Heterologous Expression in *Aspergillus nidulans* of a *Trichoderma longibrachiatum* Endoglucanase of Enological Relevance

Adela Villanueva,[†] Daniel Ramón,[†] Salvador Vallés,[†] Maria Angeles Lluch,[‡] and Andrew P. MacCabe*[,†]

Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Apartado Postal 73, Burjassot, 46100 Valencia, Spain, and Departamento Tecnología de Alimentos, ETSI Industriales, Universidad Politécnica de Valencia, 46071 Valencia, Spain

An *Aspergillus nidulans* transformant expressing the *Trichoderma longibrachiatum* endoglucanase 1 gene (*egl1*) has been constructed. The extracellular production of EGL1 in different culture media has been studied, and a medium has been found in which EGL1 is the predominant extracellular protein produced. The enzymatic properties of the heterologously produced EGL1 are very similar to those of the native enzyme. Grape maceration in the presence of culture filtrate enriched in EGL1 resulted in increased release of aroma precursors, particularly in the case of aromatic grapes. Cryoscanning electron microscopy of the flesh of grapes treated with EGL1-enriched culture filtrate revealed degradation of the cell wall matrix.

Keywords: *Aspergillus nidulans; endoglucanase; aroma; wine; cryo-SEM*

INTRODUCTION

Wine aroma is an important enological parameter of considerable complexity, which is determined by three main factors: (i) the grape—its variety, growth climate, and soil type; (ii) the wine-making process—maceration and fermentation; and (iii) wine aging. The principal components of wine aroma comprise higher alcohols, esters, and terpenes. Whereas the alcohols and esters are derived largely, although not entirely, from chemical transformations occurring during the fermentation and aging processes, terpenes are intrinsic to the grape (Strauss et al., 1986). The latter, and to a much lesser extent higher alcohols, occur in grapes as both free volatile forms (aglycons), which contribute directly to aroma, and glycosidically bound odorless compounds (glycons), which constitute a potential pool of aroma precursors (Williams et al., 1981, 1982; Günata, 1994).

The extraction of compounds that ultimately contribute to aroma, as well as flavor and color, occurs during the maceration step in which grape juice is left in contact with skins, seeds, and pulp. Traditionally, maceration was effected by enzymes liberated from the crushed grapes, but the use of microbial-culture-derived commercial pectinolytic preparations to improve juice release is now widespread (Villettaz and Dubourdieu, 1991; Colagrande et al., 1994). Such commercial preparations are mainly concentrated culture filtrates that contain a variety of activities, including cell wall degrading enzymes. Both free and glycosidically bound terpenes occur in grape skins, juice, and pulp (Wilson et al., 1986). Apart from juice release, cell wall degrading enzymes potentially provide a means of increasing the availability of aroma compounds and their glycosylated precursors in the wine-making process.

* Author to whom correspondence should be addressed (telephone +34-96-3900022; fax +34-96-3636301; e-mail andrew@iata.csic.es).

[†] Departamento de Biotecnología.

[‡] Departamento Tecnología de Alimentos.

The use of specific, microbially produced enzymatic activities to facilitate the release of aroma precursors from cell walls during maceration could improve the quality of wines derived from grapes with apparently poor aromatic profiles. Previous work in our laboratory (González et al., 1992) has reported the cloning of the *Trichoderma longibrachiatum egl1* gene, which encodes the cell wall degrading enzyme endoglucanase I (EGL1), and its expression in a commercial wine yeast strain (Pérez-González et al., 1993). The liberation of monoterpenyl diglycosides by EGL1 would augment the aroma precursor pool in grape juice available to glucosidase activities, which would subsequently result in the production of aroma.

In this work we have investigated (i) the heterologous extracellular production of EGL1 in transformants of *Aspergillus nidulans* and (ii) the release of aroma precursors after the addition of EGL1-containing culture filtrate to macerations of three grape varieties. The use of *A. nidulans* is particularly advantageous because it is an extensively studied genetic model organism, in which context we have used it previously to study xylanase production (Piñaga et al., 1994; MacCabe et al., 1999; Orejas et al., 1999), and it is closely related to the industrially important fungus *Aspergillus niger*.

MATERIALS AND METHODS

Chemicals and Substrates. Azo-CM-cellulose was supplied by Megazyme Pty. Ltd. (Sydney, Australia). Carboxymethylcellulose (CMC; sodium salt, low viscosity), birchwood xylan, polygalacturonic acid (sodium salt), *p*-nitrophenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-α-L-arabinofuranoside (pNPA), *p*-nitrophenyl-β-D-xylopyranoside (pNPX), *p*-nitrophenyl-α-L-rhamnopyranoside (pNPR), and Amberlite XAD-2 were obtained from Sigma Chemical Co. (St. Louis, MO.). Allyl alcohol was obtained from Merck KGaA (Darmstadt, Germany).

