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Separate and Combined Disruptions of Two Exo-β-1,3-Glucanase Genes Decrease the Efficiency of *Pichia anomala* (Strain K) Biocontrol Against *Botrytis cinerea* on Apple

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The modes of action of the antagonistic yeast *Pichia anomala* (strain K) have been studied; however, thus far, there has been no clear demonstration of the involvement of exo-β-1,3-glucanase in determining the level of protection against *Botrytis cinerea* afforded by this biocontrol agent on apple. In the present study, the exo-β-1,3-glucanase-encoding genes *PAEXG1* and *PAEXG2*, previously sequenced from the strain K genome, were separately and sequentially disrupted. Transfer of the *URA3*-Blaster technique to strain K, allowing multiple use of *URA3* marker gene, first was validated by efficient inactivation of the *PaTRP1* gene and recovery of a double auxotrophic strain (uracil and tryptophan). The *PAEXG1* and *PAEXG2* genes then were inactivated separately and sequentially with the unique *URA3* marker gene. The resulting mutant strains showed a significantly reduced efficiency of biocontrol of *B. cinerea* when applied to wounded apple fruit, the calculated protection level dropping from 71% (parental strain) to 8% (mutated strain) under some experimental conditions. This suggests that exo-β-1,3-glucanases play a role in the biological control of *B. cinerea* on apple. Furthermore, biological control experiments carried out in this study underline the complexity of the host–antagonist–pathogen interaction. Two experimental parameters (yeast inoculum concentration and physiological stage of the fruit) were found to influence dramatically the protection level. Results also suggest that, under some conditions, the contribution of exo-β-1,3-glucanase to biological control may be masked by other modes of action, such as competition.

Additional keywords: gene inactivation, postharvest.

Pichia anomala (strain K) has been isolated from the surface of Golden Delicious apple fruit as an efficient and reliable antagonist of *Botrytis cinerea* and *Penicillium expansum*, two worldwide postharvest pathogens of apple (Jijakli and Lepoivre 1993). Thus, strain K currently is investigated, among a wide range of other agents, for its ability to control postharvest diseases (Janisiewicz and Korsten 2002). It may constitute an effective solution to be included in an integrated pest management program aiming to reduce the environmental damage caused by synthetic fungicides (Wilson and Wisniewski 1994). Various studies have contributed to increasing knowledge on the biological properties of strain K. The biocontrol activity of strain K is preserved during mass production in a fermentor (De Clercq et al. 2003), and a molecular monitoring system has been developed and used to study strain K adaptation after its application under realistic conditions (Pujol et al. 2003). The modes of action of *Pichia anomala* have been studied in the *B. cinerea*–Golden Delicious model. A microbiological approach has highlighted the close link between fruit colonization by the yeast strain and the level of protection against *B. cinerea* (Jijakli et al. 1999). A biochemical approach leading to the purification and characterization of an exo-β-1,3-glucanase produced by strain K has underlined a close positive correlation between glucanase activity and protective ability (Jijakli and Lepoivre 1998). These results constitute the basis of a genetic study of *P. anomala*. Different marker genes of strain K have been identified, such as *PAURA3*, encoding orotidine monophosphate decarboxylase (Grevesse et al. 1998), *PALEU2*, encoding β-isopropylmalate dehydrogenase, and *PATRP1*, encoding phosphoribosyl anthranilate isomerase (Friel et al. 2003). Two genes encoding exo-β-1,3-glucanases (*PAEXG1*, accession number AJ002195, and *PAEXG2*, accession number AJ222862) have been cloned from strain K genomic DNA and sequenced. On the basis of targeted disruption with a *URA3* selection marker, Grevesse and associates report having ruled out the involvement of *PAEXG2* in the biocontrol activity of stain K (Grevesse et al. 1998, 2003). Yet, given the biochemical data, we felt that it was worth investigating further the contribution of *PAEXG1* and *PAEXG2* to antagonism of *B. cinerea* by strain K. We did this with the help of *paexg1*$^-$ and *paexg2*$^-$ single-disruption mutants and with a double-mutant strain obtained by sequential inactivation of both genes. To produce these mutants, we adapted existing molecular tools for use in *P. anomala*.

Given the complexity of interactions between strain K, *B. cinerea*, and apple wounds, multiple genes are likely to contribute to biocontrol. This is why we focused on a strategy allowing sequential gene disruption with the same marker gene. The *URA3*-Blaster technique was developed for *Saccharomyces cerevisiae* to allow multiple gene inactivations with the *URA3* gene (Alani et al. 1987). Taking advantage of the high frequency of mitotic recombination between nontandem direct repeats, the authors developed a disruption cassette in which the *URA3* gene is flanked by direct repeats of the bacterial *hisG* sequence. Spontaneous recombination between the two *hisG* sequences results in a *ura3*$^-$ phenotype that is selected on minimal medium supplemented with uracil and fluoro-orotic acid (FOA). FOA is toxic for cells expressing the *URA3* gene. In 1993, the technique was transferred success-

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fully to the diploid yeast *Candida albicans* (Fonzi and Irwin 1993), leading to disruption of various genes such as *LIG4* (Andaluz et al. 2002), *UBI3* (Roig and Gozalbo 2002), *UBI4* (Roig and Gozalbo 2003), and *ERG6* (Jensen-Pergakes et al. 1998). The aims of the present work were to transfer the *URA3*-Blaster mutagenesis technique to *P. anomala* (strain K), apply it to the sequential inactivation of the *PAEXG1* and *PAEXG2* genes potentially related to antagonistic activity, and investigate the biocontrol efficiency of the resulting mutated strains in relation to the yeast inoculum concentration and apple maturity.

RESULTS

Transfer of the *URA3*-Blaster strategy.

