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RESEARCH PAPER

Acyl substrate preferences of an IAA-amido synthetase account for variations in grape (*Vitis vinifera* L.) berry ripening caused by different auxinic compounds indicating the importance of auxin conjugation in plant development

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Abstract

Nine Gretchen Hagen (*GH3*) genes were identified in grapevine (*Vitis vinifera* L.) and six of these were predicted on the basis of protein sequence similarity to act as indole-3-acetic acid (IAA)-amido synthetases. The activity of these enzymes is thought to be important in controlling free IAA levels and one auxin-inducible grapevine GH3 protein, GH3-1, has previously been implicated in the berry ripening process. *Ex planta* assays showed that the expression of only one other *GH3* gene, *GH3-2*, increased following the treatment of grape berries with auxinic compounds. One of these was the naturally occurring IAA and the other two were synthetic, α-naphthalene acetic acid (NAA) and benzothiazole-2-oxyacetic acid (BTOA). The determination of steady-state kinetic parameters for the recombinant GH3-1 and GH3-2 proteins revealed that both enzymes efficiently conjugated aspartic acid (Asp) to IAA and less well to NAA, while BTOA was a poor substrate. *GH3-2* gene expression was induced by IAA treatment of pre-ripening berries with an associated increase in levels of IAA-Asp and a decrease in free IAA levels. This indicates that GH3-2 responded to excess auxin to maintain low levels of free IAA. Grape berry ripening was not affected by IAA application prior to veraison (ripening onset) but was considerably delayed by NAA and even more so by BTOA. The differential effects of the three auxinic compounds on berry ripening can therefore be explained by the induction and acyl substrate specificity of GH3-2. These results further indicate an important role for GH3 proteins in controlling auxin-related plant developmental processes.

Key words: Auxin, GH3 proteins, ripening, *Vitis vinifera*.

Introduction

The ripening of fruit is a complex process whose regulation depends on the action of plant hormones. The first and most prominent hormone to be associated with softening, colouring, and sweetening of some fruit was ethylene (Porritt, 1951; Burg and Burg, 1962). Ethylene has been shown to be crucial for the ripening of so-called climacteric fruit like tomato (*Solanum lycopersicum* Mill.) and banana (*Musa paradisiaca* L.), which are characterized by a sharp increase in respiratory activity (Seymour, 1993; Tucker, 1993). The role of ethylene in the ripening process of non-climacteric fruit such as strawberry (*Fragaria ananassa* Duch.) and grape (*Vitis vinifera* L.), that lack the ethylene and respiration burst (Tucker, 1993), is less obvious and still a matter of debate (Davies and Böttcher, 2009). However, in recent years it has become evident that various other plant hormones exhibit either promoting or inhibiting effects on the ripening of climacteric as well as non-climacteric fruit (Vendrell and Palomer, 1998; Davies and Böttcher, 2009). In both types of fruit the best studied inhibitor of ripening is indole-3-acetic acid (IAA), the most abundant member of the auxin class of plant hormones. Coinciding

