Note

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Antifungal Activity of a Recombinant Carnation Cystatin, rDC-CPIn

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Abstract

We investigated the effect of a recombinant cystatin (proteinaceous cysteine proteinase inhibitor) cloned from carnation and expressed in *Escherichia coli* (rDC-CPIn) on the growth of phytopathogenic fungi. rDC-CPIn inhibited the growth of *Phytophthora nicotianae, Botrytis cinerea*, and *Sclerotiana sclerotiorum*. These results suggest that the cDNA for carnation cystatin could be useful for the generation of transgenic plants with increased defenses against fungal plant pathogens.

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Cystatins are proteinaceous inhibitors of cysteine proteinases and are present in both animals and plants. Plant cystatins (phytocystatins) are grouped into a specific subfamily separate from their animal homologs (Margis *et al.*, 1998). They have been identified in monocotyledonous plants, such as rice (Abe *et al.*, 1987; Kondo *et al.*, 1990) and corn (Abe *et al.*, 1992), as well as in dicotyledonous plants, such as soybean (Hines *et al.*, 1991; Misaka *et al.*, 1996; Zhao *et al.*, 1996) and chestnut (Pernas *et al.*, 1998). Two possible roles have been proposed for these plant cystatins: (1) they probably act as regulators of proteolysis during seed maturation and germination (Salmia, 1980; Abe *et al.*, 1992), and (2) they could contribute to plant defenses by inhibiting exogenous proteinases, such as those from insect pests (Misaka *et al.*, 1996).

Cystatins have been shown to have antiviral activity (Kondo *et al.*, 1992; Aoki *et al.*, 1995), and cystatins of soybean and rice have been shown to inhibit *in vitro* digestive proteinases from coleopteran insects (Hines *et al.*, 1991; Liang *et al.*, 1991; Zhao *et al.*, 1996). These findings led to the generation of transgenic plants, transformed with exogenous cystatin cDNAs, which overexpressed cystatins and thereby obtained increased resistance against insects (Lepe *et al.*, 1995; Irie *et al.*, 1996), nematodes (Urwin *et al.*, 1995, 1997), and potyviruses (Gutierrez-Campos *et al.*, 1999).

Recently, Pernas *et al.* (1999) demonstrated that chestnut cystatin inhibited the growth of the phytopathogenic fungi *Botrytis cinerea*, *Colletotrichum graminicola*, and *Septoria nodorum*, but not that of the phytopathogenic bacteria *Erwinia chrysanthemi* and *Clavibacter michiganense*. Most recently, genes for cystatins were identified in carnation plants and shown to be expressed abundantly in petals at the full opening stage of flowers (Kim *et al.*, 1999; Sugawara *et al.*, 2002). Furthermore, Sugawara *et al.* (2002) showed that the carnation cystatin DC-CPIn is involved in the regulation of petal wilting in senescing flowers. When the cDNA for the DC-CPIn gene was expressed in *E. coli*, the resultant recombinant DC-CPIn protein (rDC-CPIn) strongly inhibited the activities of a proteinase (cysteine proteinase) extracted from carnation petals, and papain: 50% inhibition was attained at about 5 ng ml^{-1} (ca. 0.4 nM) for both the carnation proteinase and papain. The inhibitory activity of rDC-CPIn seemed to be higher than that reported for the chestnut cystatin (Ki for papain: 29 nM) (Pernas *et al.*, 1998), which has an inhibitory activity comparable to that of thionin and many antibiotics (Pernas *et al.*, 1999).