Transfer of the *URA3*-Blaster strategy was validated on *P. anomala* by targeted disruption of the *PATRP1* gene and elimination of the *URA3* marker gene. *TRP1* disruption was achieved in the *ura3*$^-$ strain KU5 by transformation with the *Hpa*I-linearized *pBLAST3-TRP1* plasmid (Fig 1A). In all, 38 *URA*$^+$ colonies selected on tryptophan-supplemented minimal synthetic-defined (SD) medium were directly analyzed by comparing their growth on minimal and tryptophan-supplemented SD medium. This phenotypic approach enabled us to select four potential *trp1*$^-$ strains, suggesting a high level of ectopic integration of the *pBLAST3-TRP1* plasmid. The four putative *trp1*$^-$ strains were polymerase chain reaction (PCR) analyzed, on the one hand, with primers SP6/TRPREV, specific to the integration site and, on the other hand, with primers URA3F/URA3R (surrounding the *URA3* marker gene) and K7F/K7R (surrounding the complete *URA3*-Blaster module) (Fig 1B). In each case, amplification products of the expected size were obtained (717 bp for targeted integration, 1.6 kb for the *URA3* gene, and 4.5 kb for the complete module) (data not shown). The *Trp1*$^-$ strains were named KT1 to KT4. Strain KT4 was randomly selected for *ura3*$^-$ on FOA medium according to the *URA3*-Blaster strategy. In all, 150 colonies were analyzed in order to select for spontaneous recombination between the two *hisG* sequences surrounding the *URA3* marker. A first phenotypic approach, targeting the incapacity to grow on tryptophan- and uracil-free SD medium, allowed selection of 15 double-auxotrophic colonies named KTU1 to KTU15. These were analyzed with primers RK7 and FK7, already used to amplify the complete *URA3*-Blaster module. Two different profiles were obtained upon migration of the amplification products (Fig 2). A band corresponding to a fragment of approximately 4.4 kb, as detected with control pBLAST3-TRP1, was observed for five strains (KTU 4, 5, 6, 10, and 11). On the other hand, 10 KTU strains displayed a single amplification band corresponding to a fragment size of approximately 1,500 bp, and no longer showed any sign of the complete *URA3*-Blaster module. Sequence analysis of the 1,500-bp fragments confirmed homologous recombination (Fig. 1B) because a unique resulting *hisG* fragment was found to be surrounded by the 5′ and 3′ extremities of the complete module.

Separate and sequential inactivation of two exo-β-1,3-glucanase-encoding genes.

Since the *URA3*-Blaster strategy was validated on *P. anomala*, it was used for separate and sequential inactivation of the *PAEXG1* and *PAEXG2* genes. Plasmids *pBLAST3-EXO1* (linearized with *Bsm*I) and *pBLAST3-EXO2* (linearized with *Pme*I) were used to disrupt the coding sequences of *PAEXG1* and *PAEXG2*, respectively. This yielded 74 and hundreds of colonies, respectively, upon selection for *URA*$^+$ transformants

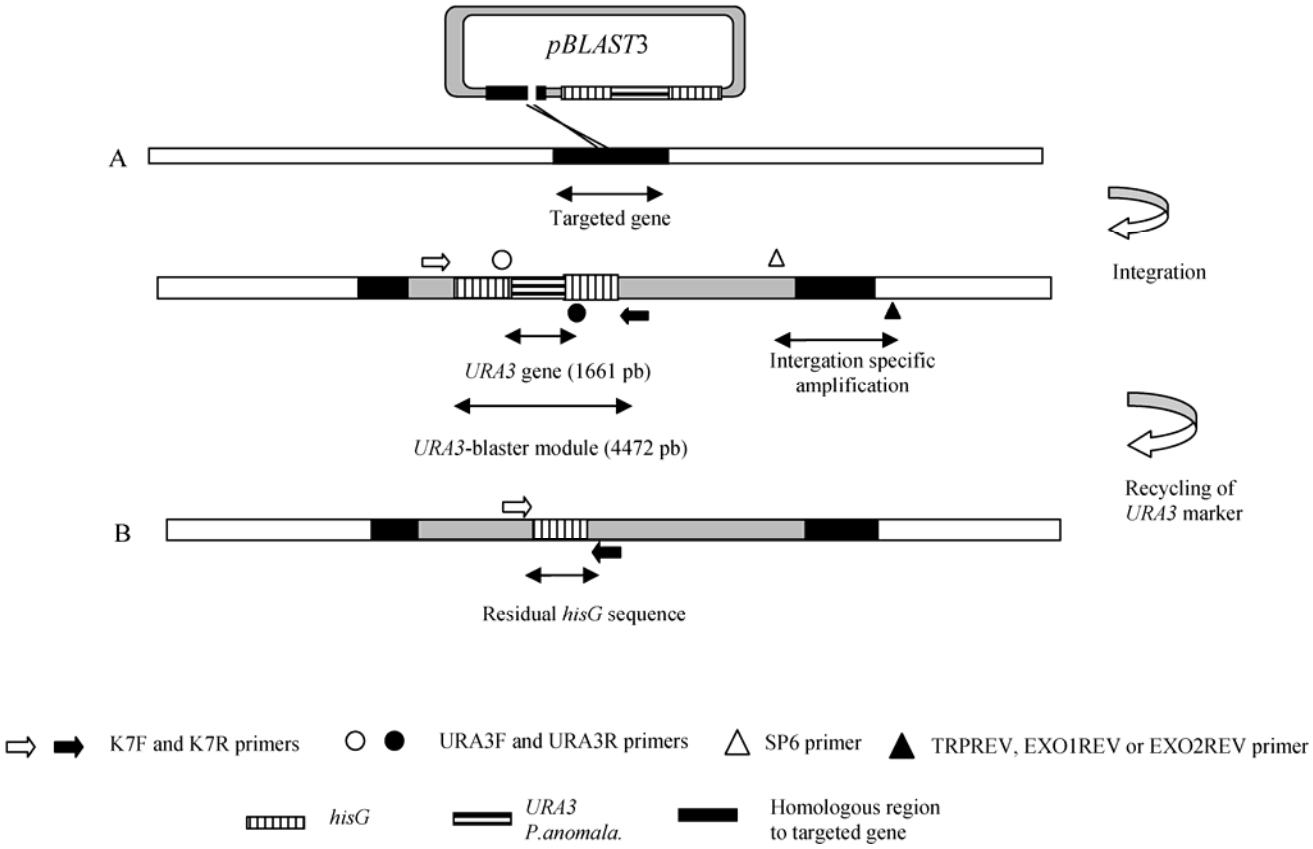

Fig. 1. A, Scheme of plasmid *pBLAST3* integration into the targeted gene of *Pichia anomala* and **B,** result obtained after removal of the *URA3* marker. Primers used to detect targeted insertion (SP6 and TRPREV, EXO1REV, or EXO2REV) or to amplify the *URA3* gene (URA3F and URA3R), the complete cassette, or the residual *hisG* sequence (K7F and K7R) are indicated.

