

Small secreted proteins from *Trichoderma virens* and their role in mycoparasitism and plant defense resistance

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Abstract

The biological control agent *Trichoderma virens* 29-8 is known to metabolize small secreted proteins (SSPs) that induce plant defense mechanisms in monocots and dicots. Also, the expression of three genes (*Tv93159*, *Tv79522* and *Tv110650*) that encode SSPs have been detected during mycoparasitic interactions against fungal pathogens (*Botrytis cinerea*, *Pythium ultimum* and *Rhizoctonia solani*) *in vivo* and *in vitro*. Purification of these proteins from *T. virens* results in low yield limiting their use commercially. To increase yield we cloned *Tv93159*, one small secreted gene expressed in mycoparasitism conditions, into the pPIC9K vector for transformation into *Pichia pastoris* SMD1168 strain. Expression conditions were optimized in a time course assay with methanol induction. Purified protein was detected in a dot blot analysis by an anti-FLAG antibody and a BCIP/NBT color development substrate.

Introduction

For more than a decade some *Trichoderma* spp. have been described as biological alternatives to chemical fungicides because of their antagonistic or mycoparasitic interactions with plant pathogens. Additionally, their ability to enhance growth and elicit plant defense responses has been demonstrated [1]. The majority of elicitors from plant pathogens have one common feature, they are small secreted proteins. So far, only one elicitor of plant defense responses has been described in fungal biocontrol agents (Sm1 from *T. virens*) [2]. These small proteins have an important role in the control of plant diseases either directly through mycoparasitism or indirectly by inducing a resistant response following treatment of plants with these active elicitors [3]. To use these elicitors on a commercial basis, their production must be enhanced and new products formulated for application. As a demonstration of enhanced production, the *P. pastoris* expression system represents an efficient alternative to the purification of the native protein from *T. virens*. The aim of this work is to express three small secreted proteins from *T. virens* in *P. pastoris* and optimize the conditions for maximum yield.

Small secreted proteins in *T. virens* 29-8

Previously, up-regulated expression of three genes (protein ID: 93159, 79522 and 110650), with homology to small cysteine secreted proteins, was detected in a RNA-Seq analysis using the Illumina® platform. Tissue was obtained after growing *T. virens* 29-8 in MS (Murashige & Skoog) medium in hydroponic system in the presence of maize roots as previously described (Djonovic et al., 2007).

Table 1. Characteristics of three SSPs from *T. virens* 29-8

	Gene length (bp)	Number exons	Conserved domains
<i>Tv93159</i>	342	1	N/I
<i>Tv79522</i>	660	3	N/I
<i>Tv110650</i>	767	2	N/I

Expression studies in *T. virens* 29-8

Expression analysis of the three selected genes was performed after growing *T. virens* 29-8 in Vogel's minimal medium (VM) supplemented with different carbon sources (Fig. 1) or in dual confrontation (Fig. 2A and 2B). Confrontation plates with *T. virens* 29-8 opposite pathogens (*B. cinerea*, *P. ultimum* or *R. solani*) on VMS agar plates overlaid with cellophane.