We examined the effects of the rDC-CPIn against various phytopathogenic fungi, on the assumption that the cDNA for DC-CPIn could be used as a transgene for generating transgenic plants overexpressing the cystatin protein.

rDC-CPIn was prepared as described previously (Sugawara *et al.*, 2002). *In vitro* assays for the inhibition of fungal growth were performed using

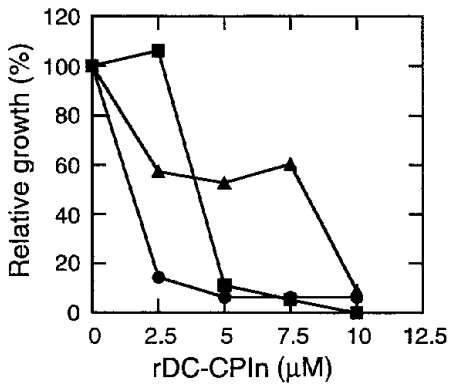

Fig. 1 Inhibition of the growth of *Phytophthora nicotianae* (●), *Botrytis cinerea* (■), and *Sclerotiana sclerotiorum* (▲) by recombinant carnation cystatin (rDC-CPIn). For the bioassay, 10^3-10^4 spores were incubated in 100 μl of 1/3 x potato dextrose broth at 22 ℃ in the dark in the presence of rDC-CPIn at different concentrations. Each incubation consisted of four separate cultures. Growth was monitored by measuring absorbance at 492 nm after 7 days for *P. nicotianae*, 3 days for *B. cinerea*, and 5 days for *S. sclerotiorum*. The means of the four separate cultures for each fungus were calculated, and data are expressed as the percentage growth relative to that in the absence of rDC-CPIn.

Phytophthora nicotianae, *B. cinerea*, and *Sclerotiana sclerotiorum* according to the method described previously (Broekaert *et al.*, 1990; Molina *et al.*, 1993; Pernas *et al.*, 1999). We used these fungi as model microorganisms because of their polyxenic pathogenecity against diverse plant species. Briefly, 10^3-10^4 spores were inoculated in 100 μl of 1/3 x potato dextrose broth in the presence of rDC-CPIn at different concentrations, and cultured at 22 ℃ in the dark with gentle reciprocating shaking. Growth was monitored by measuring absorbance at 492 nm after a prescribed numbers of culture days depending on the fungus, and is expressed as a percentage of the growth in the absence of rDC-CPIn. Experiments were repeated twice with similar results, and typical results for each fungus are shown in **Fig. 1**. rDC-CPIn inhibited the growth of three fungi, but the inhibitory effect varied depending on fungal species. rDC-CPIn at 2.5 μM inhibited the growth of *P. nicotianae* by 85%. The growth of *B. cinerea* was not inhibited at 2.5 μM, but was inhibited by 90% at 5 μM. With *S. sclerotiorum*, the growth was inhibited by 91% at 10 μM. These findings clearly indicate that the carnation cystatin has antifungal activity at micromolar concentrations, in agreement with the activity reported for chestnut cystatin (Pernas *et al.*, 1999).

rDC-CPIn at 10 μM did not inhibit the growth of a phytopathogenic bacterium *Pseudomonas syringae* (data not shown), which was in agreement with no inhibition by chestnut cystatin of the growth of the phytopathogenic bacteria *E. chrysanthemi* and *C. michiganense* (Pernas *et al.*, 1999). These observations indicate that the inhibition of the fungal growth did not result from the general toxic effect of the cystatins. Pernas *et al.* (1999) found that chestnut cystatin inhibited the *in vitro* activity of proteinase extracted from *B. cinerea*, and suggested that it arrested fungal growth by indirect inhibition of fungal cell wall development through the inhibition of a proteinase activity required for the processing of the membrane-bound chitin synthase precursor (Georgopapadakou and Smith, 1985; Machida and Saito, 1993). Whether carnation cystatin also exerts its inhibitory activity on the growth of the phytopathogenic fungi through its inhibitory effect on cysteine proteinases remains to be determined.

The present findings show that rDC-CPIn has an antifungal activity, and raises the possibility of using its cDNA to generate transgenic plants with increased defenses against fungal plant pathogens. Whether transgenic plants harboring the carnation cystatin gene (*DC-CPIn*) are more resistant against phytopathogenic fungi remains to be determined.

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