on minimal SD medium. In the case of the control (KU5 electroporated without any plasmid), no colonies were formed. For each targeted gene, 20 transformants were PCR analyzed with the primer pairs SP6/EXO1REV (*PAEXG1*, 1,434 bp if targeted integration) and SP6/EXO2REV (*PAEXG2*, 1,255 bp if targeted integration) (Fig 1B). In each case, four strains exhibited a correct amplification product, indicative of the presence of the corresponding *pBLAST3* plasmid in the coding sequence of the targeted gene (data not shown). One strain was randomly selected for each transformation and named KE_1 (*paexg1*$^-$) or KE_2 (*paexg2*$^-$). The presence of the complete *URA3*-Blaster module in the genomic DNA of the mutated strains was confirmed by PCR amplification as explained above (data not shown). Strains KE_1 and KE_2 were grown further on FOA medium in order to eliminate the *URA3* marker. In all, 9 and 24 colonies were obtained and PCR analyzed with primers RK7 and FK7, respectively. This yielded the uracil auxotrophic strains KE_1U (*ura3*$^-$, *paexg1*$^-$) and KE_2U (*ura3*$^-$, *pae*xg2$^-$), containing a residual hisG sequence in the coding sequence of the targeted gene.

PAEXG2 then was disrupted in strain KE_1U, in order to obtain a double-mutant strain (*paexg1*$^-$, *paexg2*$^-$). The pBLAST3-EXO2 plasmid was linearized with *Pme*I before a second transformation step, leading to selection of 141 uracil-prototrophic colonies on minimal SD medium. Fifty-three colonies were screened by PCR with the specific primer pair SP6/EXO2REV described above, and a 1.2-kb amplification product was obtained as proof of targeted integration. A PCR was carried out with primers SP6/EXO1REV to check that the gene *PAEXG1* remained disrupted. An amplification product of the expected size was obtained. The double-mutant strain was named KE_1E_2 (*paexg1*$^-$, *paexg2*$^-$) (Fig. 3).

Evaluation of the mutant strains.

Mutation stability. Mutation stability was evaluated in situ. Forty-five colonies of each mutant strain (KE_1, KE_2, and KE_1E_2), obtained after 96 h of growth on an apple wound, were PCR analyzed with the primer pair SP6/EXO1REV (*PAEXG1*), SP6/EXO2REV (*PAEXG2*), or both specific to the integration site. In all cases, the genes were found to have remained disrupted, as confirmed by an amplification product of the expected size (1,434 bp for *PAEXG1* and 1,248 bp for *PAEXG2*).

Growth ability. Inoculated at the same concentration (5×10^4 CFU/wound) on apple wounds, mutant and wild-type strains displayed similar colonization ability (Fig 4). Between 5.1 (KH6) and 6.1 (K) generations were completed over a period of 24 h. The double mutant grew slowly for the first 12 h, but reached a population size similar to that of the other strains at 24 h, corresponding to the *B. cinerea* inoculation time. After 48 h of growth (72 h for strain KH6), all strains reached a similar saturation level of approximately 5×10^6 CFU/wound.

Activity of secreted glucanases. Activity of secreted exo-β-1,3-glucanase was quantified in rinse water from apple wounds pretreated with 4.5×10^6 CFU of strain K, KH6, KE_1, KE_2, or KE_1E_2. The size of the yeast inoculum was chosen as conferring a high protection level. Exo-β-1,3-glucanase activity measured for each strain is shown in Figure 5. Statistical analysis revealed two significantly different groups according to Duncan's multiple range test. On the one hand, strains K, KH6, and KE_1 produced a similar level of secreted exo-β-1,3-glucanase. On the other hand, the enzyme activity measured for strains KE_2 and KE_1E_2 was close to zero and not significantly different from that of the control.

Biological control efficiency. The biological control efficiency of the mutated strains (KE_1, KE_2 and KE_1E_2) was assessed in comparison with parental strains K and KH6. The experimental design included an evaluation of the influence of yeast inoculum concentration and apple physiological stage on the protection level.

A three-way analysis of variance (ANOVA) (yeast inoculum concentration, apple physiological stage, and strain) was carried out in order to evaluate the influence of each studied factor on biocontrol (Table 1). Considered individually, each factor was found to have a significant impact on the results. This suggests that the individual and combined mutations have an impact on biocontrol and it underlines the importance of taking the various experimental parameters into account when drawing a final conclusion.

The yeast strain–inoculum concentration double interaction also showed a significant impact on the lesion diameter. No significant impact was found for the other double interactions or for the triple interaction.

One-way ANOVA then was applied to the lesion diameters measured for each strain–concentration combination, at each physiological stage considered (Table 2). For each apple stock, the difference in lesion diameter observed between apple fruit treated with a parental or a mutated strain was maximal at the lowest yeast inoculum concentration. Under these conditions,

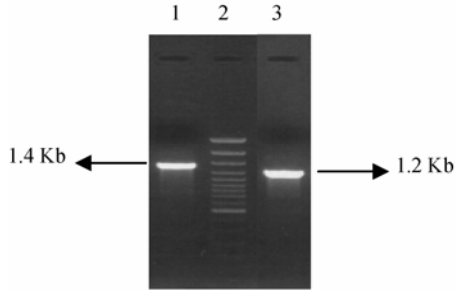

Fig. 3. Polymerase chain reaction amplification performed on genomic DNA of strain KE_1E_2 with primers SP6/EXO1REV (1.4 kb, lane 1) and SP6/EXO2REV (1.2 kb, lane 3). Lane 2 is a 100-bp DNA marker. Arrows indicate an amplification product of the expected size.

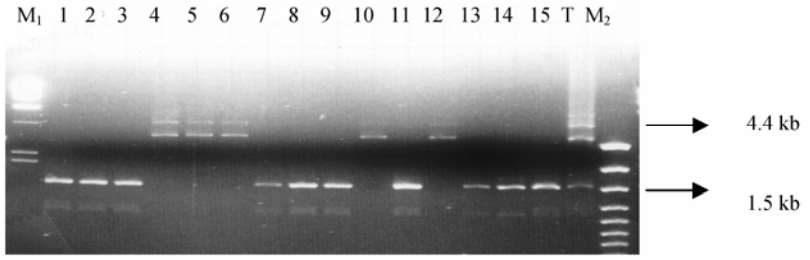

Fig. 2. Polymerase chain reaction amplification of residual *URA3*-Blaster cassette with primers K7F and K7R in strains KTU1 to KTU15. Lane 1, marker lambda/*Hin*d3; lanes 2 to 16, strains KTU1 to KTU15; lane 17, plasmid *pBLAST3-TRP1*; lane 18, 100-bp DNA marker (Fermentas, Burlington, Canada). Arrows indicate the band corresponding to the complete *hisG-URA3-hisG* cassette and the resulting *hisG* sequence after recombination.

when the apple fruit were fresh, no statistically significant difference was detected between strain KE_1 or KE_1E_2 and the untreated control. The calculated protection level, collapsing from 68% (KH6) to 2% (KE_1), emphasized these observations. The difference in biocontrol efficiency between the parental and mutated strains was reduced when the yeast inoculum size was increased, vanishing completely when an inoculum of 10^4 CFU (mature apple) or 10^5 CFU (fresh apple) was applied per wound. When 10^5 CFU of strain KH6 or KE1 was applied, the calculated protection level reached 89 and 87%, respectively, on fresh apple fruit and 97 and 96%, respectively, on mature apple fruit.

