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PREFORMED DEFENSE RESPONSES IN A POWDERY MILDEW-RESISTANT HUNGARIAN CHERRY PEPPER CULTIVAR

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ABSTRACT
A Hungarian cherry pepper (*Capsicum annuum* var. *cerasiforme*) cultivar ('Szentesi') displays resistance to pepper powdery mildew caused by *Leveillula taurica*. Resistance also develops in susceptible sweet pepper (*C. annuum*) when grafted on resistant cherry pepper cv. Szentesi rootstocks. Powdery mildew (PM) resistance is correlated with high levels of the defense regulator reactive oxygen species superoxide ($O_2^{\cdot-}$) even in healthy plants. In order to further elucidate the mechanisms of preformed defense responses in cherry pepper cv. Szentesi we have monitored levels of salicylic acid (SA), a key molecule of plant defense signaling and expression of so-called pathogenesis/defense related (PR) genes in healthy pepper plants. Assays of free and bound (glycosylated) SA by high performance liquid chromatography (HPLC) revealed that in leaves of PM-resistant pepper levels of free SA are ca. twice as high compared to that of PM-sensitive plants. No difference occurred in levels of bound (glycosylated) SA. Expression of the *CaPR-1* gene was several times higher in leaves of PM-resistant pepper than in sensitive plants as assayed by real time reverse transcription quantitative polymerase chain reaction (real time RT-qPCR). On the other hand, high expression levels of the *CaPR-2* (glucanase) gene did not entirely correlate with PM-resistance, being detectable only in PM-resistant cv. Szentesi plants but neither in PM-resistant sweet pepper cv. Totál grafted on cv. Szentesi rootstocks nor in susceptible controls (cv. Totál). It seems that graft-transmissible PM-resistance of the cherry pepper cv. Szentesi is correlated not only with high levels of superoxide but also with elevated levels of free salicylic acid and enhanced expression of the defense-related *CaPR-1* gene.

Keywords: cherry pepper, powdery mildew resistance, salicylic acid, pathogenesis-related genes

INTRODUCTION

The endoparasitic powdery mildew fungus *Leveillula taurica* (anamorph: *Oidiopsis taurica*) is a serious threat to pepper and tomato production. Under a temperate climate, heavy epidemics may cause a significant yield loss of up to 2-4 kg/m^2 in greenhouse pepper production even though mildew symptoms develop only on leaves (CERKAUSKAS AND BUONASSISI, 2003). In Hungary, pepper powdery mildew is present since 1972, causing economic losses primarily in greenhouse production (forcing) of pepper (GLITS AND FOLK, 2000). Besides extensive fungicide applications, pepper powdery mildew can be controlled by using resistant cultivars containing R genes introgressed from related wild species but this race/cultivar specific resistance is often overcome by newly emerging pathogen races (see e.g. ZHENG ET AL., 2013).
A Hungarian cherry pepper (*Capsicum annuum var. cerasiforme* L.) cultivar ('Szentesi') bred from selections of wild-grown Mexican genotypes is highly resistant to pepper powdery mildew and has been used in production for over several years (see e.g. in LANTOS, 2011). Our previous experiments have shown that cv. Szentesi exhibits a symptomless resistance to powdery mildew infection and displays high levels of the reactive oxygen species superoxide ($O_2^{\cdot-}$), a key factor of plant disease resistance, even in healthy leaves. Both superoxide accumulation and resistance can be transmitted to

susceptible sweet pepper cultivars by grafting (KIRÁLY ET AL., 2013; KÜNSTLER ET AL., 2013). Superoxide has been shown to be associated with the establishment of plant resistance to various pathogens (DOKE, 1983; DOKE AND OHASHI, 1988; ÁDÁM ET AL., 1989). Superoxide can be converted to hydrogen-peroxide (H_2O_2) that induces the accumulation of salicylic acid, a key molecule of plant defense signaling, and expression of so-called pathogenesis (defense) related genes, processes which ultimately lead to plant defense responses and resistance to pathogenic infections (WARD ET AL., 1991; CHEN ET AL., 1993; TORRES ET AL., 2006; LEHMANN ET AL., 2015).

In order to further elucidate the mechanisms of preformed defense responses in the powdery mildew (PM) resistant cherry pepper cv. Szentesi we have monitored levels of free and bound (glycosylated) salicylic acid, and expression of so-called pathogenesis/defense related (PR) genes in healthy pepper plants. Our results suggest that high levels of salicylic acid and elevated expression of PR genes in cherry pepper cv. Szentesi may have a role in its symptomless resistance response to powdery mildew.