with extensive cell division and expansion, endogenous levels of IAA were reported to be high in young fruit, after which they declined to be low at the initiation of ripening, not only in climacteric fruit such as tomato (Mapelli *et al.*, 1978; Buta and Spaulding, 1994), banana (Purgatto *et al.*, 2002), and date (*Phoenix dactylifera* L. cv. Hillawi; Abbas *et al.*, 2000) but also in non-climacteric fruit like strawberry (Archbold and Dennis, 1984) and grape (Inaba *et al.*, 1976; Cawthon and Morris, 1982; Zhang *et al.*, 2003; Deytieux-Belleau *et al.*, 2007; Böttcher *et al.*, 2010*a*). The reduction of auxin levels before the initiation of ripening has been suggested to be a prerequisite for ripening to occur (Frenkel, 1972; Given *et al.*, 1988; Buta and Spaulding, 1994; Chen *et al.*, 1999; Purgatto *et al.*, 2002; Böttcher *et al.*, 2010*a*), and this proposal is supported by the results of auxin application experiments. In a range of climacteric fruit including avocado (*Persea americana* Mill.; Tingwa and Young, 1975), banana (Vendrell, 1969; Purgatto *et al.*, 2002), kiwifruit (Fabbroni *et al.*, 2006), tomato (Cohen, 1996), and pear (Frenkel and Dyck, 1973) the application of natural or synthetic auxins has been shown to delay ripening. Reports about an acceleration of at least parts of the ripening process by auxin treatment in some other climacteric fruit (Ohmiya, 2000; Agustí *et al.*, 2003, 2004; Kondo *et al.*, 2004; Yuan and Carbaugh, 2007) can be interpreted as an indirect auxin effect mediated through the induction of ethylene biosynthesis (Kondo *et al.*, 2004; Trainotti *et al.*, 2007; Li and Yuan, 2008) and demonstrates the relevance of the type and developmental stage of fruit at the time of auxin exposure for the outcome of the treatment. The application of the synthetic auxin α-naphthalene acetic acid (NAA) also delayed the ripening of non-climacteric strawberry fruit whereas removal of the achenes, the natural source of IAA in this fruit, enhanced ripening processes such as anthocyanin accumulation, softening, and chlorophyll breakdown (Given *et al.*, 1988). More recent studies provide evidence that, in strawberry fruit, auxin application suppresses the expression of many ripening-associated genes related to pigmentation, stress response, cell wall metabolism, and the synthesis of flavour and aroma compounds (Manning, 1994, 1998; Harpster *et al.*, 1998; Aharoni *et al.*, 2002). Another non-climacteric fruit with a well-documented ripening delay in response to the application of natural or synthetic auxins is grape. When applied before the initiation of ripening (veraison) auxins caused a reduction in the accumulation of sugars and anthocyanins and a delayed decrease in acidity and chlorophyll levels (Weaver, 1962; Hale, 1968; Hale *et al.*, 1970; Davies *et al.*, 1997; Yakushiji *et al.*, 2001; Ban *et al.*, 2003; Jeong *et al.*, 2004; Fujita *et al.*, 2006; Deytieux-Belleau *et al.*, 2007; Böttcher *et al.*, 2010*b*). The above-mentioned auxin application experiments involved the use of a wide range of auxins and auxin-like compounds including IAA, indole-3-butyric acid (IBA), NAA, benzothiazole-2-oxyacetic acid (BTOA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenoxypropionic acid (2,4-DP), and 3,5,6-trichloro-2-pyridyloxyacetic acid (TPA). In studies that compared the effects of two or more auxinic compounds, varying efficacies in delay or enhancement of ripening were reported, for example, IAA was less efficient than 2,4-D in delaying respiratory climacteric, ethylene production, yellowing of peel, softening of pulp, production of volatiles, and conversion of starch in banana slices (Vendrell, 1969) and 2,4-DP increased the colouring of apple skin whereas NAA and 2,4-D had no effect (Stern *et al.*, 2009). In auxin application experiments with grape berries, BTOA was generally found to be very effective in inhibiting ripening (Weaver, 1954, 1955, 1962; Hale, 1968; Coombe and Hale, 1973; Davies *et al.*, 1997), seemingly by maintaining berries in the preveraison state delaying softening, anthocyanin accumulation, and sugar accumulation as well as the expression of ripening-related genes (Davies *et al.*, 1997). The mechanisms by which auxins delay fruit ripening and the reason for the varying effectiveness of different auxinic compounds are not known, but recent findings regarding the role of auxin conjugation in ripening fruit might provide the first insight into the underlying mechanisms. A potential importance for IAA-amido synthetases termed GH3 proteins, which catalyse the conjugation of IAA to amino acids (Staswick *et al.*, 2002, 2005), has been reported for the ripening process of pungent pepper (CcGH3, Liu *et al.*, 2005) and grape (GH3-1, Böttcher *et al.*, 2010*a*), possibly by inactivating endogenous IAA through the formation of the non-cleavable IAA–Asp conjugate (Böttcher *et al.*, 2010*a*). It is known from *in vitro* studies with *Arabidopsis* (*Arabidopsis thaliana* L.) GH3 proteins that a broad range of auxin substrates, including IBA, indole-3-propionic acid (IPA), phenylacetic acid (PAA), and NAA, can be conjugated by these enzymes, but with varying efficiencies (Staswick *et al.*, 2005). For some auxinic compounds (4-chloro-3-indole acetic acid, 2,4-D and dicamba) there was no detectable conjugation by the *Arabidopsis* enzymes. The selective activity of GH3 proteins might therefore not only explain why auxinic compounds like 2,4-D and dicamba are effective growth regulator herbicides (Kelley and Riechers, 2007), but could also be of importance in the inactivation of auxins applied to fruit, thereby leading to different degrees of ripening inhibition.

Data on the varying effectiveness of IAA, NAA, and BTOA in delaying grape berry ripening are presented here. The identification and characterization of five novel grapevine group II IAA-amido synthetase genes expressed in berries revealed that the transcript levels of one of them, *GH3-2*, were increased in response to auxin treatments as has previously been demonstrated for *GH3-1* (Böttcher *et al.*, 2010*a*). Detailed analyses of the *in vitro* activities of both GH3-1 and GH3-2, the levels of their main *in vitro* reaction product (IAA–Asp) and the expression of the corresponding *GH3* genes in IAA-treated berries indicated that different preferences for the acyl substrates of GH3-2 might be causative for the observed variations in auxin-induced ripening delay.