DISCUSSION

We have demonstrated for the first time that the biocontrol efficiency of *P. anomala* (strain K) is affected by inactivation of the *PAEXG1* gene, the *PAEXG2* gene, or both. Furthermore,

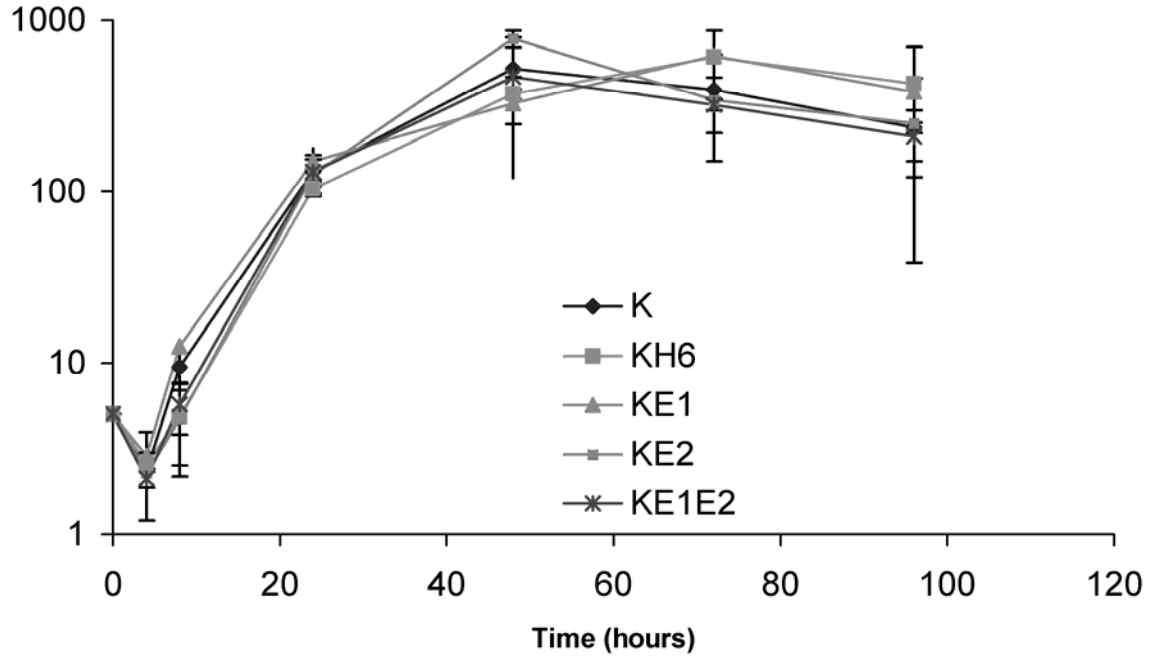

Fig. 4. Growth kinetics of strains K, KH6, KE_1, KE_2, and KE_1E_2 after inoculation of apple wounds with 5×10^4 CFU of each strain, resuspended in isotonic water. Quantification was done after 4, 8, 12, 24, 48, and 72 h.

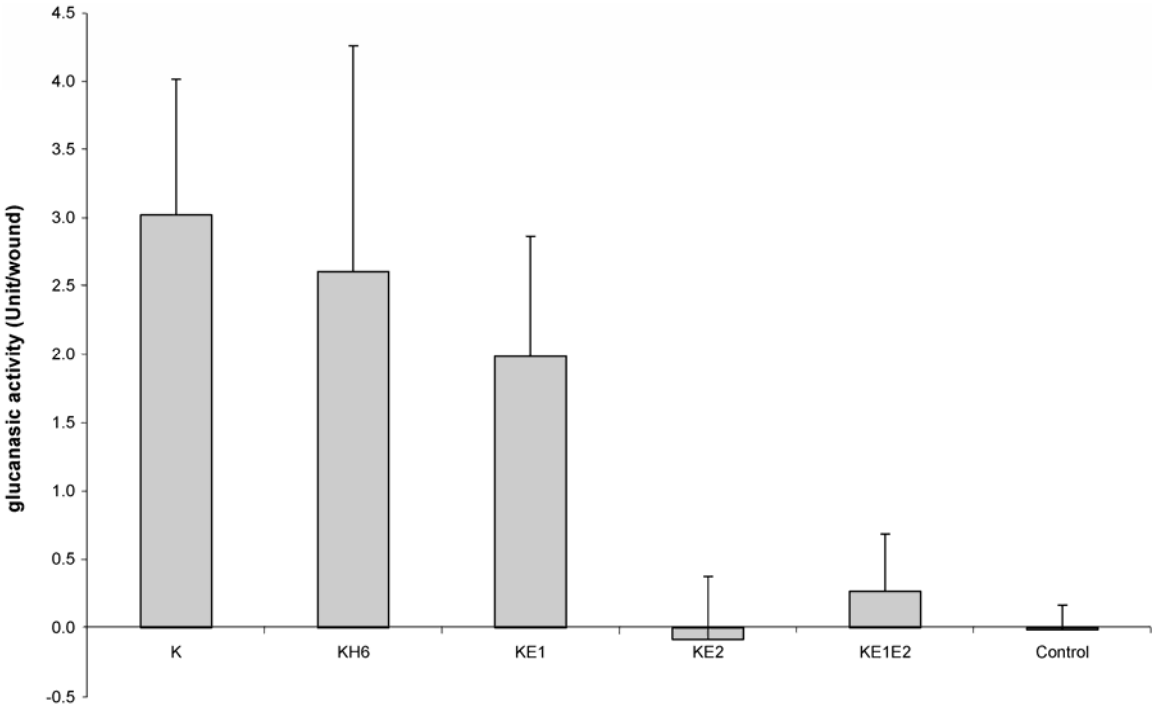

Fig. 5. Exo-glucanase activity calculated from the rinse water of wounds treated with strain K, KH6, KE_1, KE_2, or KE_1E_2 (untreated control). Wounds (18 mm in diameter) were treated with 450 µl of a 10^7 CFU/ml suspension. Three apple fruit (six wounds) were used per treatment. After 48 h, wounded sites were rinsed with distilled water (three wounds/strain). The exolytic activity was quantified with the glucose HK kit (values with the same letter are not significantly different, Duncan test, $P < 0.05$).

our data highlight the complexity of the antagonistic relationship established within the host–antagonist–pathogen system, because differences in protection level between mutated and wild-type strains are modulated by apple maturity and yeast inoculum size.

