

SHORT COMMUNICATION
**TRANSFORMATION OF *METSCHNIKOWIA PULCHERRIMA* 320,
 BIOCONTROL AGENT OF STORAGE ROT,
 WITH THE GREEN FLUORESCENT PROTEIN GENE**

F. Nigro, M.M. Finetti Sialer and D. Gallitelli

Dipartimento di Protezione delle Piante dalle Malattie, Università degli Studi di Bari and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via G. Amendola 165/A, I-70126 Bari, Italy

SUMMARY

The yeast *Metschnikowia pulcherrima* (Pitt) M.W. Miller, isolate 320, showing antagonistic activity against *Botrytis cinerea*, causal agent of storage rot of table grapes, strawberry and kiwifruit, was transformed with the yeast-enhanced green fluorescent protein (yEGFP) gene, using plasmid pACT2.yEGFP and the lithium acetate protocol. Colonies expressing yEGFP were selected using an epifluorescence microscope. The antagonistic activity of five GFP transformants was evaluated on table grapes, cvs 'Matilde' and 'Italia', and compared to that of the *M. pulcherrima* 320 wild type. The activity of two transformants was indistinguishable from that of the parental strain, significantly reducing *Botrytis* storage rot; however, three transformants lost antagonistic activity completely. GFP-transformants and *M. pulcherrima* wild type showed the same growth rates in vitro. This appears to be the first report of *M. pulcherrima* transformation with the GFP gene.

RIASSUNTO

TRASFORMAZIONE DELL'ISOLATO 320 DI *METSCHNIKOWIA PULCHERRIMA*, ANTAGONISTA DEL MARCIUME BOTRITICO IN POSTRACCOLTA, CON IL GENE PER L'ESPRESSIONE DELLA PROTEINA FLUORESCENTE. *Metschnikowia pulcherrima* (Pitt) M.W. Miller, isolato 320, attivo in postraccolta contro il marciume botritico dell'uva da tavola, della fragola e dell'actinidia, è stato trasformato con un gene per l'espressione di proteina fluorescente (GFP), utilizzando il plasmide pACT2.yEGFP ed il protocollo del litio acetato. Le colonie esprimenti GFP sono state selezionate mediante osservazione al microscopio a fluorescenza. L'attività antagonista di cinque trasformanti è stata valutata su bacche di uva da tavola (cvs 'Matilde' e 'Italia') comparandola con

quella dell'isolato originale di *M. pulcherrima* 320. Due ceppi dei trasformanti hanno mostrato una attività antagonistica non differente da quella dell'isolato originale, fornendo significative riduzioni del marciume botritico; i restanti tre ceppi, invece, hanno completamente perso l'attività antagonistica. La crescita in vitro dei trasformanti è risultata non differente da quella del ceppo originario. Al presente, questa è la prima volta che viene riportata la trasformazione di *M. pulcherrima* con GFP.

Key words: postharvest disease, biological control, *Botrytis cinerea*, table grapes, GFP expression.

In the past 10 years, use of naturally occurring antagonistic yeasts and yeast-like fungi to protect fresh fruit and vegetables against postharvest diseases has gained increasing importance. The large body of information now available shows that these organisms have characteristics of an 'ideal antagonist' (Wilson and Wisniewski, 1989), such as the ability to survive under adverse environmental conditions, resistance to chemicals (Droby et al., 1993; Ippolito et al., 1998), competition for nutrients and space, and absence of metabolites deleterious to human health (Castoria et al., 1997; Ippolito et al., 1997a; Piano et al., 1997). Furthermore, preharvest application of yeasts and yeast-like fungi is becoming increasingly popular since this results in colonization of fruit surfaces and wounds prior to the establishment of postharvest pathogens (Ippolito et al., 1997b; Leibinger et al., 1997; Lima et al., 1997; Ippolito et al., 1998). However, little information is available on the ecology of these microorganisms on fruit surfaces and wounds (Mercier and Wilson, 1994). Using the green fluorescent protein (GFP) as a reporter gene (Chalfie et al., 1994) provides an effective means of studying gene expression (Cheng et al., 1996), following development of pathogens within their host plants (Spellig et al., 1996), and, more recently, identifying microorganisms on the leaf surface (Vanden Wymelenberg et al., 1997). These results indicate that GFP expression can provide a powerful means of studying plant-microorganism relationships in nature.

Corresponding author: F. Nigro
 Fax: +39.080.5442911
 E-mail: gallitel@agr.uniba.it

In several assays, the yeast *M. pulcherrima* (Pitt) M.W. Miller (anamorph: *Candida pulcherrima*), isolate 320, gave promising results in the control of *Botrytis* storage rot of strawberries, table grapes and kiwifruit (Salerno, unpublished results). However, better understanding of microbial ecology on fruit surfaces and modes of action seems fundamental for this organism to be utilized effectively. Since availability of yeast strains carrying a reporter gene could greatly help these investigations, a study was carried out to obtain GFP transformants of *M. pulcherrima* 320 and to investigate whether these retain the antagonistic activity of the wild strain.