Materials and methods

Plant material

Treatments with different auxins were conducted with *Vitis vinifera* L. cv. Shiraz fruit (Adelaide Hills, South Australia, 35.018223 S, 138.838220 E). Berries were sprayed twice (29 December 2009, 13 January 2010) during the preveraison period with 80 mg l^{-1} IAA (Sigma-Aldrich, St Louis, USA), 50 mg l^{-1} NAA (Gibco BRL Life Technologies, Grand Island, USA) or 20 mg l^{-1} BTOA (American Cyanamid Company, Princeton, USA) in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton, Australia). Control fruit were sprayed with a 0.1% (v/v) Chemwet 1000 solution. The trial was of a randomized triplicate design, the sample size per replicate and treatment was 30 bunches. Samples of 60 randomly harvested berries per replicate were taken 1, 2, and 7 days post the initial spray (dpis), followed by weekly sampling throughout development. Sampling was completed between 09.30 h and 14.30 h, berries were weighed, immediately deseeded, frozen in liquid nitrogen, and stored at –80 °C until used. Anthocyanins and total soluble solids (TSS) were measured for each of these replicates.

For the analysis of developmental changes in gene expression *Vitis vinifera* L. cv. Cabernet Sauvignon flowers (at the 50% cap fall stage: anthesis (0 wpf)) and berries from 2–16 weeks post-flowering (wpf) were collected at fortnightly intervals, (23 November 2004–16 March 2005) from a commercial vineyard (Clare Valley, South Australia, 33.794364 S, 138.628353 E) in the 2004/2005 season. Sampling was completed between 09.30 h and 14.30 h, berries (100–150 berries sampled at each time point) were immediately deseeded and the tissue was frozen in liquid nitrogen and stored at –80 °C until used.

For the *ex planta* berry induction experiment grape berries (*Vitis vinifera* L. cv. Shiraz) were sampled from a vineyard in the Adelaide Hills, South Australia (35.018223 E, 138.838220 S) at either 22 d (12 January 2009) or 12 d (22 January 2009) before veraison between 09.00–10.00 h and kept on ice until used.

Determination of anthocyanin and total soluble solid (TSS) levels

Frozen berries were ground to a powder using an IKA A11 basic analytical mill (IKA, Staufen, Germany). For the measurement of TSS (degrees Brix) 100 mg of berry powder was thawed on ice, the tissue was pelleted by centrifugation at 18 000 *g* for 5 min and the supernatant was analysed with an RFM710 digital refractometer (Bellingham Stanley, Tunbridge Wells, UK). For anthocyanin determination, 300 mg of powdered sample was added to 1.5 ml of MeOH containing 1% (v/v) HCl. Anthocyanins were extracted at room temperature in the dark on a rotating mixer for 1 h. The tissue was pelleted by centrifugation at 18 000 *g* for 15 min and the supernatant retained. Depending on the developmental stage the supernatant was diluted up to 20-fold with MeOH, 1% (v/v) HCl. Total anthocyanins were measured spectrophotometrically by reading absorbance at 520 nm immediately following centrifugation.

Phylogenetic analysis

GH3-related plant sequences were identified by BLASTP searches of the non-redundant protein database (*P* value $\leqslant 10^{-20}$) using the *Vitis vinifera* L. GH3-1 protein sequence as the query. Non-redundant sequences that, based on comparison with the grapevine GH3-1 protein, were considered full-length were included in the analysis. NCBI or GenBank accession numbers for all sequences used in the phylogenetic analysis are listed in Supplementary Table S1 at *JXB* online. The sequences were aligned using the ClustalX (version 2.0.10) program (Larkin *et al.*, 2007) in the multiple alignment mode and the Neighbor–Joining unrooted tree was generated with PHYLIP 3.6 (Felsenstein, 1989). The Invitrogen Vector NTI AlignX software (version Advanced 10) was used to determine sequence similarities and identities.

RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA was extracted from grape berry tissues according to Davies and Robinson (1996) and further purified as described by Symons *et al.* (2006). First-strand cDNA for quantitative real-time PCR was synthesized with Superscript III enzyme (Invitrogen, Carlsbad, USA) using 1 μg of RNA and the Oligo $(dT)_{20}$ primer in a reaction volume of 20 μl following the manufacturer's instructions. Quantitative RT-PCR was conducted as described by Symons *et al.* (2006) using the following gene-specific primer pairs: *GH3-1* (XM_002271216), 5′-ATCTACGAGCGCAAACAAGTC-C-3′ and 5′-GTGTGAGTTGGTGCCAGTTGAG-3′; for *GH3-2* (XM_002283850), 5′-CTGAGTTGTGGAACCCAGTGAC-3′ and 5′-GCGGATGTAGAAGTTGGGAAAG-3′; for *GH3-3* (XM_002283193), 5′-ACATTCTTCCGGCATACGAAGT-3′ and 5′-TGAGCTGGTCGTCACCACTTAT-3′; for *GH3-4* (XM_002263317), 5′-TGATGCTCCTATGCAGATCCTC-3′ and 5′-CAAGTGACTGGATTCCACAACC-3′; for *GH3-5* (XM_002276205), 5′-GGAACATTTGACAAGCTCATGG-3′ and 5′-CCCTTGAATTCAGAAGCTCGAT-3′; for *GH3-6* (XM_002268242), 5′-TAGGATAGTGAAGCCTGGCACA-3′ and 5′-CTCTTTGGATTTGATGCACCTG-3′ and, for normalization of cDNA levels, *Actin2* (AM465189), 5′-GCACCCTTCG-CACGATATGA-3′ and 5′-TGACGCAAGGCAAGGACTGA-3′. Each PCR was performed in triplicate. To calculate the copy number of the *GH3* genes in each reaction, the purified gene fragments used for the standard curves were quantified using PicoGreen (AGRF, Adelaide, South Australia) and the number of molecules in each standard dilution was determined according to Whelan *et al.* (2003). The specificity of the reactions was confirmed by melt curve analysis as well as separation on agarose gels and the identity of each product was verified by sequencing (AGRF, Adelaide, South Australia).

Ex planta *berry induction assay*

Berries were sampled from 40 bunches of 10 vines at two time points prior to veraison (22 d and 12 d), sterilized in 0.05% (v/v) Tween 20 containing one Milton antibacterial tablet l^{-1} (Milton Australia, Laverton North, Victoria) for one hour and washed three times with sterile nanopure water. All the following procedures were carried out in a laminar flow under sterile conditions. A thin slice was cut off horizontally around the brush area of each berry to facilitate compound absorption and 20 berries were placed on Petri dishes filled with 25 ml of Gamborg's media, 0.025% (w/v) casein hydrosylate, 0.8% (w/v) agar, pH 5.7–5.8 and one or more of the following additives (final concentrations), respectively: IAA (0.5 μM), NAA (0.5 μM), BTOA (0.5 μM), 12% (w/v) sucrose.

Berries were placed on the plates with the brush facing the agar, the plates were sealed with Parafilm and kept in the dark at room temperature. After 24 h the berries were harvested, deseeded, and frozen in liquid nitrogen.

Chemical synthesis of auxin amino acid conjugates

[Indole-D_5]IAA and [1,4-$^{13}C_2$]-L-aspartic acid were from Cambridge Isotope Laboratories (Andover, USA), L-tryptophan methyl ester hydrochloride and L-aspartic acid dimethyl ester hydrochloride were purchased from Sigma-Aldrich (St Louis, USA).

IAA–amino acid conjugates were synthesized according to Ilić *et al.* (1997) using either unlabelled IAA or [indole-D_5]-labelled IAA as the starting substrate. The same protocol was used for the synthesis of NAA–Asp and BTOA–Asp, but since no labelled forms of NAA and BTOA were commercially available [1,4-$^{13}C_2$]-L-aspartic acid was used to obtain labelled conjugates of these

compounds. Conjugate-containing fractions were further purified by reversed-phase HPLC using an Agilent 1100 series system equipped with a Luna C18 column [250×10 mm, 5 µm (Phenomenex, Torrance, CA)], using isocratic elution [40% (v/v) MeOH, 0.2% (v/v) acetic acid (IAA–Asp, Rt=7.8 min; BTOA–Asp, Rt=13.2 min) or 60% (v/v) MeOH, 0.2% (v/v) acetic acid (IAA–Trp, Rt=8.4 min; NAA–Asp, Rt=6.2 min)] at a flow rate of 4.5 ml min^{-1}. The column eluent was monitored using a DAD detector observing at a single wavelength (280 nm).

Protein purification and enzyme assay

The coding region of *GH3-2* was amplified by PCR from a Cabernet Sauvignon berry cDNA template using gene-specific primers (5'-TAT**CATATG**GCAGTTGATTCCGGTCTGTC-3', 5'-ATA**GCGGCCGC**GTCCGTACGTCGTCGCTCTG-3') with additional *Nde*I and NotI sites (in bold). Cloning, heterologous expression, and purification of GH3-2-His (C-terminal fusion) as well as TLC-based assays for IAA–amino acid conjugate formation were performed as described by Böttcher *et al.* (2010*a*) for GH3-1.