To obtain our double mutant, we chose a reliable gene inactivation technique allowing multiple disruptions with a single marker gene. We chose the *URA3*-Blaster technique because it allows both positive and negative selection in a *ura3*$^-$ background. We used the strategy to construct *trp1*$^-$, *paexg1*$^-$, and *paexg2*$^-$ single mutants and a *paexg1*$^-$, *paexg2*$^-$ double mutant derived from the uracil-auxotrophic strain KU5. The number of targeted integrations obtained in this work (Table 3) was very low compared with what can be obtained in other species, such as *C. glabrata* (Cormack and Falkow 1999), *C. tropicalis* (Roher and Picataggio 1992), or other *Pichia* spp. (Klinner and Schäfer 2004). However, such comparisons are often hazardous because of different experimental conditions, different yeast strains, or different targeted genes. Furthermore, transformation and homologous recombination are integral parts of a complex process that depends on both the physical properties and the genetic characteristics of the strain. Homologous recombination, well described in *S. cerevisiae* (Haber 2000), involves the expression of several genes. Many other factors also can influence the transformation efficiency (and, hence, the number of targeted insertions), including the transformation technique, the structure of the integrated genetic construct (circular, ends-in, ends-out vector), and the chromosomal location of the targeted gene (Klinner and Schäfer 2004).

The *URA3*-Blaster strategy was used successfully for sequential inactivation of *PAEXG1* and *PAEXG2*, but a major decrease in homologous recombination efficiency was observed. Under our experimental conditions, we obtained 20% targeted integration for separate inactivation of *PAEXG1* and *PAEXG2* and only 1.8% for the second disruption, targeting *PAEXG2* in the KE_1U genome. This may be due to the presence of three conserved nucleotide sequences in *PAEXG1* and *PAEXG2*. The *pBLAST3-EXO2* plasmid, used for the second inactivation, was linearized within one of those conserved regions, creating a bias in the disruption efficiency. Disruption of the *PAEXG1* gene ultimately led to its replacement by two inactivated copies, each containing the conserved targeted sequence. Hence, the *pBLAST3-EXO2* plasmid used for the second inactivation was faced with three potential integration sites. It is interesting that the percentage of targeted integration in strain K, as in many other species, increased with the length of the homologous sequence, as seen in Table 3 for the genes *TRP1*, *PAEXG1*, and *PAEXG2*.

The results of our biological control tests highlight the contribution of glucanase to antagonism of *B. cinerea* by *P anomala* on apple. They also underline the complexity of in situ experiments and the need to consider the various experimental parameters in order to ensure the reproducibility of the results and to draw valid conclusions about modes of action of the antagonist. In addition to overall statistical analysis (three-way ANOVA), each strain–yeast concentration–apple physiological stage combination was analyzed separately (Table 2). Depending on whether the yeast concentration was high or low and on whether the apple fruit were fresh or mature, the conclusions were different. Both high yeast concentration and maturation appeared to compensate for inactivation of the *PAEXG1* or *PAEXG2* gene. This observation is strengthened by the calculated percentage of protection: the mutated strains exerted no protective effect when low concentrations were applied to fresh apple fruit, but their protective effect was similar to that of the parental strain KH6 when they were applied to mature apple fruit at medium or high concentration or to fresh apple fruit at high concentration.

Thus, a higher yeast population (a greater inoculum size) appears to counterbalance the effect of the glucanase-minus mutations. A relation previously has been suggested between wound colonization by strain K (and, thus, yeast cell density) and the level of protection (Jijakli and Lepoivre 1993). On the other hand, it is commonly established that the protective effect resulting from the application of a biocontrol agent results from multiple modes of action operating simultaneously or sequentially. Thus, the relative contribution of exo-β-1,3-glucanases to biocontrol might be greater under conditions where that of other modes of action, such as competition for

Table 2. Lesion diameter measured with parental (K and KH6) and mutated strains KE_1 (*paexg1*$^-$), KE_2 (*paexg2*$^-$), and KE_1E_2 (*paexg1*$^-$, *paexg2*$^-$) applied to wounded Golden Delicious apple fruit, 24 h before inoculation with *Botrytis cinerea* (5×10^4 spores/wound)[y]

	Diameter, status		
Strain[z]	**10^3 CFU/wound**	**10^4 CFU/wound**	**10^5 CFU/wound**
A1			
K	1.06 a	0.45 a	0.14 a
KH6	1.18 a	0.41 a	0.37 a,b
KE_1	3.6 c	1.17 b	0.47 a,b
KE_2	2.94 b	1.69 b	0.55 a,b
KE_1E_2	3.38 b,c	1.44 b	0.78 b
Témoin	3.68 c	3.68 c	3.68 c
A2			
K	1.04 a	0.45 a	0.09 a
KH6	1.15 a	0.68 a	0.11 a
KE_1	2.09 b	1.1 a	0.14 a
KE_2	2.38 b	1.2 a	0.4 a
KE_1E_2	2.28 b	1.1 a	0.43 a
Témoin	3.67 c	3.67 b	3.67 b

[y] Yeasts were inoculated at three different concentrations (10^3, 10^4, and 10^5 CFU per wound). Five fruits (10 wounds) were used for each combination. For each apple stock and each concentration, values with the same letter are not significantly different (Duncan test, $P < 0.05$).

[z] Lesion diameter measured with parental (K and KH6) and mutated strains KE_1 (*paexg1*$^-$), KE_2 (*paexg2*$^-$), KE_1E_2 (*paexg1*$^-$,*paexg2*$^-$) applied to wounded Golden Delicious apples, 24 h before inoculation with *B. cinerea* (5×10^4 spores per wound). Yeasts were inoculated at three different concentrations (10^3, 10^4, and 10^5 CFU per wound). Five apples (10 wounds) were used for each combination. For each apple stock and each concentration, values with the same letter are not significantly different (Duncan test, $P < 0.05$). A1 = Fresh apples, A2 = Matured apples.