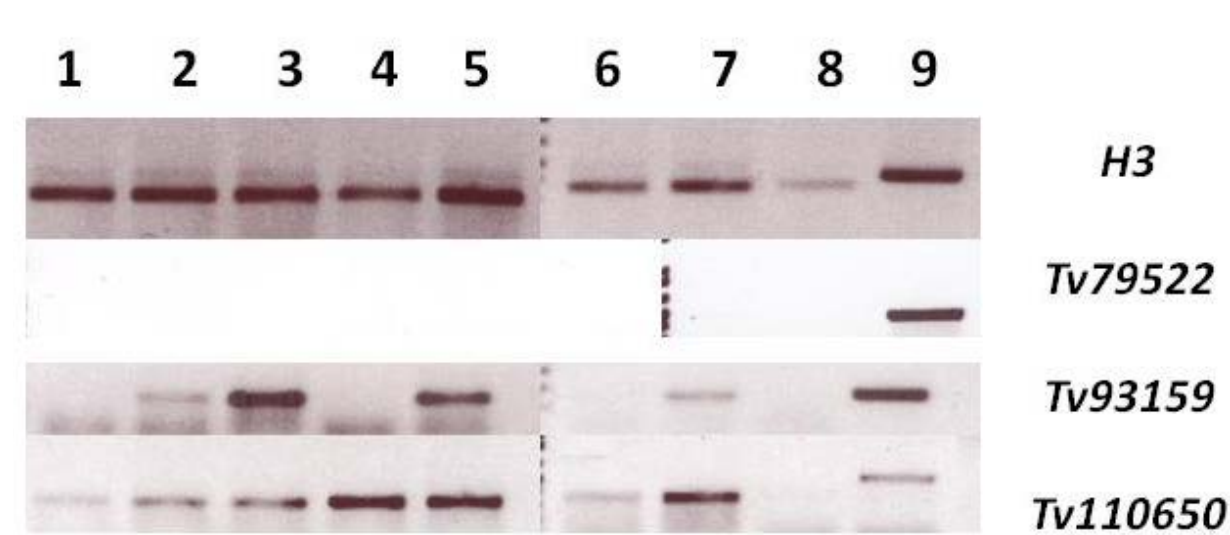


Fig. 1. Expression analysis of selected genes *in vitro* was analyzed in VM (7) or VM supplemented with 1.5% sucrose (8), 0.2% maize roots (6), 1% colloidal chitin (5), 0.5% *B. cinerea* (4), 0.5% *P. ultimum* CWs (3), 0.5% *R. solani* CWs (2) or 1% lignin (1) by RT-PCR. *T. virens* 29-8 gDNA (9) was used as a positive control. Histone (H3) gene was used as loading control.

