Secreted expression in E. coli of P. infestans elicitin using the FLAG system
Kamoun Lab (2000)

Kamoun, S., P. van West, A.J. de Jong, V.G.A.A. Vleeshouwers, and F. Govers. 1997. A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. Molecular Plant-Microbe Interactions. 10:13-20.

Results

Bacterial expression of cloned inf1 gene.

In order to determine whether the product of the inf1 gene is biologically active as an elicitor molecule, the coding sequence of the processed form of the INF1 protein was cloned in pFLAG-ATS, a vector which allows isopropyl-b-D-thiogalactopyranoside (IPTG) induced expression of the inserted gene in E. coli. The product is secreted by E. coli as a fusion protein containing the signal peptide of the ompA gene followed by the epitope tag, FLAG, at the amino-terminus. In addition to pFB53 (FLAG-INF1), a second plasmid, pFB52, was constructed, in which the cysteine codon at position 23 (or position 3 of the processed protein) of INF1 was mutated into a serine. After induction with IPTG, cultures of E. coli strains containing either plasmid were centrifuged and the supernatants collected and filter sterilized. Infiltration in tobacco (cv. Xanthi) leaves with ten-fold diluted supernatants of strain pFB53 (FLAG-INF1) consistently induced a HR, whereas supernatants of strain pFB52 (FLAG-INF1S3) did not induce any visible response (data not shown).

To further investigate the secretion of the fusion proteins, western blots containing soluble cellular extracts and culture supernatants of E. coli strains bearing pFB52 or pFB53 were incubated with FLAG monoclonal antibodies (Fig. 7A). Two bands of approximately 12 and 14 kDa reacted with the FLAG antibody in the cellular fraction, whereas only the lower band was present in the supernatant fractions suggesting that secretion of the fusion proteins is accompanied by the proper removal of the ompA signal peptide. Affinity chromatography purification of the fusion proteins was conducted from E. coli supernatant fractions using beads coated with FLAG monoclonal antibodies. Only a single band corresponding to either the FLAG-INF1 or the FLAG-INF1S3 fusion proteins was detected in the purified fractions after silver staining or western blot analysis (data not shown). Infiltration of tobacco leaves with 100 nM of the purified proteins FLAG-INF1 and FLAG-INF1S3 confirmed the results obtained with the crude supernatants, as only FLAG-INF1 induced a HR (Fig. 7B). Infiltration with the various buffers and culture supernatant from E. coli bearing the cloning vector induced no visible response. These results suggest that the cloned inf1 gene encodes an active elicitor protein and that cys23 is crucial for the activity of the elicitor.

Methods

Plasmids

Plasmids pFB52 and pFB53 were constructed by cloning polymerase chain reaction (PCR) amplified DNA fragments corresponding to the inf1 ORF into the HindIII site of pFLAG-ATS (IBI-Eastman Kodak, New Haven, CT). The oligonucleotides used in the PCR are SK-F1 and SK-R1 for the fragment cloned in pFB53, and SK-F1S and SK-R1 for the fragment cloned in pFB52 (Table 4). Partial nucleotide sequencing of the cloned fragments (including the mutated site) in pFB52 and pFB53 fully matched the predicted sequence. The N-terminal sequence of the processed recombinant FLAG-INF1 protein of pFB53 is "DYKDDDDKDKVKI
TTCTTSQQT...". The FLAG antibody binding site is underlined, and the first ten amino acids of mature INF1 are shown in bold with the mutated cysteine of pFB52 shown in bold italics.

Expression of inf1 in E. coli.

Expression of inf1 in pFLAG-ATS and immuno-affinity purification of FLAG fusion proteins were conducted following the protocols provided by the manufacturer (IBI-Eastman Kodak, New Haven, CT). Overnight cultures of E. coli DH5a containing either pFB52 or pFB53 were diluted (1:100) in LB medium containing ampicillin (50 mg/ml) and incubated at 37oC. When the OD600 of the cultures reached 0.6, IPTG was added to a final concentration of 0.4 mM. The cultures were further incubated for 3-4 hours before processing. Immuno-affinity purification was performed using a FLAG M2 antibody affinity gel.

Elution of fusion proteins from the affinity column was obtained after treatment with 0.1 M glycine (pH 3.0).

Summary:

FLAG-INF1 Expression

Strain: DH5alpha (pFB53)

Induction:

1. Inoculate 3-5 ml LB amp with pFB53
2. Grow overnight
3. Inoculate 100 ml LB amp with2 ml overnight culture
4. Grow until OD600 reaches 0.5-0.8
5. Add 200 ul 0.2M IPTG (final cc is 0.4 mM)
6. Grow for at least 4 hours (overnight is OK)
7. Centrifuge, collect supernatant, filter sterilize to eliminate E. coli cells
8. Store at -80oC
9. FLAG-INF1 protein can be detected using M2 AntiFLAG monoclonal