Organisms and Culture Conditions. Plasmids were propagated in *Escherichia coli* strain DH5α [*endA1, hsdr17, gyrA96, thi1, recA1, supE44,* Δ*lacU169, (Φ80 lacZΔM15)*]

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grown on LB medium (Sambrook et al., 1989). The *Aspergillus nidulans argB2, metG1, biA1* strain used for transformation was grown from a conidial inoculum of 1×10^6 conidia/mL in *Aspergillus* minimal medium (AMM) (Pontecorvo et al., 1953) containing 1% (w/v) glucose, supplemented with arginine, methionine, and biotin to final concentrations of 1 mg/mL, 100 μg/mL, and 10 ng/mL, respectively. Cultures were incubated in an orbital shaker at 37 °C and 200 rpm for 14 h. Growth of *A. nidulans* for enzyme production was carried out in *Aspergillus* complete medium (ACM) (Pontecorvo et al., 1953) and several *Aspergillus* minimal media containing 1% (w/v) glucose as carbon source and supplemented with 1% (w/v) yeast extract (AMMY), 1% (w/v) casamino acids (AMMC), or 1% (w/v) peptone (AMMP), as detailed in the text. All cultures were incubated in an orbital shaker at 37 °C and 200 rpm and collected at various times after inoculation.

AMM: 1% glucose, 1.6 mM ammonium tartrate, 1× trace elements solution, 1× salt solution, pH 6.8. CMA: 1% glucose, 1× trace elements solution, 1× salt solution, 1× vitamin solution, 0.2% peptone, 0.1% yeast extract, 0.15% casamino acids, pH 6.8. 50× salt solution: 0.4 M KH_2PO_4, 0.35 M KCl, 0.1 M $MgSO_4$. 100× vitamin solution: 50 mg of thiamin, 10 mg of biotin, 50 mg of pyridoxine hydrochloride, 100 mg of riboflavin, 100 mg of nicotinic acid, 200 mg of calcium pantothenate made to 1 L and filter sterilized. 1000× trace elements solution: 8 mg of $ZnSO_4$, 40 mg of $Na_2B_4O_7$, 400 mg of $CuSO_4$, 800 mg of $FeSO_4 \cdot 7H_2O$, 800 mg of $MnSO_4$, 800 mg of Na_2MoO_4 made to 1 L and autoclaved.

Transformation. Protoplasts from the *A. nidulans argB2* strain were obtained following standard protocols as described in Ventura and Ramón (1991). Cotransformation was performed using 5 μg of plasmid pIJ16 (5.7 kb) (Johnstone et al., 1985), which contains the *A. nidulans argB* gene as the selectable marker, and 25 μg of plasmid pPGPDEGL1 (6.4 kb) containing the *egl1* cDNA (1.53 kb) of *Trichoderma longibrachiatum* under the control of the constitutive *A. nidulans* glyceraldehyde-3-phospate dehydrogenase (*gpdA*) gene promoter (Ganga et al., 1997). Arginine prototrophic transformants were selected on *Aspergillus* selective minimal medium (ASMM) containing 1 M sucrose as the osmotic stabilizer and 1% (w/v) glucose. Transformants were picked onto ASMM plates containing 1% (w/v) glucose. The plates were incubated at 37 °C for 3 days and then overlaid with 5 mL of 1% (w/v) CMC, 0.7% (w/v) agar in 50 mM sodium acetate buffer (pH 4.5) and incubated at 50 °C for 2.5 h. The surface of the plates was flooded with 1% (w/v) Congo Red, left to stand for 10 min, and then washed several times with 1 M NaCl. Endoglucanase-producing colonies were identified by virtue of the production of clear halos.

DNA Manipulations. DNA manipulations were carried out following standard protocols (Sambrook et al., 1989).

Enzyme Assays. Endoglucanase (endo-β-1,4-D-glucanase, EC 3.2.1.4) activity was assayed using CMC or azo-CM-cellulose as substrates. CMCase activity was determined by incubating a 250 μL reaction mixture containing 50 μL of the appropriately diluted enzyme and 100 μL of a 2.8% (w/v) CMC suspension in 50 mM sodium acetate buffer (pH 4.5) (150 μL) for 15 min at 62 °C. Reducing sugars released were quantified according to the Nelson−Somogyi method (Spiro, 1966). One unit of CMCase activity was defined as the amount of enzyme that released 1 μmol of glucose equivalents/min. When the dye-modified substrate azo-CM-cellulose was used, a 0.5 mL reaction mixture containing 0.25 mL of the commercial substrate solution and 0.25 mL of the appropriately diluted enzyme were incubated at 62 °C for 10 min. The reaction was stopped by adding 1 mL of precipitation solution (prepared as described by the substrate solution manufacturer), and samples were centrifuged for 5 min in a microfuge. The absorbance of the supernatant was measured at 590 nm. One unit of endoglucanase activity was defined as the amount of enzyme that yields one unit of absorbance at 590 nm in 1 h.