MATERIAL AND METHOD

Seeds of cherry pepper (*Capsicum annuum* var. *cerasiforme*) cv. Szentesi and sweet pepper (*C. annuum*) cv. Totál are commercially available in Hungary and were sown in a laboratory greenhouse. Grafts were carried out by cutting both rootstock and scion plants above the cotyledons in a 45° angle and pairing with the aid of a grafting clips. At least two weeks were allowed for development of graft unions. Plants were about 70 days old when used for experiments.

The presence of free and bound (glycosylated) forms of SA in healthy pepper leaves was detected by high performance liquid chromatography (HPLC) analysis as described by MEUWLY AND MÉTRAUX (1993) and COLE ET AL. (2004).

To assay expression of pathogenesis/defense related (PR) genes in healthy pepper leaves total RNA was extracted in liquid nitrogen with a plant RNA extraction minicolumn kit followed by reverse transcription and real-time quantitative polymerase chain reaction (qPCR) as described earlier (HAFEZ ET AL., 2012). Expression of a pepper actin gene (*CaAct*, GenBank accession AY572427) was used as an internal control.

Oligonucleotide primers used in qPCR were the following: 5'-ATCCCTCCACCTCTTCACTCTC-3' (5' primer) and 5'-GCCTTAACCATTCCTGTTCCATTATC-3' (3' primer) for a 128 bp pepper actin *(CaAct)* cDNA fragment (GenBank AY572427); 5'-GTTGTGCTAGGGTTCGGTGT-3' (5' primer) and 5'- CAAGCAATTATTTAAACGATCCA-3' (3' primer) for a 301 bp pepper PR gene *(CaPR-1)* cDNA fragment (GenBank AF053343); 5'-ACAGGCACATCTTCACTTACC-3' (5' primer) and 5'-CGAGCAAAGGCGAATTTATCC-3' (3' primer) for a 226 bp pepper PR glucanase *(CaPR-2)* cDNA fragment (GenBank AF227953).

RESULTS

HPLC assays of free and bound (glycosylated) salicylic acid (SA) in healthy pepper leaves demonstrated that in leaves of PM-resistant pepper (i.e. cv. Szentesi and sweet pepper cv. Totál grafted on cv. Szentesi rootstocks) levels of free SA are ca. twice as high as in PM-sensitive plants (*Fig. 1A*). However, no difference occurred in levels of the bound (glycosylated) form of SA: it was essentially the same in both PM-resistant and PM-susceptible pepper (*Fig. 1B*).

Monitoring transcriptional changes of so-called pathogenesis/defense related (PR) genes in healthy pepper leaves by real time RT-qPCR revealed that expression of the *CaPR-1* gene is several times higher in leaves of PM-resistant pepper than in sensitive plants. Interestingly, however, *CaPR-1* expression was by far the highest in PM-resistant cv. Szentesi plants, while it was markedly lower in PM-resistant sweet pepper cv. Totál grafted on cv. Szentesi rootstocks (*Fig. 2A*). On the other hand, high expression levels of the *CaPR-2* (glucanase) gene did not entirely correlate with PM-resistance, being detectable only in PM-resistant cv. Szentesi plants but neither in PM-resistant sweet pepper cv. Totál grafted on cv. Szentesi rootstocks nor in susceptible controls (cv. Totál) (*Fig. 2B*).