M. pulcherrima isolate 320 was transformed with yeast-enhanced GFP (yEGFP) using plasmid pACT2.yEGFP. The yEGFP was excised from pUC19.yEGFP (kindly provided by Dr. J.P. Brown, University of Aberdeen, UK) using PstI/HindIII restriction sites. This fragment, optimized for expression in *Candida albicans* (Cormack et al., 1997), was ligated to the SmaI site of pACT2 (Clontech) after appropriate modification of the protruding ends, and cloned in *E. coli*, strain DH5 α . The resulting construct contained the coding region for GFP downstream of the promoter sequence for the *Saccharomyces cerevisiae* alcohol-dehydrogenase 1 (ADH1). Several clones containing the GFP fragment were obtained and the correct orientation in the pACT2 vector was verified by electrophoresis of the HpaI-digested plasmids.

Cells of *M. pulcherrima* were transformed using the lithium acetate protocol (Gietz et al., 1992). Cells were collected by centrifugation (10,000 g, 5 min) and, after appropriate dilution, plated on nutrient yeast dextrose agar (NYDA). The plates were incubated at 25°C for at least 4 days and chimaeric colonies expressing yEGFP were identified using a Zeiss HBO50 fluorescence microscope with the filter combination BP450-490/FT510/LP520 (excitation filter-dichroic-emission filter).

The efficiency of transformation of *M. pulcherrima* 320 was comparable to that reported by Vanden Wymelenberg et al. (1997). Seven GFP-transformed strains were obtained out of 6×10^3 yeast colonies examined. Five transformants exhibited a stronger green fluorescence than the remaining two, as assessed visually. Transformants were morphologically indistinguishable from the wild type and retained the fluorescence after six subculturings on yeast-peptone-dextrose (YPD) agar plates at monthly intervals.

The growth of the five GFP transformants (denoted gfp1, gfp2, gfp3, gfp4, gfp5) and *M. pulcherrima* 320 wild type (wt) was also evaluated. Fifty ml of YPD broth were inoculated with a cell suspension (1.0×10^5

final concentration) and incubated at 25°C, 150 rpm, for 72 h. Cell numbers were determined at 12 h intervals using a haemocytometer. Growth rates of the transformed strains were found to be the same as that of *M. pulcherrima* 320 wt (Fig. 1).

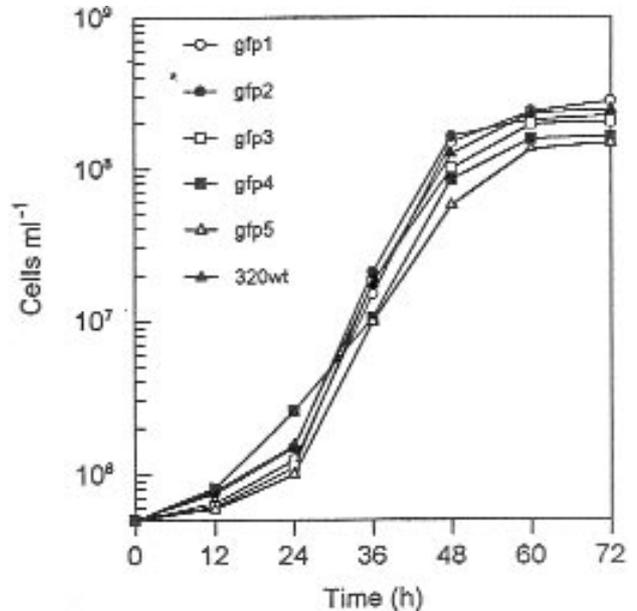


Fig. 1. In vitro growth of five GFP-transformed *M. pulcherrima* strains as compared to the wild type (320 wt). Strains were grown in YPD broth at 25°C, 150 rpm, for 72 h and the number of cells number was determined at 12 h intervals.

Possible transfer of the GFP gene to other microorganisms was also investigated. In particular, 1.0 - 1.2×10^5 cells both of gfp4 and a hygromycin B-resistant (HygB^R) strain of *Aureobasidium pullulans* (de Bary) Arnaud (Nigro, Ippolito and Salerno, unpublished information) were co-cultured in YPD broth at 25°C, 150 rpm, up to the stationary phase (72 h). Ten μl of cell suspension were plated onto YPD agar amended with HygB (100 ppm), after appropriate dilution. Plates were incubated at 25°C and the resulting colonies were examined under the fluorescence microscope after 7 days. No cells of HygB^R *A. pullulans* expressing GFP were found among 2.5×10^3 examined. Similar results were obtained after co-culturing gfp4 and conidia of *Botrytis cinerea* Pers.