Table 1. Results of three-way analysis of variance carried out on the observed lesion diameter, following the four biological control experiments[z]

Source	DF	Mean square	Pr > *F*
Concentration	2	65.91	<0.0001
Physiological stage	1	8.97	0.0002
Strain	5	74.37	<0.0001
Concentration × physiological stage	2	1.14	0.1637
Strain × concentration	10	5.37	<0.0001
Strain × physiological stage	5	1.17	0.1013
Strain× concenrtation × physiological stage	10	0.63	0.4404

[z] Analysis was performed with the SAS software (SAS institute, Cary, NC, U.S.A.).

nutrients or space, is reduced. The observed variations of lesion diameter according to population size (Table 2) suggest that the contribution of exo-β-1,3-glucanase to biocontrol is less when the applied yeast concentration is high, even though the glucanase activity in each cell remains the same. At low yeast cell density, competition for space or nutrients may be reduced, increasing the relative contribution of the glucanases to biocontrol. Another explanation might lie in the fact that competition inhibits germination of *B. cinerea* conidia, as previously described (Jijakli and Lepoivre 1998); at low concentration, competition is reduced, allowing conidium germination and release of glucan oligomers into the medium as a consequence of major cell wall modifications. In the parental strains, glucan oligomers could induce the expression of both glucanase genes and stimulate glucanase-mediated biocontrol.

The lesion size measured after application of parental strains was unaffected by the physiological stage of the fruit, but the mutant trains exerted a higher biological control capacity when applied to mature apple fruit (Table 2). We hypothesize that ripening leads to a modification of nutrient composition. Increased nutrient availability might reduce the need of glucanase production, if the enzymes are required only for nutrient uptake. Fruit maturation also might modify the balance between antagonist and pathogen, reinforcing competition-mediated biocontrol. Our results suggest that the physiological stage of the fruit is a critical constraint for the reliability of biological control tests. El Ghaouth and associates (2003) have made similar observations regarding the effect of host maturity on the biocontrol capacity of *C. saitoana*, highlighting the inability of this agent to induce systemic resistance in ripened apple fruit.

In a previous study, Grevesse and associates (2003) ruled out any involvement of the *PAEXG2* gene in the biocontrol activity of strain K. Yet this work was carried out on riper fruit and with a starting yeast inoculum five times higher than the lowest one used here. Thus, the apparent discrepancy between their results and ours is likely to be attributable to both yeast population density and physiological stage of the fruit, because our three-way ANOVA highlights their significant influence. On the basis of experiments involving disruption of the *Coexg1* gene in the biocontrol agent *C. oleophila* (in vitro assays showed no exo-glucanase activity in the mutant), Yehuda and associates (2003) likewise ruled out any involvement of the encoded exo-β-1,3-glucanase in the protection of kumquat against *Penicillium expansum*. Yet their biological control tests were carried out with large yeast inocula (10^7 cells/wound), likely to mask the glucanase contribution.

The calculated protection levels obtained after application of strains KE_2 and KE_1E_2 correlate positively with the measured activity of secreted glucanase. Previous works indicate that Paexg2p is secreted, whereas Paexg1p is cell wall anchored (Grevesse et al. 2003). Consequently, the glucanase activity quantified in our experiments is limited to the secreted enzyme, related to expression of *PAEXG2* gene. No exo- or endoglucanase activity was detected in the rinse water of wounds treated with mutated strain KE_2 (*paexg2*$^-$) or KE_1E_2 (*paexg1*$^-$, *paexg2*$^-$), confirming inactivation of *PAEXG2* and also confirming that paexg2p is the sole secreted glucanase. The activity of the anchored Paegx1p protein was not quantified, but disruption of the *PAEXG1* coding sequence and the stability of the mutation were established. Thus, the gene, which is present in a single copy (Grevesse et al. 2003), is not expressed in strain KE_1.

In the studied model, the residual capacity to protect against *B. cinerea* was similar for all mutants studied, both single and double. We had rather expected a cumulative effect for the double mutant, given the decreased protection level observed after application of each single mutant. In the literature, β-1,3-glucanases are suggested to play a role in yeast morphogenesis (apical growth, budding, mating, ascospore formation, and release), mobilization of glucan for use as a fuel, and hydrolysis of exogenous material for use as a nutrient. It appears here that both exo-β-1,3-glucanases are required for glucanase-mediated biological control of *B. cinerea* on apple. The combined inactivation of both *PAEXG1* and *PAEXG2* genes in the KE_1E_2 double-mutant strain might be compensated by another enzymatic pathway, by enhanced endo-glucanase activity, or by expression of another gene encoding an anchored exo-β-1,3-glucanase. Furthermore, glucanase activity is not the most important mode of action, because inactivation of both genes together can be offset by increasing the yeast inoculum concentration.

In conclusion, we have shown, for the first time in the field of biological control, an in situ decrease in the protection level afforded by a yeast strain as a result of glucanase inactivation. The growth ability of the mutated antagonistic strains was unaffected; therefore, it seems very likely that the glucanases themselves play a role in biological control. Interestingly, it is possible to compensate for the effect of the mutations on the protection level by adjusting the yeast cell density and apple maturity. This suggests the involvement of other factors. In our experiments, the effect of the mutations was most significant at low cell density and on freshly harvested apple fruit.

In this work, the *URA3*-Blaster strategy has been successfully transposed and applied to *Pichia anomala* strain K. To our knowledge, this is the first report of a marker rescue technique for this species. We now intend to use the *URA3*-Blaster strategy to inactivate new genes of interest, recently identified by cDNA amplified fragment length polymorphism (Massart et al. 2006).

MATERIALS AND METHODS

Microorganisms and growth conditions.

P. anomala strain KU5 (*ura3*$^-$) derives from strain KH6, an isogenic segregant obtained after sporulation of *P. anomala* (Hansen) Kurtzman (strain K). The *URA3* gene has been inactivated by integrative disruption (Grevesse et al. 2003). KU5 was grown at 30ºC in uracil-supplemented SD medium (0.175% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate, 2% dextrose) prior to transformation and at 30ºC on SD + agar, supplemented if necessary with tryptophan, for regeneration and *URA3*$^+$ selection.

Escherichia coli strain TOP10F′ (Invitrogen, Carlsbad, CA, U.S.A.) was used for plasmid construction and propagation.

