance against different pathogens such as fungi, bacteria or viruses. This resistance is expressed locally at the site of infection or treatment and systemically in uninfected parts of the plant (Ryals et al., 1996; Hammerschmidt, 1999).

It has become clear that resistance responses are not mediated by a single genetic program and signalling molecules are involved in response to different pathogens (Century et al., 1997). Recently, studies of defense signalling pathways on *Arabidopsis* revealed that induced defenses against microbial pathogens and herbivorous insects are regulated by a network of interconnecting signalling pathways in which the plant signal molecules like SA, JA/ET play a dominant role (Dong, 1998; Feys & Parker, 2000). *PR-1* is a marker gene in the SA-dependent signalling pathway. *PDF1.2* is a marker gene in the JA /ET-dependent signalling pathway. JA/ET are involved in ISR (induced systemic resistance) and defense gene expression both SAR and ISR require key regulatory protein *NPR1* (Van wees et al., 2000). Oxidative burst, including H_2O_2 production, is one of early events that are associated with HR (hypersensitive response) in many plant-pathogen interactions (Lamb & Dixon, 1997), in which ROIs (reactive oxygen intermediates) produced damage the cell via uncontrolled oxidation of cellular components. APX is found throughout the cell, and is believed to be one of the main scavengers of peroxides (Mittler R et al., 1998).

The *Sclerotinia* disease is the most important fungal disease in oilseed rape. Applying fungicides and crop rotation are currently major methods of controlling this disease. But they are not always effective. Recently, molecular biological methods had been used to research the resistance response mechanism of *S. sclerotiorum*. Yanar & Miller (2004) treated Pepper with Benzothiadiazole derivative and non-pathogenic *Xanthomonas campestris* pathovars reduced the level of disease caused by *S. sclerotiorum.* But the mechanism of resistance responses to *S. sclerotiorum* is not clear yet.

In this research, we pretreated oilseed rapes with MeJA, BTH and low concentration oxalic acid (OA) to investigate the resistance response to *S.sclerotiorum* and detected the level of H_2O_2 and expression of defense-related genes, in order to look at the mechanism of resistance responses to *S.sclerotiorum* in oilseed rape.

Material and Methods

Chemical treatment and challenge inoculation: Up to four-leaf-old oilseed rape plants were grown in a growth room. First and second leaves were sprayed with chemicals, including 0.1mmol L^{-1} Benzothiadiazole (Syngerta) dissolved in water, 0.1 mmol L^{-1} methyl jasmonate (Sigma) in 0.9% ethanol, 5 mmol L^{-1} oxalic acid dissolved in water, until run-off.Then a half of each treated plants were used to challenge-inoculate with *S. sclerotiorum* mycelia (cultured on PDA media at 23°C for 2 days).

Disease resistance investigation: 30 plants were evaluated for each treatment with MeJA, BTH, OA and water, respectively. Length and width of lesions were assessed in local and systemic leaves every 6 h after inoculation.

Trypan blue staining: 12 h post inoculation leaves were excised using a scalpel and decolourised in a 3:1(v/v) mixture of ethanol and chloroform for 24 h, then stained in 0.025% trypan blue in lactophenol for 4 h. The stained leaf tissue was mounted in 70% glycerol in distilled water on a glass slide. Penetration assessments were done using a light microscope at 200 magnifications.

Histochemical detection of H_2O_2 in oilseed rape leaves: The excised leaves were placed in 1 mg ml^{-1} DAB-HCL (pH 3.8, Amresco, USA) and incubated in growth room for 8 h prior to sampling. DAB reactions were examined in leaves which were cleared in boiling ethanol (96%) for 10 min. The latter samples were stored and examined in 96% ethanol. H_2O_2 is visualized as a reddish-brown colouration. The decolorized leaves were photographed using a fluorescence microscope (LEICA DMRE, Gernany).

Semi-quantitave RT-PCR: The first-strand synthesized cDNA was used for RT-PCR amplification with the primers of *Actin* (β-*actin*), *Glu* (*β-1, 3-glucanase*), *Chi* (*chitinase*), *PR-5*(Pathogenesis related protein 5)*, PR-1, PDF1.2*, *APX and NPR1*. The internal control, β-*actin*, was used for correcting loading amount of PCR-amplified cDNA.

Results

The investigation showed that lesion sizes were significantly reduced in all pretreated leaves compared with water pretreated control plants at 24 h after treatment (Fig.1a and b). Sizes of lesions in both local and systemic leaves significantly reduced compared with control plants at 36 h after inoculation. Among these three chemical treatments, there was not significant difference each other.