Antagonistic activity of the five selected GFP transformants was evaluated on table grapes and compared to that of *M. pulcherrima* 320 wt. Single grapes (cvs 'Matilde' and 'Italia') were wounded at one site in the equatorial zone and 20 μl of a suspension (1.0 - 1.2×10^8 cells ml^{-1} in water) of GFP transformants or wt were

pipetted into the wound; controls received only water. Two hours later the wounds were inoculated with a spore suspension (1.0×10^5 spores ml^{-1}) of *B. cinerea*. The number of wounds developing lesions was recorded after 3 days at 20°C (RH = 95-97%). Disease severity was evaluated using a scale from 0 (healthy berries) to 4 (100% fruit surface rotten). Trials were arranged in a randomized design. Each treatment consisted of three replicas and each replica consisted of 20 berries. The data were compared using Duncan's Multiple Range Test (DMRT).

Strains gfp4 and gfp5 showed the same antagonistic activity as *M. pulcherrima* 320 wt, providing a significant ($P \leq 0.01$) reduction in percentage of infected berries on both 'Matilde' and 'Italia' cultivars. In contrast, strains gfp1, gfp2 and gfp3 behaved like the untreated control (Figs 2 and 3). Gfp4 and gfp5 also induced, on both cultivars, a significantly ($P \leq 0.01$) lower disease severity than the control and the other GFP-transformed strains. However, berries treated with *M. pulcherrima* 320 wt were significantly less diseased than berries that had received other treatments (Figs 2 and 3).

These results suggest that transformation with the GFP gene may lead to complete loss of the antagonism or a reduction of the activity but does not modify growth. This needs more investigation since some papers report differences in epiphytic fitness of mutants that completely lost antagonistic activity under field and laboratories conditions (Beattie and Lindow, 1994).

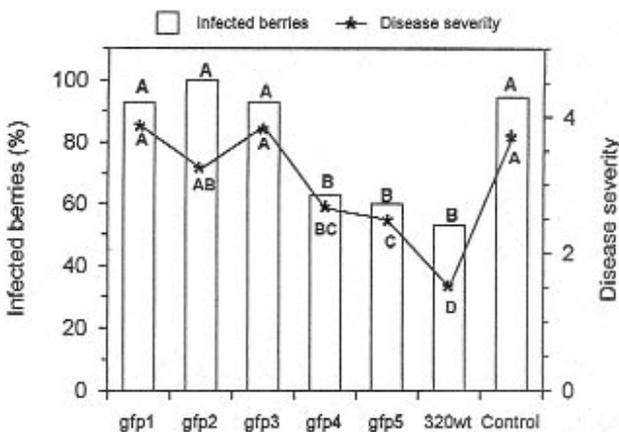


Fig. 2. Effect of five GFP-transformed *M. pulcherrima* strains on the development of Botrytis rot of table grapes (cv. 'Matilde') as compared to the wild type (320 wt) and untreated control. Disease assessment was made on berries incubated at 20°C (RH = 95-97%) for 3 days. Mean separation according to Duncan's multiple range test ($P \leq 0.01$).

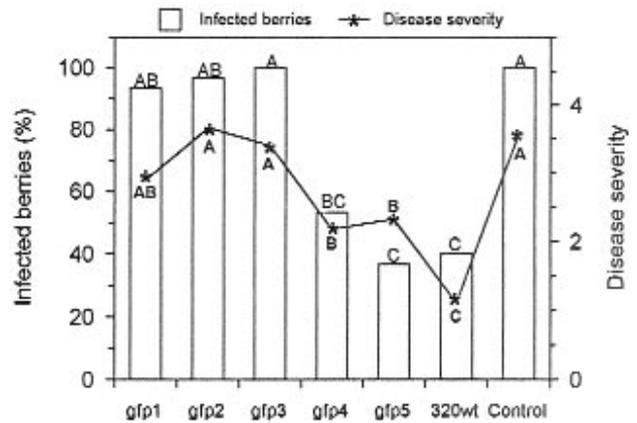


Fig. 3. Effect of five GFP-transformed *M. pulcherrima* strains on the development of Botrytis rot of table grapes (cv. 'Italia') as compared to the wild type (320 wt) and the control. Parameters as in Fig. 2.

The availability of variant strains of *M. pulcherrima* 320 carrying a genetically stable reporter gene but still endowed with useful antagonistic activity would help studies on the modes of action of this yeast under standard storage and field conditions. Moreover, GFP expression may provide a powerful method for ecological studies on plant-microbe relationships in nature and a method to assess colonization and dispersal of populations of biocontrol agents released into the environment (Gullino et al., 1995; Thrane et al., 1995; Lo et al., 1998). The understanding of population dynamics should result in a more effective technology for the biological control of postharvest disease of fruit and vegetables, and, eventually, provide information for a better manipulation of the microbial community on fruit surfaces.