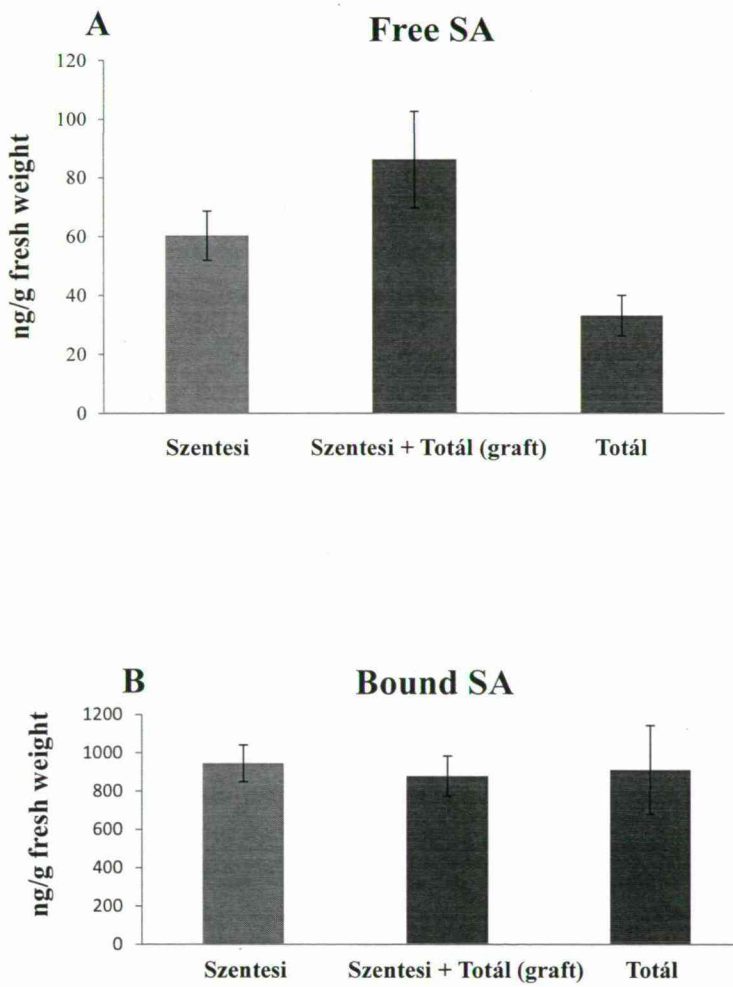

Figure 1. Levels of free (A) and bound (glycosylated) (B) salicylic acid (SA) in leaves of healthy pepper plants assayed by HPLC in powdery mildew-resistant cherry pepper cv. Szentesi and sweet pepper cv. Totál grafted on cv. Szentesi rootstocks and in powdery mildew-susceptible sweet pepper cv. Totál

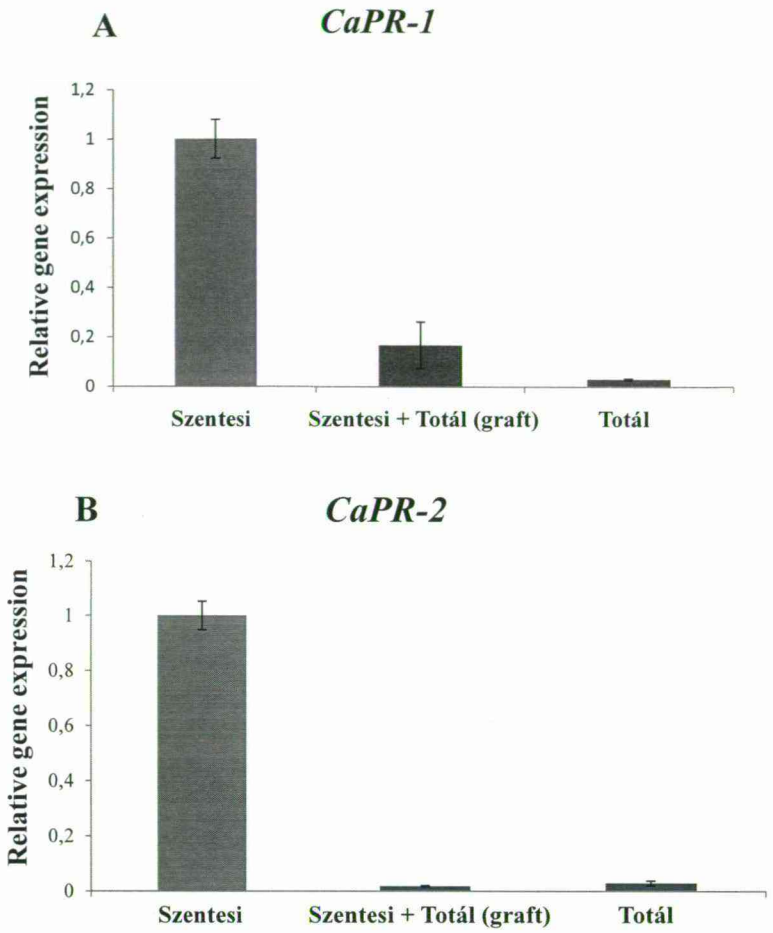

Figure 2. Expression of the pathogenesis/defense related genes *CaPR-1* (A) and *CaPR-2* (glucanase) (B) in leaves of healthy pepper plants assayed by real time RT-qPCR in powdery mildew-resistant cherry pepper cv. Szentesi and sweet pepper cv. Totál grafted on cv. Szentesi rootstocks and in powdery mildew-susceptible sweet pepper cv. Totál

DISCUSSION

Grafting may facilitate stable transmission of certain genotypic and phenotypic traits (RAPD DNA profiles, fruit shape and color) from e.g. pepper rootstocks to scions (see e.g. TALLER ET AL., 1998). However, only a few cases are mentioned in the literature when plant disease resistance has been transferred by grafting (ŠUTIĆ, 1965; AL-MAWAALI ET AL., 2012; VULIĆ ET AL., 2013), but the biochemical/genetic mechanisms were not described in any case. Our previous research has shown that resistance to pepper powdery mildew (PM) also develops in susceptible sweet pepper (*C. annuum*) when grafted on resistant cherry pepper cv. Szentesi rootstocks. This graft-transmissible PM resistance is correlated with high levels of the defense regulator reactive oxygen species superoxide ($O_2^{\cdot-}$) even in healthy plants (KIRÁLY ET AL., 2013; KÜNSTLER ET AL., 2013). It is known that in barley leaves artificial generation of superoxide by external treatment with e.g. riboflavin and methionine confers resistance to powdery mildew (*Blumeria graminis* f.sp. *hordei*) (EL-

ZAHABY ET AL., 2004). Also, sufficient expression of a NADPH-oxidase gene responsible for superoxide production is required for penetration resistance of barley to its powdery mildew pathogen (PROELS ET AL., 2010).