Mycelia growth was inhibited in chemical pretreated leaves with BTH, MeJA or OA. The mycelia became cured, short and thicker (Fig.2) while mycelial growth in untreated leaves looked well like that in PDA media.

The results of H_2O_2 detection showed that inoculated leaves had a red-brown staining localized on the cell walls of mesophyll cells, which diffused rapidly through the minor veins of the leaf, while less DAB staining in water pretreated plants. These results implicated that there are more H_2O_2 being produced in chemical treated local leaves.

Fig.1 lesions at 36 h after inoculation with *Sclerotinia sclerotiorum* in oilseed rape.

a: local leaves, pretreated with MeJA, BTH, OA or water (CK) 24 h before inoculation, b: systemic leaves. Black scale bar =1cm

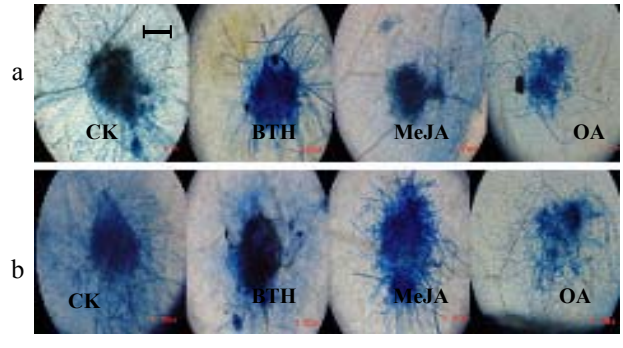

Fig.2 Change of mycelia growth of *Sclerotinia sclerotiorum* at 12 h in chemical pretreated oilseed rape plants. Pretreatments with water (CK), BTH, MeJA or OA 24 h before inoculation. a: local leaves, b: systemic leaves. Black scale bar = 0.05 mm

Different chemical treatments significantly altered the expression levels of these defense related genes (Fig.3). In MeJA treated leaves, *PDF1.2* was up-regulated (Fig. 3a). In BTH treated leaves, the expression of *PR-1, PR-5* and *APX* were coincidently increased. The expression of *PDF1.2* was up-regulated only at 6 h and 12 h after application of BTH, andthen back to the control level (Fig.3b). In OA treated leaves, *APX* was up-regulated (Fig.3c). The expression level of *NPR1* is low in either chemical treated leaves or water pretreated controls. The steady-state levels of these defense related genes were very low in the uninfected leaves. However, expression of these genes was significantly induced in infected oilseed rape leaves by *S. sclerotiorum*.

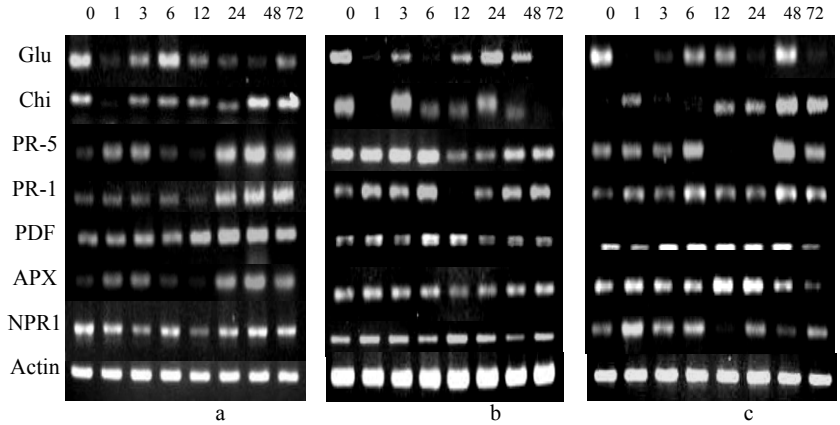

Fig.3 mRNA levels of defense-related genes in oilseed rape leaves in 0,1,3,6,12,24,48 and 72 h after treatment with chemical (MeJA, BTH or OA). *ACTIN*: a reference gene, a: MeJA induced, b: BTH induced, c:OA induced.

Discussion

Trypon blue staining results showed that mycelium growth was suppressed in chemical-treated leaves. Since chemical like SA does not inhibit fungal germination and growth (He & Wolyn, 2005), and thus we assume induced resistance may come from subsequence of defense gene expression.

RT-PCR results showed these treatments significantly altered the expression levels of these genes in different pathways (JA- and SA-dependent and oxidative burst signalling pathways), possibly indicating that the three main defense pathways also existed in oilseed rape. In this research, we suggest that OA produced by *S. sclerotiorum* may involve in induced resistance to *S. sclerotiorum* in oilseed rape.

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