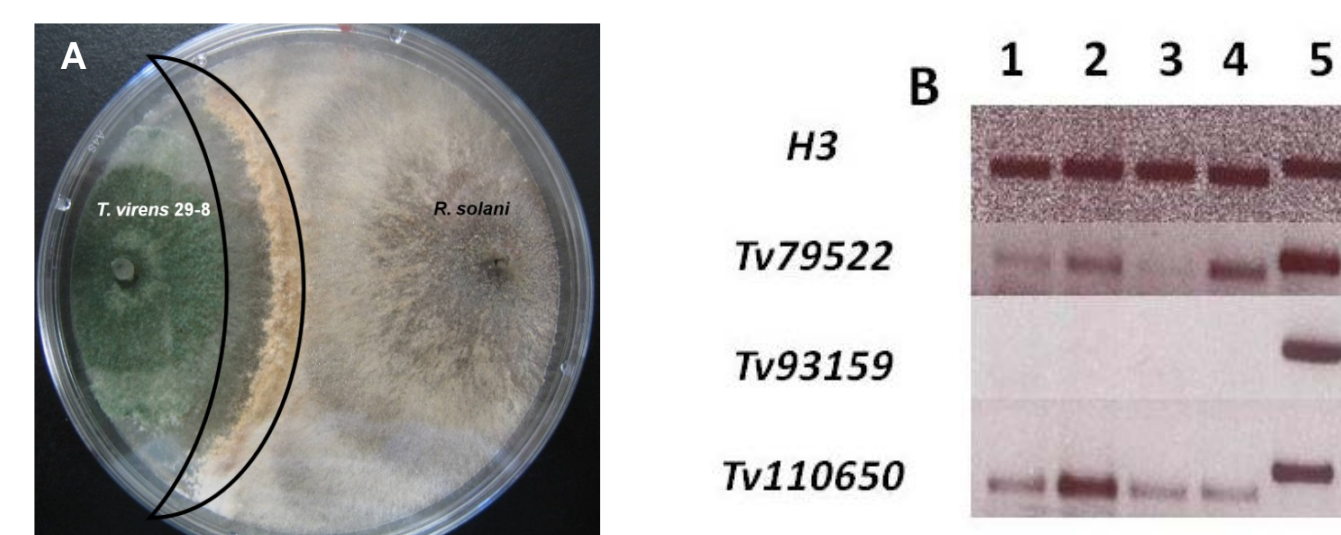


Fig. 2. (A) *T. virens* 29-8 and *R. solani* dual confrontation VMS plate. Contact tissue was collected for RNA extraction. (B) Expression analysis of selected genes *in vivo* was analyzed in *T. virens* 29-8 vs *T. virens* 29-8 (1), *T. virens* 29-8 vs *B. cinerea* (2), *T. virens* 29-8 vs *P. ultimum* (3) or *T. virens* 29-8 vs *R. solani* (4) by RT-PCR. *T. virens* 29-8 gDNA (5) was used as a positive control. Histone (H3) gene was used as loading control.

Plasmid design and *P. pastoris* transformation

The *P. pastoris* system offers a broad range of possibilities for the heterologous production of secreted proteins. The experiments were performed following protocols from Invitrogen.