Table 3. Percentage of targeted integration obtained for the genes already studied in *Pichia anomala* strain K, according to the length of the homologous region

Gene	Analyzed transformant	Homologous region (pb)	Targeted integration	Reference
TRP1	38	437	10.5	This work
PAEXG1	20	1,301	20	This work
	26	317	0	Grevesse et al. 2003
PAEXG2	20	962	20	This work
	8	352	0	Grevesse et al. 2003

The strain was cultured in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) and plated on low-salt LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing zeocin (50 μg per 100 ml) for positive selection of transformants.

B. cinerea Pers.:Fr (strain V) was isolated from rooting strawberry (Plant Pathology Unit, FUSAGx, Gembloux, Belgium). For long-term storage, the strain was placed at –70ºC in 25% glycerol. Before inoculation, the fungus was grown for 10 ± 2 days at 24ºC on potato dextrose agar medium. Spores were quantified with a Bürker cell.

General nucleic acid techniques.

Yeast genomic DNA was prepared by a glass-bead disruption method (Ausubel et al. 1991). If not otherwise stated, PCR experiments were performed on 100 ng of genomic DNA, with classical *Taq* polymerase (Roche, Basel, Switzerland). When mentioned, PCR experiments were carried out directly on a yeast colony according to a rapid protocol. In this case, colonies were deposited in PCR tubes containing the polymerase-free PCR mix. After 3 min in the microwave oven (700 W), the polymerase was added and the tubes were placed in the thermocycler.

Vector construction.

All the primers used in this study are listed in Table 4. All restriction enzymes were obtained from Fermentas (Burlington, Canada). The three steps leading to the integrative plasmids used in this work are summarized in Figure 6. Plasmid *pNKY51* containing the *hisG-URA3-hisG* module was kindly provided by Dr E. Alani (Cornell University, Ithaca, NY, U.S.A.). The *S. cerevisiae URA3* gene contained in *pNKY51* was removed by *Hin*dIII digestion and replaced with the coding sequence of the *P. anomala URA3* gene, available in the GenBank database (no. Y09221). *PaURA3* was PCR amplified with the expanded high-fidelity enzyme (Roche) using primers URA3F, annealing at positions 24 to 42 of the complete database sequence, and URA3R, annealing at positions 1,651 to 1,670. Both primers contain an engineered *Hin*dIII site at their 5′ extremities (underlined). *pNKY51*, renamed *pBLAST1*, contains a *URA3*-Blaster module specific to *P. anomala* (Fig. 6).

An internal part of each *P. anomala* gene to be disrupted (*TRP1, PAEXG1*, *and PAEXG2*) was amplified and ligated into the *p-ZERO* plasmid in order to construct the *pBLAST2* plasmid series. Each internal sequence was amplified with primers designed by DNAMAN software (Lynnon Biosoft, Quebec, Canada) and containing a *Kpn*I (forward primer) or *Sac*I (reverse primer) restriction site at the 5′ extremity. The internal sequence and the *p-ZERO* vector were digested with *Kpn*I and *Sac*I restriction enzymes and both purified products were ligated according to the manufacturer's protocol (Invitrogen).

Finally, the complete *hisG-URA3-hisG* module of *pBLAST1* was amplified with primers K7F, spanning positions 7,531 to 7,550 on the pBLAST1 sequence, and K7R, spanning positions 3,914 to 3,927. Primer K7F contains an artificial *Xba*I restriction site and K7R an artificial *Xho*I restriction site at the 5′ extremity (underlined). The PCR product was purified, digested with both enzymes, and ligated into the previously digested and dephosphorylated *pBLAST2* series vectors. The resulting plasmids were called *pBLAST3-TRP1*, *pBLAST3-EXO1*, and *pBLAST3-EXO2,* according to the targeted gene, and each displays a unique restriction site in the homologous region.

Transformation and selection.

Exponentially growing KU5 (*ura3*$^{-}$) cells were transformed using the AcLi/DTT/TE method (Thompson et al. 1998). Electroporation was carried out with a Bio-Rad (Hercules, CA, U.S.A.) Gene Pulser equipped with Pulse Controller Plus (0.75 kV, 200 ohms, 25 μF). Electrocompetent cells (50 μl, 6×10^9 CFU/ml) were mixed with 3 μl of linearized plasmid (1 μg/μl). Positive selection of transformants was based on restoration of uracil prototrophy by growth at 30ºC on minimal SD medium.

After *TRP1* targeting, ectopic and targeted insertions first were distinguished phenotypically, by comparing growth on SD medium supplemented or not with tryptophan. Suspected *trp1*$^{-}$ strains and all transformants obtained after targeting of the *PAEXG1* and *PAEXG2* genes were further analyzed by PCR. Forward primer SP6, spanning positions 239 to 259 of the *pBLAST3* sequence, is homologous to the coding strand of the integrative plasmid. Reverse primers TRPREV, EXO1REV, and EXO2REV are homologous to the noncoding strand of the *P. anomala TRP1*, *PAEXG1*,and *PAEXG2* genes and located 202, 70, and 224 bp, respectively, after the homologous region (Fig. 1A). The expected sizes of the PCR products were 702 bp (*TRP1*), 1,434 bp (*PAEXG1*), and 1,255 bp (*PAEXG2*).

Table 4. Primers used in the present work (Eurogentec, Liege, Belgium)

Primer name	Primer sequences
FTRP1	5′-ttGGTAACaaagccaggtcaagatag-3′
RTRP1	5′–ttGAGCTccctccagaaacatcaactc–3′
FEXO1	5′-TTGGTACCTCATTGGGTGGTTGGTTT-3′
REXO1	5′-TTGAGCTCGGATTTCCAGAGGCAGC-3′
FEXO2	5′-TTGGTACCaaccctatcccttcaaga-3′
REXO2	5′-TTGAGCTCTCATAACGAGAACCACGA-3′
URA3F	5′-ttAAGCTTcttccatctcttaacgtc-3′
URA3R	5′-ttAAGCTTtattaccgtaatcacaatag-3′
K7F	5′-ttCTCGAGatcttcagcatcttttactt-3′
K7R	5′-ttTCTAGAcgatataggcgccagc-3′
SP6	5′-atttaggtgacactatagaa-3′
TRPREV	5′-tttgatgaatctctgctgcttc-3′
EXO1REV	5′-tgaaagaattgtatatctagt-3′
EXO2REV	5′-aagttaatggggatgggaa-3′