Xylanase activity was measured in a 250 μL reaction mixture containing 100 μL of 3% (w/v) birchwood xylan suspension in 50 mM sodium acetate buffer (pH 4.5) (150 μL) and 50 μL of the appropriately diluted enzyme solution. For polygalacturonase activity determinations, 250 μL of reaction mixture containing 62.5 μL of 1% (w/v) polygalacturonic acid suspension in 50 mM sodium acetate buffer (pH 4.5) (137.5 μL) and 50 μL of the appropriately diluted enzyme were used. For β-glucanase activity determinations, 75 μL of β-glucan suspension containing 0.1% (w/v) of substrate in 50 mM sodium acetate buffer (pH 4.5) (125 μL) was added to 50 μL of appropriately diluted enzyme solution. All reaction mixes were incubated at 62 °C for 15 min, and reducing sugars liberated were determined as noted above. One unit of xylanase, polygalacturonase, or β-glucanase activity was defined as the amount of enzyme that released 1 μmol of glucose equivalents/min.

Hydrolytic enzyme activities including β-glucosidase, α-arabinofuranosidase, β-xylosidase, and α-rhamnosidase were determined by measuring the release of *p*-nitrophenol (pNP) at 400 nm during incubation in 50 mM sodium succinate buffer (pH 5.5) at 62 °C for 15 min in the presence of the substrates pNPG (0.02% w/v), pNPA (0.02% w/v), pNPX (0.1% w/v), or pNPR (0.05% w/v), respectively. All reactions were of 500 μL final volume and contained 25 μL of enzyme mix. One unit of enzyme activity was defined as that amount releasing 1 μmol of pNP/min.

Electrophoresis and Western Analysis. SDS−polyacrylamide gel electrophoresis was performed according to the procedure of Smith (1984) using a total acrylamide concentration of 10% (w/v) and an *N*,*N*′-methylenebis(acrylamide) concentration of 3.3%. Silver staining was performed as described by Merril et al. (1981). The low molecular mass calibration mixture from Pharmacia was used as standard. Western blotting was carried out as detailed by Towbin et al. (1979). Antibody against EGL1 of *Trichoderma reseei* was kindly provided by Dr. C. P. Kubicek (Abteilung für Mikrobielle Biochemie, Institut für Biochemische Technologie und Mikrobiologie, Technische Universität Wien, Austria). Western blotting analysis was carried out by incubation of nitrocellulose blots with this antiserum followed by staining with alkaline phosphatase linked goat anti-mouse immunoglobulin (IgG) as described by the manufacturer (Boehringer, Mannheim. Germany).

Influence on Enzyme Activity of Temperature, pH, and Compounds of Enological Relevance. The optimum temperature for endoglucanase activity was determined by performing the standard assay over the temperature range 25−70 °C. The pH optimum for endoglucanase activity was measured under standard conditions in universal buffer (Stauffer, 1989) across the pH range 4.5−8.0 at 62 °C. In view of the potential for wine flavor enhancement by recombinant β-1,4-endoglucanase, the influence on its activity of glucose, ethanol, and SO_2 at concentrations normally occurring in wine was examined.

Macerations and Analyses. Crushed white grapes of the Macabeo and Muscat varieties were incubated with constant gentle agitation under the following conditions: 14 °C for 8 h, 14 °C for 24 h, 21 °C for 3 h, and 21 °C for 12 h. The Bobal (red) grape was similarly treated at 21 °C for 12, 24, and 48 h with constant agitation. Macerations in the presence of 51EA culture filtrate were conducted using a CMCase activity of ∼0.053 unit/mL. The extraction of free and bound compounds was performed following the protocol of Günata et al. (1985) as modified by Versini et al. (1994). Samples were filtered and supernatants were then fractionated on Amberlite XAD-2 columns. Free volatile compounds were eluted using a pentane/dichloromethane (2:1 v/v) mixture, and glycosylated aroma precursors were eluted with an ethyl acetate/methanol (9:1 v/v) mixture. The latter fractions were dried in a rotary evaporator and redissolved in 75 mM citrate phosphate buffer (pH 5.0) (Gomori, 1955). Aglycons were removed from glycons by enzymatic hydrolysis at 40 °C for 24 h using the commercial glycolytic preparation AR2000 (Gist-Brocades, The Netherlands), which contains the following activities: β-glucosidase, β-xylosidase, α-arabinofuranosidase, and α-rhamnosidase (Granata, 1994; our unpublished data). Sugars released were analyzed by HPLC (Waters model 410) using a Sugar Pack column (Millipore) equilibrated with degassed Milli-Q H_2O run

Table 1. Endoglucanase Activities of *A. nidulans* Transformants

transformant	endoglucanase activity[a] (units/mL) av	SD
argB2 + pIJ16	0.00	0.00
8EA	1.19	0.10
10EA	0.64	0.20
13EA	0.58	0.13
20EA	0.26	0.19
21EA	0.20	0.10
24EA	0.00	0.00
28EA	0.27	0.06
31EA	0.09	0.06
39EA	0.31	0.04
40EA	0.32	0.00
41EA	0.60	0.03
45EA	0.48	0.12
46EA	0.48	0.11
47EA	0.08	0.08
50EA	0.40	0.00
51EA	1.31	0.07
53EA	0.11	0.05
54EA	0.45	0.05
55EA	0.08	0.02
56EA	0.01	0.00

[a] All strains were grown in triplicate for 24 h at 37 °C and 200 rpm under glucose-repressing conditions [1% (w/v) glucose] in 10 mL of liquid AMM supplemented with 0.05% (w/v) yeast extract, biotin, and methionine by inoculation with 1×10^8 conidia.

at 85 °C at a constant flow rate of 0.5 mL/min, and detection was done with a differential refractometer. L-Arabinose, D-glucose, and L-rhamnose were used as standards.