Determination of kinetic parameters

To identify suitable protein amounts and incubation times to be used for the determination of steady-state kinetic parameters for GH3-1 and GH3-2 the following standard assay conditions were used: 50 mM TRIS/HCl (pH 8.6), 3 mM $MgCl_2$, 3 mM ATP, 1 mM DTT, 1 mM aspartic acid (Asp) or tryptophan (Trp), 1 mM IAA, 25 °C, 50 µl volume. The reactions were initiated by adding 0–1 µg of purified GH3-1 or GH3-2 from glycerol stocks [10% (v/v) glycerol, 1 mM DTT, 5–10 mg ml^{-1} protein, stored at –80 °C for up to one month] and stopped after 0–20 min by adding 10 µl 50% (v/v) HCl. After the addition of 250–750 pmol of labelled IAA–-Asp or IAA–Trp as internal standards the samples were extracted twice with 150 µl ethyl acetate. The extract was dried and the residue resuspended in 30 µl 60% (v/v) MeOH, 1% (v/v) acetic acid to be analysed by LC-MS. Steady-state parameters for both enzymes were determined using the standard assay but varying the concentration of one substrate at a time: (0–20 mM) Asp, (0–20 mM) Trp, (0–5 mM) ATP, (0–2 mM) auxins (IAA, NAA or BTOA). Reactions were stopped after 0, 2, 5, and 10 min, appropriate labelled standards were added and the samples were acidified to pH 1–2. Samples were extracted as described above and subjected to LC-MS analysis (below). The reactions products were quantified, initial velocities were calculated from the linear range and fit to the Michaelis–Menten equation using non-linear regression (SigmaPlot 11.0).

LC-ESI-MS/MS analysis of auxins and conjugates

For the quantification of IAA and IAA–Asp in grapes, auxins were extracted from 100 mg of grape berry tissue, spiked with 500 pmol of [indole-D_5]IAA and [indole-D_5]IAA–Asp as internal standards, as described by Kowalczyk and Sandberg (2001). After extraction and diethyl ether partitioning the aqueous phase was acidified to pH 1–2 and applied to a 50 mg Env^+ SPE column (Isolute, Uppsala, Sweden). The column was washed with water (1 ml) and then eluted with 80% (v/v) MeOH, 1% (v/v) acetic acid (2.5 ml). The dried residue was resuspended in 40 µl 60% (v/v) MeOH, 1% (v/v) acetic acid to be analysed with an Agilent LC-MS system (1200 series HPLC coupled with a 6410 triple quad mass spectrometer). The sample (10 µl) was first separated on a Luna C18 column [75×4.6 mm, 5 µm, (Phenomenex, Torrance, CA)] held at 30 °C using the following solvent conditions: 0–8 min isocratic 60% (v/v) MeOH, linear gradient from 60% (v/v) to 95% (v/v) MeOH in 1 min, held for 5 min, from 95% (v/v) to 60% (v/v) in 1 min, held for 6 min, 0.4 ml min^{-1}. The effluent was introduced into the ESI ion source (nebulizer pressure 35 psi) with a dissolvation gas temperature of 300 °C at a flow of 8 l min^{-1}, with the capillary voltage set to 4 kV. The detection was performed by multiple reaction monitoring (MRM) in positive ion mode. The optimization of fragmentation was done with purified IAA–Asp and IAA as well as the labelled standards using Agilent MassHunter Optimizer software. With the collision energy ranging between 14–46 eV, quinolinium ions were the major fragments for all analysed compounds (*m/z* 130 for unlabelled IAA and IAA–Asp, *m/z* 134 for labelled IAA and IAA–Asp) and were used for quantitation.

For the analysis of conjugates produced by the *in vitro* reactions with recombinant GH3-1 and GH3-2, 10 µl of the dissolved residues were introduced into the ESI ion source as described above. After optimization of the fragmentation conditions, the fragments used for quantitation were as follows: for labelled and unlabelled IAA and IAA–Asp as described above, *m/z* 130 for unlabelled IAA–Trp, *m/z* 134 for labelled IAA–Trp, *m/z* 141 for NAA, labelled and unlabelled NAA–Asp, *m/z* 161 for BTOA, labelled and unlabelled BTOA–Asp. For the analysis of BTOA and BTOA–Asp the chromatography conditions were changed to: 0–11 min isocratic 60% (v/v) MeOH, linear gradient from 60% (v/v) to 95% (v/v) MeOH in 1 min, held for 5 min, from 95% (v/v) to 60% (v/v) in 1 min, held for 6 min, 0.4 ml min^{-1}.