REFERENCES

- Beattie G.A, Lindow S.E., 1994. Comparison of the behaviour of epiphytic fitness mutants of *Pseudomonas syringae* under controlled and field conditions. *Applied Environmental Microbiology* **60**: 3799-3808.
- Castoria R., De Curtis F., Lima G., De Cicco V., 1997. β -1,3-glucanase activity of two saprophytic yeasts and possible mode of action as biocontrol agents against postharvest diseases. *Postharvest Biology and Technology* **12**: 293-300.
- Chalfie M., Tu Y., Euskirchen G., Ward W.W., Prasher D.C., 1994. Green fluorescent protein as a marker of gene expression. *Science* **263**: 802-805.
- Cheng L., Fu J., Tsukamoto A., Hawley R.G., 1996. Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. *Nature Biotechnology* **14**: 606-609.

- Cormack B.P., Bertram G., Egerton M., Gow N.A.R., Falkow S., Brown A.J.P., 1997. Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*. *Microbiology* **143**: 303.
- Droby S., Hofstein R., Wilson C.L., Wisniewski M.E., Fridlender B., Cohen L., Weiss B., Daus A., Timar D., Chalutz E., 1993. Pilot testing of *Pichia guilliermondii*: a bio-control agent of postharvest diseases of citrus fruit. *Biological Control* **3**: 47-52.
- Gietz D., St. Jean A., Woods R.A., Schiestl R.H., 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Research* **20**: 1245.
- Gullino M.L., Migheli Q., Mezzalama M., 1995. Risk analysis in the release of biological control agents. Antagonistic *Fusarium oxysporum* as a case study. *Plant Disease* **79**: 1193-1201.
- Ippolito A., El Ghaouth A., Wilson C.L., 1997a. Indagini su alcuni dei possibili meccanismi di azione dell'isolato L47 di *Aureobasidium pullulans*, attivo contro marciumi post-raccolta di ortofrutticoli. In: Società Italiana di Patologia Vegetale (ed.). Atti V Convegno Annuale S.I.Pa.V, Legnaro (Padova) 1997, 5 (Abstract).
- Ippolito A., Nigro F., Lima G., Romanazzi G., Salerno M., 1998. Xanthan gum as adjuvant in controlling table grape rots with *Aureobasidium pullulans*. *Journal of Plant Pathology* **80**: 258 (Abstract).
- Ippolito A., Nigro F., Romanazzi G., Campanella V., 1997b. Field application of *Aureobasidium pullulans* against *Botrytis* storage rot of strawberries. Workshop Proceedings, COST 914-915, 'Non conventional methods for the control of post-harvest disease and microbiological spoilage', Bologna 1997, 127-133.
- Leibinger W., Breuker B., Hahn M., Mendgen K., 1997. Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. *Phytopathology* **87**: 1103-1110.
- Lima G., Ippolito A., Nigro F., Salerno M., 1997. Effectiveness of *Aureobasidium pullulans* and *Candida oleophila* against postharvest strawberry rots. *Postharvest Biology and Technology* **10**: 169-178.
- Lo C.T., Nelson E.B., Hayes C.K., Harman G.E., 1998. Ecological studies of transformed *Trichoderma harzianum* strain 1295-22 in the rhizosphere and on the phylloplane of creeping bentgrass. *Phytopathology* **88**: 129-136.
- Mercier J., Wilson C.L., 1994. Colonization of apple wounds by natural occurring microflora and introduced *Candida oleophila* and their effect on infection by *Botrytis cinerea* during storage. *Biological Control* **4**: 138-144.
- Piano S., Neyrotti V., Migheli Q., Gullino M.L., 1997. Bio-control capability of *Metschnikowia pulcherrima* against *Botrytis* postharvest rot of apple. *Postharvest Biology and Technology* **11**: 131-140.
- Spellig T., Bottin A., Kahmann R., 1996. Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. *Molecular General Genetics* **252**: 503-509.
- Thrane C., Lübeck M., Green H., Degen Y., Allerup S., Thrane U., Jensen D.F., 1995. Tool for monitoring *Trichoderma harzianum*: I. Transformation with the GUS gene by protoplast technology. *Phytopathology* **85**: 1428-1435.
- Vanden Wymelenberg A.J., Cullen D., Spear R.N., Schoenicke B., Andrews J.H., 1997. Expression of green fluorescent protein in *Aureobasidium pullulans* and quantification of the fungus on leaf surfaces. *Biotechniques* **23**: 686-690.
- Wilson C.L., Wisniewski M.E., 1989. Biological control of postharvest disease of fruits and vegetables: an emerging technology. *Annual Review of Phytopathology* **27**: 425-441.

Received 16 February 1999

Accepted 6 July 1999