Electron Microscopy. Whole undamaged grapes were cut into lens-like sections, parallel to the longitudinal axis, with a razor blade and incubated for 24 h at room temperature (25 °C) with either undiluted culture filtrate from strain 51EA or 50 mM sodium acetate buffer (pH 4.5). Undiluted culture filtrate corresponds to a CMCase concentration 25 times greater than that used in macerations. Tissues were then fixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 48 h at 4 °C. SEM and cryo-SEM (freezing, sublimation, and metalization) were carried out on sections of ~1 mm width as detailed by Rosell and Lluch (1998).

RESULTS AND DISCUSSION

Construction of an *A. nidulans* Transformant Expressing EGL1. The *A. nidulans argB2* strain was cotransformed with the *argB*-carrying plasmid pIJ16 (Johnstone et al., 1985) and plasmid pPGPDEGL, which carries the *egl1* cDNA of *Trichoderma longibrachiatum* under the control of the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene promoter (Ganga et al., 1997). As a primary screen for the presence of pPGPDEGL, 37 prototrophic transformants were assayed on plates for endoglucanase production in the presence of glucose, conditions under which endogenous *A. nidulans* endoglucanolytic activity is repressed. The quantification of endoglucanase activity in 20 cotransformants is presented in Table 1. The highest level of endoglucanase activity was produced by transformant 51EA, which was hence chosen for further study.

Southern blot analysis of *Bam*HI (does not cut pPGPDEGL) and *Eco*RV (cuts pPGPDEGL once) genomic digests of 51EA is consistent with the presence of a tandem multicopy of the transforming plasmid: a single band corresponding to twice the size of the linear plasmid is observed in the *Bam*HI digest (Figure 1). The presence of additional bands in the *Eco*RV digest may reflect incomplete digestion by this enzyme. No hybridizing bands are detectable in the untransformed (*argB2*) strain or in a control transformed solely with pIJ16. Transformant 24EA, which produces no EGL1 activity (see Table 1), appears to contain a disrupted segment of *egl1*.

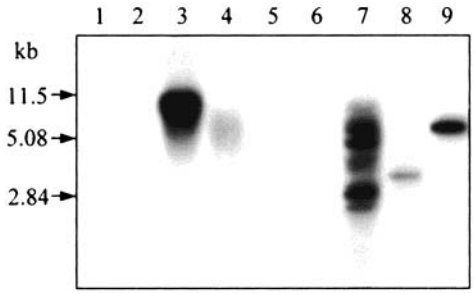

Figure 1. Southern blot. Tracks 1−8 were loaded with similar amounts (by EtBr staining) of genomic DNA from untransformed (*argB2*) and pIJ16-transformed (pIJ16::*argB2*) *A. nidulans* and the cotransformants 24EA and 51EA, digested with restriction enzymes: track 1, *argB2*/*Bam*HI; track 2, pIJ16::*argB2*/*Bam*HI; track 3, 51EA/*Bam*HI; track 4, 24EA/*Bam*HI; track 5, *argB2*/*Eco*RV; track 6, *argB*/*Eco*RV; track 7, 51EA/*Eco*RV; track 8, 24EA/*Eco*RV; track 9, 1 ng of linearized pPGPDEGL.

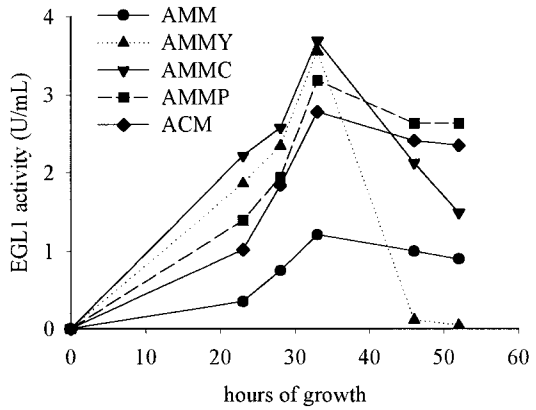

Figure 2. Extracellular EGL1 activity production profiles of 51EA grown in different culture media: production in AMM and ACM; production in AMM supplemented with different nitrogen sources (C, P, Y).

Heterologous Production of EGL1. The time course of production of EGL1 activity by 51EA at 37 °C was investigated in 25 mL cultures inoculated to a final spore titer of 10^7/mL, containing minimal medium (AMM) supplemented with the following organic nitrogen sources: yeast extract (Y), casamino acids (C), and peptone (P). All media tested produced a major peak of EGL1 activity ~33 h after inoculation of spores (Figure 2). This activity peak was ~3-fold greater in supplemented media compared to that in AMM.