Statistical data analysis

All statistical analyses were performed using SPSS 15.0 (SPSS, Chicago, Illinois, USA).

Results and discussion

Different auxins vary in their ability to delay grape berry ripening

The ripening-delaying effects of a variety of auxins have been described for many different fruit, but the molecular mechanisms causing this delay remain elusive.

In this study preveraison Shiraz berries were treated twice with either the natural auxin IAA, the synthetic auxin NAA, or the synthetic auxin-like compound BTOA, all of which have previously been reported to delay certain aspects of the ripening of grape berries (Weaver, 1962; Hale, 1968; Coombe and Hale, 1973; Davies *et al.*, 1997; Jeong *et al.*, 2004; Deytieux-Belleau *et al.*, 2007; Böttcher *et al.*, 2010*b*). Following the treatments the progress of berry development was monitored at regular intervals through to harvest (Fig. 1A–C). Veraison of the Control fruit was found to be 27 dpis, when Brix (Fig. 1A) and anthocyanin (Fig. 1B) levels as well as berry weight (Fig. 1C) increased rapidly. IAA-treated berries followed a very similar trend with no significant difference in berry weight and only minor differences in Brix (27 dpis) and anthocyanin (17 and 27 dpis) levels when compared with the Control (Table 1). The NAA and BTOA treatments led to a delayed accumulation of TSS from 27 dpis through to the harvest of Control fruit at 62 dpis (23.1° Brix) with BTOA-treated fruit having significantly lower Brix levels than NAA-treated berries throughout this period (Fig. 1A; Table 1). Berries sprayed with NAA reached a similar Brix level (23.8°) to the Control 13 d after harvest of the Control fruit, BTOA-treated fruit were collected another 6 d later and by that time had a slightly higher Brix level of 24.6°.

Anthocyanin accumulation was delayed in both NAA- and BTOA-treated fruit (Fig. 1B) with both treatments

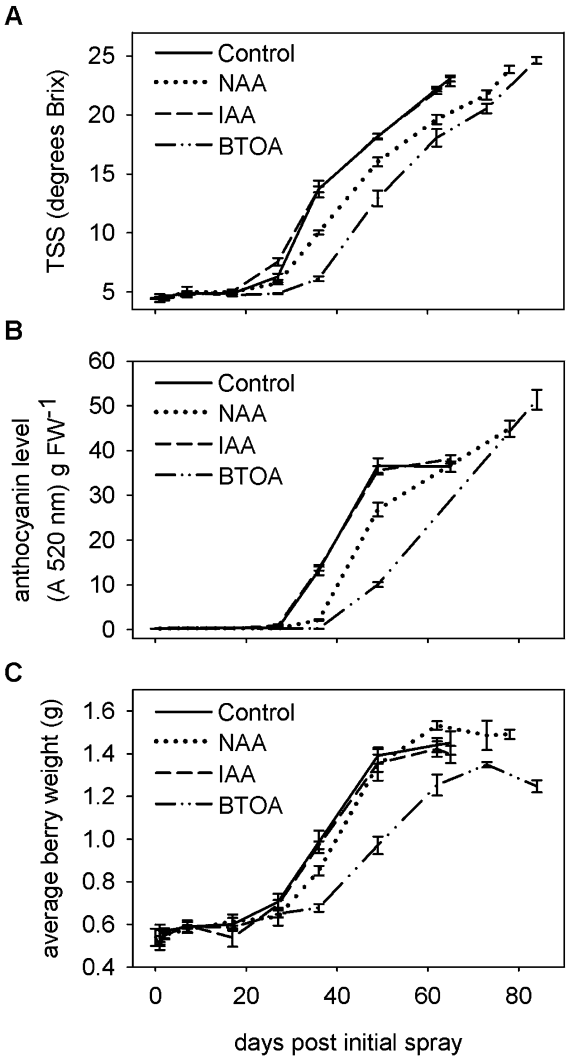

Fig. 1. Delayed ripening of Shiraz berries after treatment with different auxins. (A) Changes in TSS, measured as degrees Brix, in field-grown Shiraz berries treated twice (24 d and 9 d preveraison) with 80 mg l^{-1} IAA, 50 mg l^{-1} NAA, 20 mg l^{-1} BTOA in 0.1% (v/v) Chemwet 1000 or 0.1% (v/v) Chemwet 1000 (Control), sampled at 1, 2, 7, 17, 27, 36, 49, 62, 65, 73, 78, and 84 dpis. (B) The same berry samples were used to measure anthocyanin levels (absorbance at 520 nm) and (C) changes in berry weight. All data represent means ±STERR (n=3).