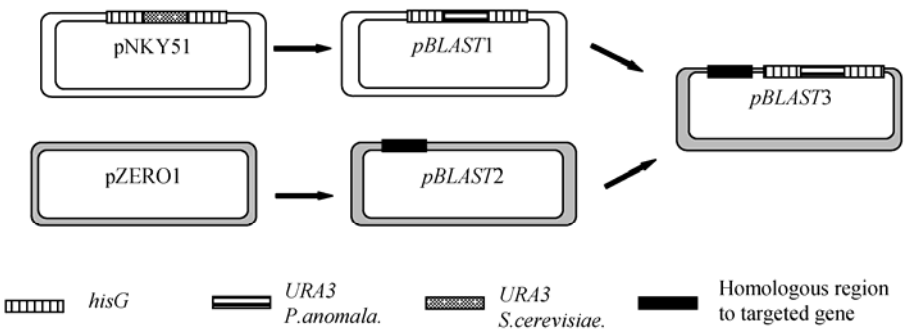

Fig. 6. General scheme used during the construction of the pBLAST3 plasmid series.

Elimination of the *URA3* gene.

Removal of the *URA3* marker gene was done on FOA medium (SD medium supplemented with uracil and fluoro-orotic acid) (Duchefa, Haarlem, The Netherlands). Mutants initially selected as *URA*$^+$ were cultured in liquid SD medium and 1 ml was plated on selective FOA medium. Both media were supplemented with tryptophan if necessary. After 5 days of growth at 30ºC, the colonies obtained were tested for spontaneous loss of the *URA3* gene. *Trp1*$^-$ colonies were analyzed first by comparative plating on SD medium supplemented with uracil or tryptophan to eliminate revertants and spontaneously resistant cells. Double-auxotrophic strains (*trp1*$^-$, *ura3*$^-$) and putative *ura3*$^-$, *paexg1*$^-$, or *ura3*$^-$, *paexg2*$^-$ colonies were further analyzed by PCR with the above-described K7F and K7R primers, surrounding the *URA3*-Blaster module, in order to amplify the resulting *hisG* sequence (Fig. 1B). The resulting PCR product was cloned with the TA-cloning kit (Invitrogen) according to the manufacturer's instructions, and sequencing was done by the GATC-Biotech company (Konstanz, Germany).

Mutation stability.

The in vivo stability of the mutations introduced was investigated. Each mutant strain (5×10^4 CFU) was applied to an apple wound and incubated for 96 h at 24ºC. Colonies subsequently were collected and spread on YEPD (10% yeast extract, 20% peptone, and 20% glucose) medium before analysis according to the rapid PCR protocol. The primer pairs used were the above-described SP6/EXO1R (*PAEXG1*) and SP6/EXO2R (*PAEXG2*), designed to amplify a specific fragment in case of targeted disruption.

Wound colonization.

The wild-type and mutant strains were compared as regards their in vivo growth capacity. Seven wounds were produced on an apple (6 mm in diameter) and 5×10^4 CFU of each strain, collected in isotonic water, was applied at the middle of each wound before incubation at 25ºC. After 4, 8, 12, 24, 48, and 72 h, the wounds were cut out and crushed with 10 ml of distilled water. After dilution, the cells were spread on petri dishes containing YEPD medium. For each strain and each incubation time, three wounds were collected and mixed. The growth capacity was expressed as the number of generations obtained between two measures, calculated as follow: $G_t = LOG_2 (C_{t2}/C_{t1})$, where G_t = number of generation at the t_2 time, C_{t1} = CFU/ml at the t_1 time, and C_{t2} = CFU/ml at t_2 time.

In situ secreted exo-glucanase activity.

Apple wounds (18 ml in diameter) were inoculated with 5×10^6 CFU of mutant or wild-type strain, or with distilled water for a control. After a 48-h incubation at 24ºC, the rinse water from three wounds made on an apple were pooled and filtered through an acrodisc (0.45 µm). Samples were extensively dialyzed against 0.05 M potassium acetate buffer (pH 5.5) at 4ºC. For enzymatic activity detection, a reaction mixture containing 100 µl of the sample in 1 ml of potassium acetate buffer (pH 5.5) with 2% laminarin and 0.02% NaN_3 was shaken at 120 rpm at 37ºC for 24 h.

The exo-β-1,3-glucanase activity was determined by monitoring the release of glucose monomers from laminarin, according to the manufacturer's instructions (glucose/HK) (Sigma-Aldrich, St. Louis). The glucose monomer concentration, proportional to the optical density at 340 nm value, was calculated from a standard curve determined with glucose concentrations ranging from 0 to 160 µg/ml.

For each sample, glucanase activity was measured in a native aliquot of the enzyme solution and in an aliquot denatured by incubation in a boiling bath for 10 min. The amount of sugar released in the enzymatic reaction was determined as the difference between the values obtained for the native and denatured samples. One unit (U) of exo-β-1,3-glucanase activity was defined as the amount of enzyme releasing 1 µg of glucose equivalents per hour. In all experiments, one apple with three wounds was tested for each strain. Three experiments were carried out.

Biocontrol efficiency.

Biological control experiments were carried out on Golden Delicious apple. Fruit were surface disinfected with 10% sodium hypochlorite for 2 min and rinsed twice in distilled water. Two wounds were made with a cork borer (8 mm in diameter and 4 mm deep) at the equator of each fruit and 50 µl of antagonist suspension was applied at 10^3, 10^4, and 10^5 CFU/wound (water was used as a control). After drying, the fruit were stored in closed plastic boxes at 24ºC for 24 h. Pathogen suspension (50 µl) then was inoculated into each wound (5×10^4 spores/wound) before storage under the same conditions for 7 days or until the lesion diameter of the control reached approximately 30 mm. The percentage of protection (P) was calculated as follows: $P = 100 (D_t - D_y)/D_t$, where D_t is the mean diameter of the lesion on apple fruit in the control group and D_y is the mean diameter in presence of the control agent.

Tests were carried out on an initial apple stock, divided in two parts: on the one hand, fresh apple fruit used directly for assessment of biocontrol efficacy and, on the other hand, apple fruit that were allowed to ripen at 24ºC for 10 days before undergoing the same assessment. Five fruit (10 wounds) were used for each concentration and each strain. Two experiments were carried out with each apple stock.

Statistical analysis.

Statistical analysis was carried out with SAS software (SAS Institute, Cary, NC, U.S.A.). ANOVA was applied, followed by Duncan's multiple range test ($P < 0.05$) to separate means.

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