Culture filtrates were analyzed by SDS−PAGE to determine the profile of extracellular proteins produced (Figure 3A). Minimal medium supplemented with peptone (track 3) was found to exhibit the least complex pattern of polypeptide bands. The same culture filtrates were analyzed by immunoblotting to identify EGL1 (Figure 3B). Tracks 1 and 7 demonstrate the specific production of EGL1 by transformant 51EA compared to the untransformed *argB*2 strain and show that the heterologously produced enzyme has a molecular mass very similar to that produced homologously (*T.l* tracks). The occurrence of an additional, lower molecular mass band in AMMY (track 5) may have arisen from proteolytic degradation of EGL1. With regard to the latter,

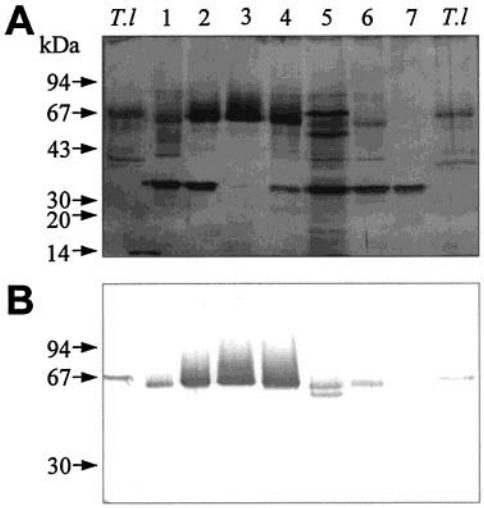

Figure 3. (A) SDS polyacrylamide gel; (B) western blot of SDS−PAGE. Tracks were loaded with 25 μL of culture filtrate from growth of *h* hours of the following strains: *T.l*, *T. longibrachiatum* multicopy transformant, 24 h in AMM; track 1, 51EA, 24 h in AMM; track 2, 51EA, 33 h in ACM; track 3, 51EA, 33 h in AMMP; track 4, 51EA, 33 h in AMMC; track 5, 51EA, 33 h in AMMY; track 6, 51EA, 33 h in AMM; track 7, *argB*2, 24 h in AMM.

Tilburn et al. (1995) have demonstrated the induction of the gene *prtA*, which encodes an alkaline protease, at alkaline pH. The pH values of the various production media were monitored, and in the case of AMMY at 33 h the pH was 8, whereas in all other media used the pH range was between 4.6 and 6.3; hence, this apparent degradation may be due to the production of an alkaline protease by *A. nidulans*. The relatively low complexity of the extracellular protein profile and the high level of EGL1 production observed for 51EA in AMMP indicates that EGL1 is the predominate extracellular protein produced. In particular, the production of a 33 kDa protein, which is present in other media, appears to be inhibited in the presence of peptone.

Enzymatic Properties of 51EA Culture Filtrate. The temperature and pH profiles of the 51EA AMMP culture filtrate (Figure 4A,B) were characterized and found to be very similar to those of homologously produced EGL1 (Ganga, 1997). Apart from endoglucanolytic activity, *Trichoderma* EGL1 has been shown to hydrolyze polymeric substrates other than CMC (Penttilä et al., 1987; Biely et al., 1991; Ganga, 1997). To assess the presence of these subsidiary activities, polymeric substrates were incubated at the CMCase optimal temperature determined above for EGL1 activity (62 °C) with 33 h AMMP culture filtrates in which 51EA and a control transformant (contains only the selection plasmid pIJ16) had been grown. Because the latter is not a producer of EGL1, the presence of any endogenous *A. nidulans* activities will be apparent. The subsidiary xylanolytic and glucanolytic activities observed in 51EA (Table 2) are similar to those detected previously in EGL1 purified from *T. longibrachiatum* (Ganga, 1997) and comprise cell wall degrading activities potentially suitable for enhancing the release of aroma precursors during grape maceration. The similarity of the polygalacturonolytic activities in the 51EA and pIJ16 culture filtrates suggests that this activity is of endogenous origin and not contributed by EGL1. Similar assays performed at 25 °C resulted in reductions of approximately 13-, 8-, and 5-fold in CMCase, xylanolytic, and glucanolytic activity levels, respectively (Table 2).