Experimental approach

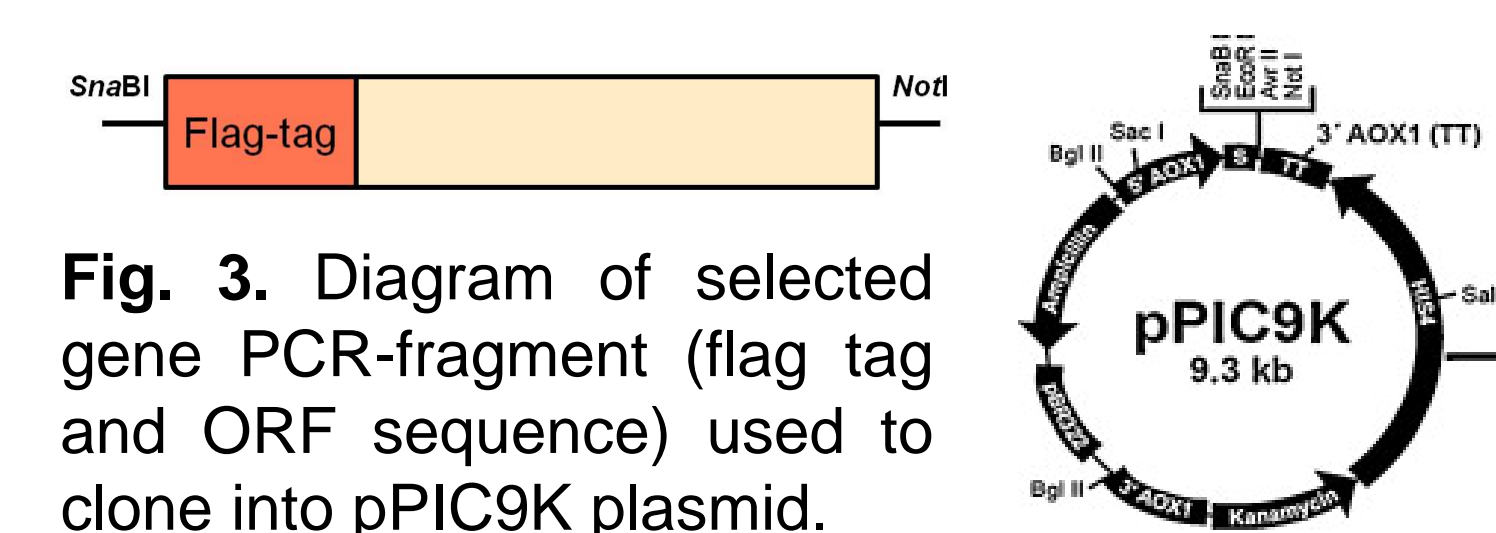
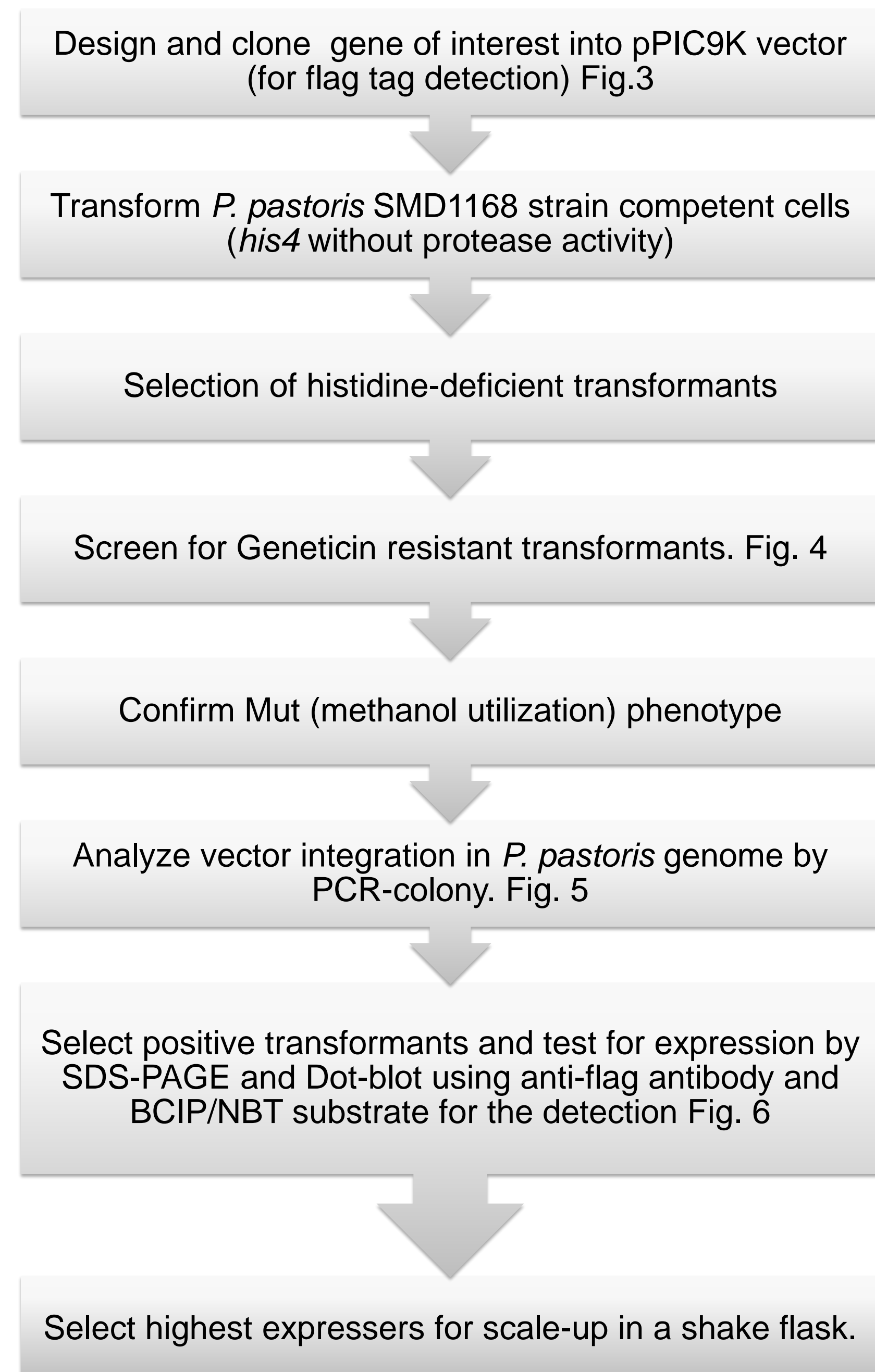


Fig. 3. Diagram of selected gene PCR-fragment (flag tag and ORF sequence) used to clone into pPIC9K plasmid.