Results of our present study show that mechanisms of preformed defense responses in the powdery mildew (PM) resistant cherry pepper cv. Szentesi could involve not only the elevated production of ROS like superoxide but also elevated levels of the free form of salicylic acid (SA), a key molecule of plant defense signaling. Levels of free SA were ca. twice as high in leaves of PM-resistant pepper as in PM-sensitive plants. Evidence for the role of SA in plant resistance responses has come from experiments with transgenic tobacco and *Arabidopsis thaliana* unable to synthesize or accumulate high levels of SA (GAFFNEY ET AL., 1993; DELANEY ET AL., 1994; WILDERMUTH ET AL., 2001). Also, overproduction of SA in transgenic and interspecific hybrid plants stimulates resistance to viral and fungal pathogens (VERBERNE ET AL., 2000; COLE ET AL., 2004).

We found, however, that no difference occurred in levels of the bound (glycosylated) form of SA, it was essentially the same in both PM-resistant and PM-susceptible pepper. This suggests that bound forms of SA may not play a direct role in defense (disease resistance) responses of pepper plants. However, one should consider that the bound (glycosylated) form of SA (SAG) is how this signaling molecule is likely stored in plants, and hydrolysis of SAG in tobacco, a close relative of pepper, may require as little as 2 hours (HENNIG ET AL., 1993; VLOT ET AL., 2009). Therefore, *in planta* SAG might have a role in conferring basal levels of preformed defense responses in e.g. cherry pepper as well.

Our results also show that elevated expression of so-called pathogenesis/defense related (PR) genes in healthy cherry pepper cv. Szentesi may be associated with the preformed defense responses leading to PM resistance. Expression of the *CaPR-1* gene was several times higher in healthy leaves of PM-resistant pepper than in sensitive plants. *CaPR-1* is encoding for a basic PR-1 protein and shows elevated expression in pepper cultivars during successful resistance to the oomycete pathogen *Phytophthora capsici* (SILVAR ET AL., 2008). Similarly, overexpression of *CaPR-1* in tobacco plants enhances tolerance to oomycete and bacterial pathogens (SAROWAR ET AL., 2005). *PR-1* genes may also contribute to penetration resistance of barley plants to powdery mildew (*B. graminis* f.sp. *hordei*) infection, as shown by transient silencing of the *PR1-b* gene in barley epidermal cells (SCHULTHEISS ET AL., 2003). Although the functional role of PR-1 proteins in plant disease resistance is not exactly known, it has been demonstrated that in leaves of broad bean (*Vicia faba*) a basic PR-1 protein can inhibit differentiation of rust (*Uromyces fabae*) infection hyphae (RAUSCHER ET AL., 1999). In pepper, the basic PR-1 protein encoded by *CaPR-1* could play a similar role in PM-resistance, considering that both pathogens (broad bean rust and pepper PM) enter plant leaves through stomatal pores.

On the other hand, we have shown in this study that in healthy pepper leaves high expression levels of the *CaPR-2* gene do not entirely correlate with PM-resistance, being detectable only in PM-resistant cv. Szentesi plants but neither in PM-resistant sweet pepper cv. Totál grafted on cv. Szentesi rootstocks nor in susceptible controls (cv. Totál). *CaPR-2* encodes for a basic β-1,3-glucanase, an enzyme that hydrolyzes β-1,3-glucans, components of fungal and oomycete pathogen cell walls (see e.g. VAN LOON ET AL., 2006). Interestingly, *CaPR-2* expression is markedly induced after *P. capsici* infection only in certain resistant pepper cultivars and a significant gene induction is also observed during successful pathogenesis (SILVAR ET AL., 2008). It seems that *CaPR-2* expression may be involved not only in disease resistance but also in general stress responses during e.g. fungal pathogenesis, therefore, it is likely not a reliable marker of (preformed) plant defense responses.

In summary, the present study has demonstrated that graft-transmissible PM-resistance of the cherry pepper cv. Szentesi is correlated not only with high levels of superoxide but also with elevated levels of free salicylic acid and enhanced expression of the defense-related *CaPR-1* gene. These findings could serve as a basis for further investigations on elucidating the precise pathophysiological mechanisms of this symptomless resistance of pepper to its devastating powdery mildew pathogen.

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