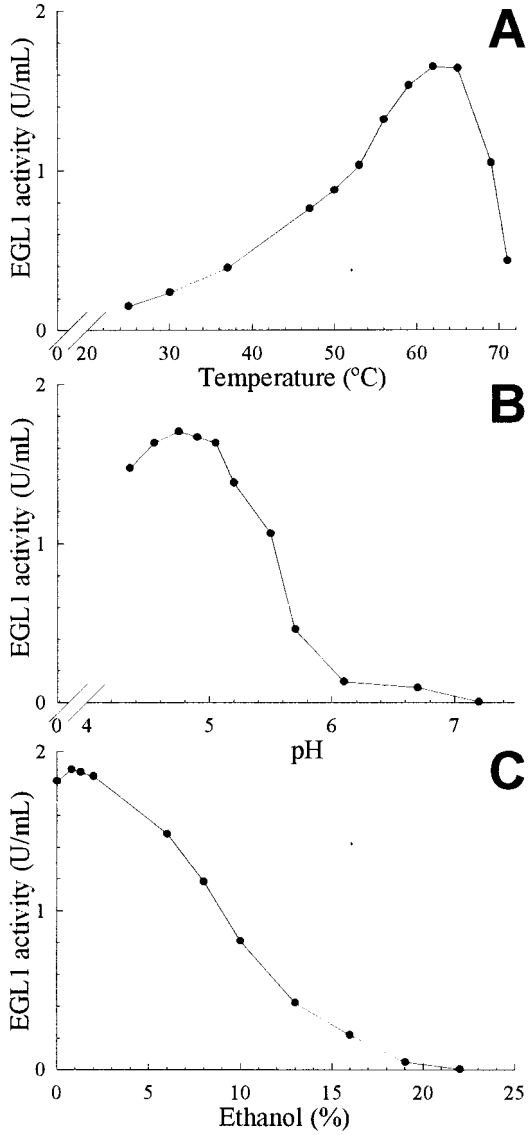

Figure 4. Activity profile of 51EA culture filtrate versus (A) temperature, (B) pH, and (C) ethanol concentration.

Table 2. Enzymatic Activities Present in 33 h AMMP Culture Filtrates of 51EA and pIJ16[a]

substrate	51EA (units/mg) at 62 °C	51EA (units/mg) at 25 °C	pIJ16 (units/mg) at 62 °C	pIJ16 (units/mg) at 25 °C
CMC	0.250 ± 0.008	0.019 ± 0.001	0.001 ± 0.001	0.0
birchwood xylan	0.047 ± 0.003	0.006 ± 0.001	0.001 ± 0.001	0.0
barley glucan	0.054 ± 0.000	0.001 ± 0.001	0.000 ± 0.000	0.0
polygalacturonic acid	0.005 ± 0.001	0.0	0.006 ± 0.001	0.0

[a] Both strains were grown in triplicate; assays were done in duplicate at 62 and 25 °C and are expressed as units of activity per milligram of dry weight of mycelium. The dry weights of 51EA and pIJ16 mycelia recovered from each 25 mL culture were 154.8 ± 7.5 and 149.1 ± 8.0 mg, respectively.

Assays were also performed using chromogenic substrates including pNP-substituted glucopyranoside, arabinofuranoside, xylopyranoside, and rhamnopyranoside for hydrolytic activities, which could result in aroma release from precursors. In all cases, activity levels were either not detectable or extremely low (data not shown).

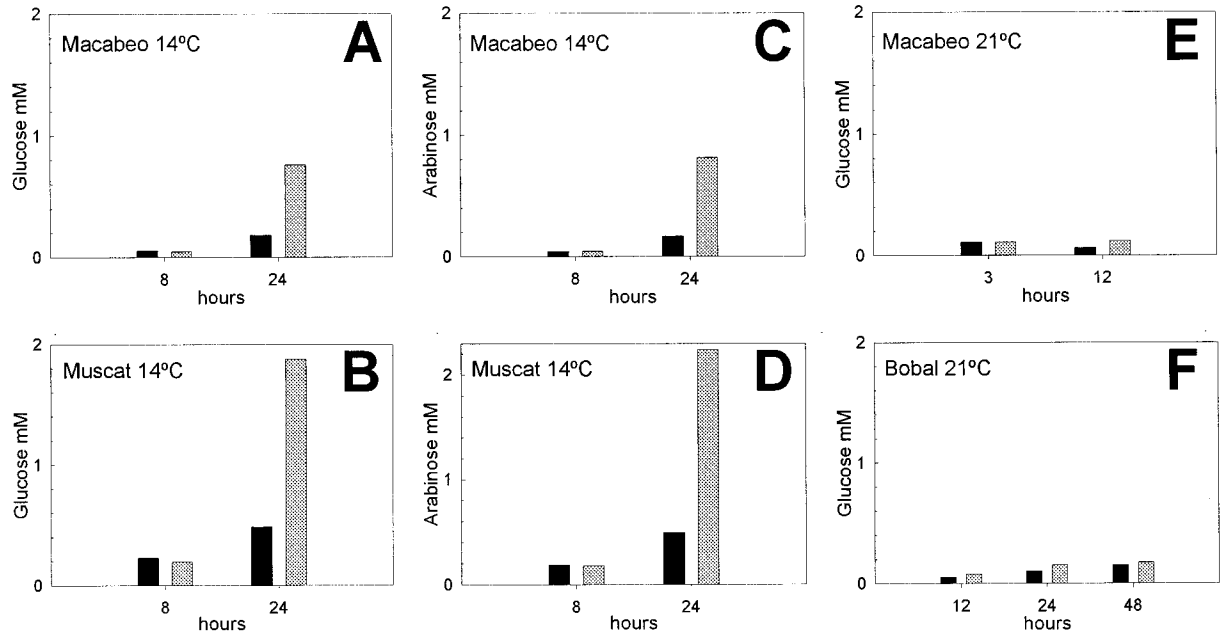

Figure 5. Monosaccharide release during macerations of Macabeo, Muscat, and Bobal grape varieties in the presence (shaded bars) and absence (black bars) of 51EA culture filtrate. The duration and temperature of the macerations are indicated in each graph.