leading to anthocyanin levels significantly lower than those of the Control fruit between 17–49 dpis (Table 1). This was mainly due to a particularly slow rate of accumulation between 27–36 dpis, when a rapid increase in anthocyanin levels was observed in Control and IAA-treated berries. Compared with all other treatments BTOA-treated berries had the lowest anthocyanin levels at 36 and 49 dpis. No sample of NAA- and BTOA-treated berries was taken at harvest of the Control fruit (62 dpis), but when harvested 13 d (NAA) and 19 d (BTOA) later the anthocyanin concentration measured in auxin-treated berries was 1.2-fold (NAA) and 1.4-fold (BTOA) higher than that of Control berries. Since sugar and anthocyanin accumulation seem to be linked in developing grapes (Böttcher *et al.*, 2010*b*) this moderate increase in anthocyanin levels might be related to the slightly higher Brix levels of the auxin-treated fruit at harvest when compared with the Control berries (Fig. 1A).

Table 1. Statistical analysis of the data presented in Fig. 1A–C
One-way ANOVA was used to compare the means followed by Duncan's *post hoc* test. In each column (Brix, anthocyanin, weight) data denoted by a different letter differ significantly (P <0.05). C, Control; I, IAA; N, NAA; B, BTOA.

dpis	Brix				Anthocyanin				Weight			
	C	I	N	B	C	I	N	B	C	I	N	B
1	a	a	a	a	a	a	a	a	a	a	a	a
2	a	a	a	a	a	a	a	a	a	a	a	a
7	a	a	a	a	b	b	b	a	a	a	a	a
17	a	a	a	a	a	b	b	ab	a	a	a	a
27	b	a	b	c	b	a	b	b	a	a	a	a
36	a	a	b	c	a	a	b	c	a	a	b	c
49	a	a	b	c	a	a	b	c	a	a	a	b
62	a	a	b	c					a	a	a	b

Interestingly, changes in berry weight (Fig. 1C) throughout development qualitatively differed between fruit sprayed with NAA or BTOA. Although statistically different from the Control at only one time point (36 dpis; Table 1), the growth of NAA-treated Shiraz berries followed a similar trend to that previously described for NAA-treated Shiraz berries (Böttcher *et al.*, 2010*b*). The rate of berry growth was delayed during the first 30–40 dpis, but then accelerated beyond the rate of Control fruit growth, leading to bigger berries at harvest (Fig. 1C) probably due to an increase in cell size (Böttcher *et al.*, 2010*b*). BTOA-treated berries were smaller than berries from any other treatment from 36 dpis through to harvest (Fig. 1C; Table 1), which is in accordance with previous studies examining the effect of BTOA on Shiraz berry development (Hale, 1968; Davies *et al.*, 1997). Although the smaller size of the BTOA-treated fruit was, in part, due to a longer delay in berry expansion post-veraison, it was also a result of a lower rate of berry size increase (Fig. 1C).

From the presented data it can be seen that the effect on grape berry ripening was different for each of the three auxinic compounds used in the experiment. Treatments with IAA had no effect on growth or colour accumulation of developing berries and in disagreement with previously published data (Deytieux-Belleau *et al.*, 2007) there was also no delay in sugar accumulation. On the other hand both NAA and BTOA delayed sugar and anthocyanin accumulation with BTOA being more effective, but were clearly distinguished in their effect on berry growth. It has been reported previously that the efficiency in the delay in fruit ripening depends on the compound used, for example, 2,4-dichlorophenoxyacetic acid (2,4-D) was a better inhibitor of the ripening of banana slices than IAA (Vendrell, 1969) and BTOA caused a greater delay in the ripening of Doradillo grapes than the auxin efflux inhibitor 2,3,5-triiodobenzoic acid (TIBA) (Coombe and Hale, 1973). As for the opposite effects of NAA and BTOA on berry growth it has been reported in several previous studies that,

depending on the physiological response assayed, BTOA does not always act as would be expected from a classical auxin. When Crane (1952) treated parthenocarpic fig (*Ficus carica* L.) achenes with BTOA they developed a completely sclerified endocarp, indistinguishable from that of pollinated fruit. This effect was not found with any other auxin tested. In another example Steward and Shantz (1956) applied BTOA to carrot root explants and artichoke tuber explants which induced growth by cell division and not, as caused by IAA treatment, by cell enlargement. It is possible that the differential effect of BTOA on berry expansion may be due to it having very strong auxin-like properties in grape but it is also possible that BTOA is acting in a pleiotropic manner.