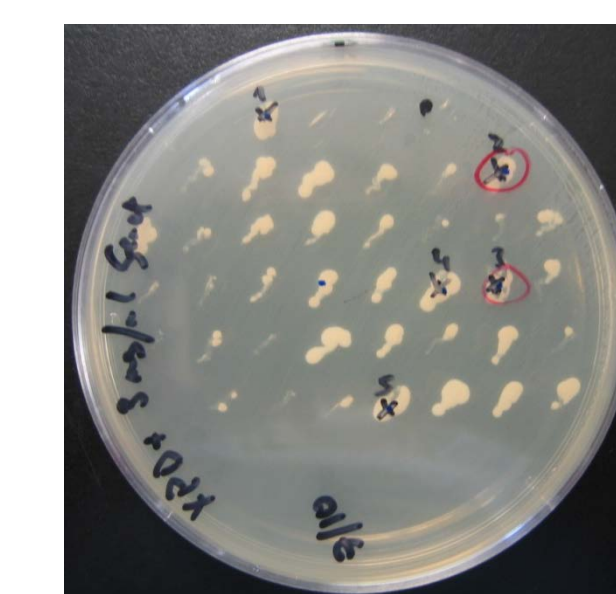


Fig. 4. Image of a YPD (Yeast extract peptone dextrose) plate supplemented with 3 mg/ml of Geneticin to select hyper-resistant transformants.

Fig. 5. PCR analysis of *Pichia* integrants with 5'AOX and 3'AOX primers. 93159-pPIC9K plasmid (1), parent plasmid (2), tested transformants (3-7).

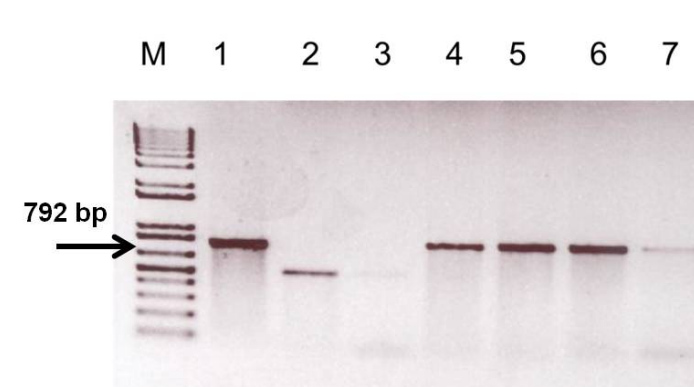
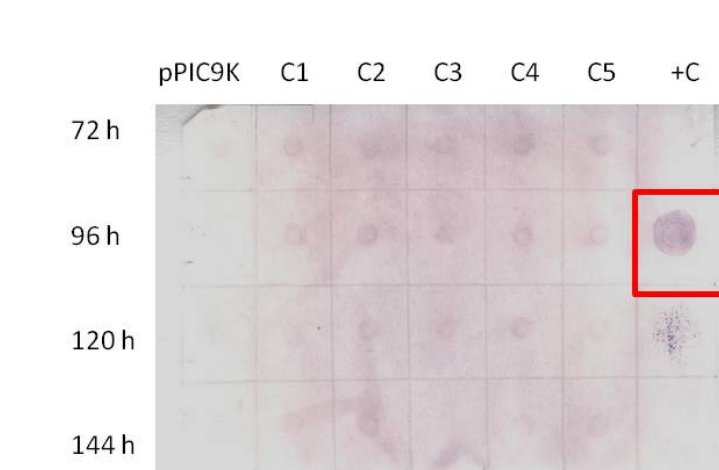


Fig. 6. Dot blotting of culture filtrates from parental plasmid (pPIC9K) and 5 transformants (C1-C5) obtained after growing *P. pastoris* in BMMY for 72, 96, 120 and 144 h. Flag tag peptide was used as a positive control (+C)



Conclusions

Many biologically important proteins have successfully been over-produced using *P. pastoris* system for agricultural, medical and industrial applications. In this study, we have explored the use of this system to enhance the production of small secreted proteins from *T. virens* 29-8 that may be involved in mycoparasitism and induction of maize defense responses.

References

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- [2] Djonovic, S; Vargas, WA; Kolomiets, MV; Horndeski, M; Wiest, A and Kenerley, CM (2007) A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. Plant Physiol. 145: 875-889
- [3] Djonovic, S; Pozo, MJ; Dangott, LJ; Howell, CR and Kenerley, CM (2006) Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Mol. Plant-Microbe Interact 19: 838-853

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