The endoglucanolytic activity of 51EA culture filtrate has also been studied to ensure its suitability for use under wine-making conditions. Although the pH and temperature optima (see Figure 4A,B) do not coincide with the conditions occurring during wine fermentation, enzymatic activity of ~20−25% is retained at pH 3.5 and 30 °C. Enzymatic activity in the presence of the enologically important compounds glucose, ethanol, and SO_2 was also assayed. Using azo-CM-cellulose as substrate, glucose concentrations up to 240 g/L showed no inhibitory effects on activity. Indeed, the presence of glucose tended to augment activity (data not shown). Increasing the level of ethanol led to inhibition, 10% (v/v) ethanol resulting in ~50% maximal activity (Figure 4C). With regard to SO_2, at 50 ppm, EGL1 retained 35% activity (30 ppm is the concentration normally used in wine).

Grape Maceration by Heterologously Produced EGL1. The potential for the application of heterologously produced EGL1 to wine-making was investigated by comparing glycon (aroma precursor) release in macerations conducted in the presence of EGL1-containing culture filtrate to macerations conducted in its absence, under conditions representative of those used in wine-making with regard to temperature, duration, and concentration of enzymatic activity. Three grape varieties, two white (Macabeo and Muscat) and one red (Bobal), were selected and macerated for various times and temperatures depending on grape type and the process normally used in the winery. Glycons were recovered by fractionation of must supernatants on Amberlite and enzymatically hydrolyzed using the commercial glycolytic preparation AR2000. Sugar fractions were subsequently analyzed and quantified by high-performance liquid chromatography (HPLC).

Sugar release, which can be taken as an indirect measure of glycosylated precursor content, was enhanced 3−4-fold in the presence of 51EA culture filtrate in the two white grape varieties macerated for 24 h at 14 °C (Figure 5A,B). Incubation at 21 °C for shorter periods did not, however, result in such enhancement (Figure 5E). That similar quantities of arabinose were released compared to glucose indicates that the major diglycosidic aroma precursor is arabinosylglucoside (Figure 5A−D). To a lesser extent the presence of rhamnosyl precusors was also indicated, rhamnose constituting ~2% of the total glucose, arabinose, and rhamnose residues released in Macabeo and Muscat musts (data not shown). Under the elevated temperature conditions typical for red grape wine-making, little or no effect of the presence of culture filtrate was noted with the Bobal grape variety, even after incubation for 48 h (Figure 5F).

Electron Microscopic Analysis of Bobal Grape Maceration by EGL1. The nature of the primary and subsidiary activities of EGL1 can be expected to result in alterations to the hemicellulosic matrix of plant cell walls. To investigate the apparent lack of precursor release from the Bobal grape after maceration with 51EA culture filtrate, a comparative electron microscopic examination was made of sectioned, undamaged, mature Bobal grape lens-like sections treated with either undiluted 51EA culture filtrate or buffer. The results of scanning electron microscopy (SEM) and cryo-SEM are presented in Figure 6. In overall views (A, B, E, and F), the fracture of 51EA-treated material can be seen to have occurred principally within the middle lamella, resulting in the appearance of cell wall surfaces (B), whereas fracture of the control (A) occurred across cells, thus revealing the sublimed residues [solute water glass (swg)] of the cellular contents. Weakening of the hemicellulosic component of the middle lamella by the action of EGL1 during incubation with 51EA culture filtrate could account for the observation that the cryo-fracture follows the plane of the weakened middle lamella. The corresponding razor-cut samples analyzed by SEM clearly show the presence of pitting (arrowed) of the cell walls in the treated sample (F) compared to the control (E). Higher magnification of cryo-SEM (C and D) and SEM (G and H) material reveals pitting (D and H) and rupture (H) of the cell wall matrix in 51EA-treated material compared to the control (C and G). That

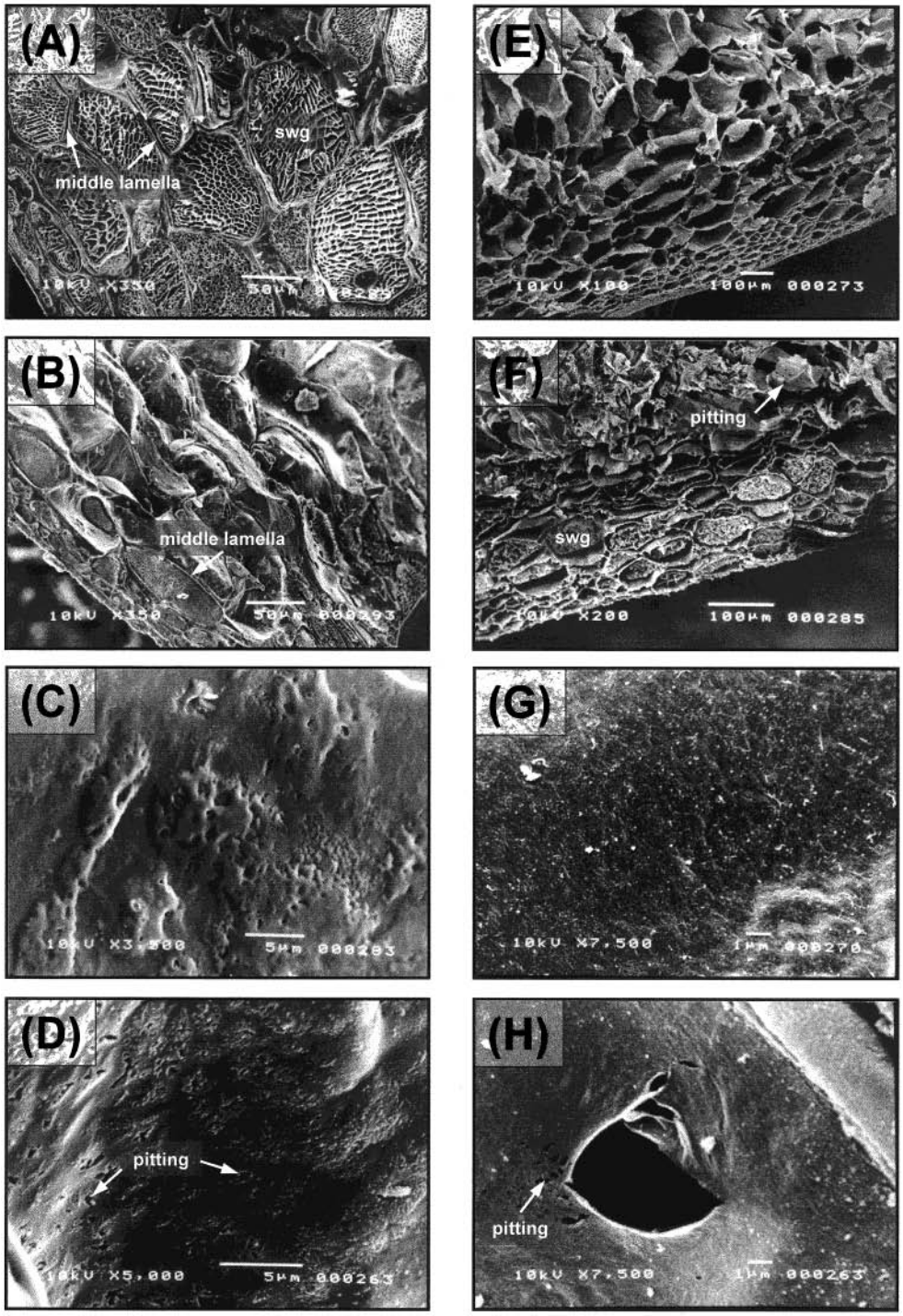

Figure 6. Comparative electron microscopic analysis of macerated grape tissue: (A−D) cryo-SEM analysis; (E−H) SEM analysis; (B, D, F, H) grape sections incubated with undiluted 51EA culture filtrate; (A, C, E, G) grape sections incubated with buffer (control). Panels A, B, E, and F show cross-sectioned cells, panels C, D, G, and H show the cell walls. The middle lamella is arrowed in panels A and B; pitting is arrowed in panels D, F, and H (see text).

increased precursor release was not observed upon maceration of Bobal grapes with 51EA culture filtrate may be due either to (i) a paucity of precursors in this grape, (ii) a need for more extensive enzymatic degradation of the hemicellulosic matrix (the treatment of the grape tissue for electron microscopy was performed using an EGL1 activity 25 times greater than that used in the macerations), or (iii) an as yet uncharacterized inhibition of the release of precursors in macerations conducted at 21 °C.

Conclusions. The *A. nidulans* cotransformant 51EA containing the *T. longibrachiatum egl1* gene under the control of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene promoter produces enzymatically active EGL1. Manipulation of the growth medium resulted in EGL1 being the predominant extracellular protein produced when 51EA was grown in peptone-supplemented minimal medium. Analysis of the enzymatic properties of 51EA culture filtrate showed no significant differences in activity from those of homologously produced EGL1, and application of this culture filtrate in white grape macerations resulted in increased release of aroma precursors, particularly in the case of the more aromatic Muscat grape. Electron microscopy

indicates that this enzymatic treatment results in physical weakening of both grape cell walls and the middle lamella.

That EGL1 can be produced heterologously as the predominant extracellular protein provides a considerable advantage over currently available crude extracts which contain a variety of enzymatic activities. The greater availability of aroma precursors during maceration can be expected to provide more substrate for the subsequent action of glycolytic preparations such as AR2000, the consequent release of aglycons, and thus enhancement of the aroma of the finished wine. That the latter glycolytic activities are not predominant in 51EA culture filtrate minimizes the premature loss of aroma during the maceration process. In this context, the use of 51EA culture filtrate in macerations of grape varieties of apparently lesser aromatic potential may provide a means of ultimately improving aroma and, hence, a re-evaluation of the quality of the final wine produced.

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