Grape GH3 genes involved in the response to exogenous auxins

There are three main families of early auxin response genes (Abel and Theologis, 1996), which are the Small Auxin Up RNA (*SAUR*) genes, the Auxin/IAA-inducible (*AUX/IAA*) genes, and the Gretchen Hagen (*GH3*) genes (Chapman and Estelle, 2009) and the expression of these genes can be used to elucidate the molecular mechanisms involved in the different responses to exogenous auxins. In the scope of this study the IAA-amido synthetase encoding *GH3* genes were of particular interest due to the known selectivity of these enzymes for their auxin substrates (Staswick *et al.*, 2005) and the implication of one grapevine GH3 protein (GH3-1) in the ripening process of berries (Böttcher *et al.*, 2010*a*). Interestingly, the expression of *GH3-1* was induced in preveraison berries treated with IAA, NAA, and BTOA (Böttcher *et al.*, 2010*a*). This raises the question if the differences in ripening delay caused by exogenous application of IAA, NAA, and BTOA (Fig. 1) might be related to the activity of GH3 proteins.

In order to identify all putative IAA-conjugating grapevine GH3 proteins that are responsive to auxins, a BLASTP similarity search on the NCBI database was performed using the grapevine GH3-1 protein as the query. From this search, eight additional non-redundant grapevine GH3-like proteins, consisting of 578–613 amino acids, were predicted and these were designated GH3-2 to GH3-9 (Table 2).

To examine the relationship between the grapevine GH3 proteins and those from other plant species, an unrooted phylogenetic tree was constructed from the alignment of 67 full-length GH3 protein sequences (Fig. 2). GH3 proteins can be divided into three groups (I–III) according to their sequence similarity and their function (Staswick *et al.*, 2002, 2005). Group I contains *Arabidopsis* GH3-11 (JAR1) which specifically adenylates jasmonic acid (Staswick *et al.*, 2002). Some members of the numerous group II proteins (Fig. 2), including Vv GH3-1, have been shown to be IAA-amido synthetases conjugating mainly IAA to a variety of amino acids (Staswick *et al.*, 2005; Ding *et al.*, 2008; Böttcher *et al.*, 2010*a*). Group III proteins seem to be restricted to the genera of *Arabidopsis*, *Brassica*, and *Gossypium* (Terol *et al.*, 2006). The first characterization of a member of this group (At GH-12) recently showed that, by conjugation of 4-substituted benzoates, this protein might be involved in the regulation of chorismate-derived pathways (Okrent *et al.*, 2009). Two grapevine proteins (Vv GH3-7, Vv GH3-9) were present in group I, Vv GH3-1–Vv GH3-6 belonged to group II, and Vv GH3-8 clustered outside of the groups together with a *Populus* sequence (Pt GH3-14) and a rice protein (Os GH3-6). This rice sequence has already been described as a GH3-related sequence with no clear phylogenetic position (Terol *et al.*, 2006).

Table 2. GH3 family in grapevine

Protein	Accession number[a]	ORF length (bp)[b]	Protein length (aa)[c]	Genomic *locus*[d]
GH3-1	XP_002271252	1797	598	AM440162
GH3-2	XP_002283886	1803	600	AM479359
GH3-3	CAN83696	1842	613	AM474945
GH3-4	XP_002263353	1791	596	AM449684
GH3-5	XP_002276241	1767	588	AM471236
GH3-6	XP_002268278	1782	593	AM431746
GH3-7	XP_002272560	1779	592	AM466064
GH3-8	XP_002271002	1737	578	AM461067
GH3-9	CAN69491	1740	579	AM439522

[a] NCBI or Genbank accession number of predicted open-reading frames.
[b] Length of predicted open-reading frame in base pairs.
[c] Length of the predicted polypeptide in amino acids.
[d] Accession number of genomic clone in which the sequence is present.

Grapevine GH3 proteins shared 31–86% identity and 51–92% similarity with the highest per cent sequence similarity found between Vv GH3-1 and Vv GH3-2. The overall identity and similarity between the grapevine sequences and other plant GH3 proteins ranged from 29–88% and 48–93%, respectively.

With GH3-1 already a potential candidate as a deactivator of exogenous auxins in grapes it was tested whether the corresponding genes of any of the five remaining group II grapevine GH3 proteins were expressed in berries (Fig. 3A) and induced by auxin treatments (Fig. 3B). All five *GH3* genes displayed high transcript accumulation in Cabernet Sauvignon flowers and young berries, peaking at flowering (*GH3-5*), 2 wpf (*GH3-6*), 4 wpf (*GH3-3*, *GH3-4*) or 6 wpf (*GH3-2*) (Fig. 3A). During this period of berry development auxin levels are high (Inaba *et al.*, 1976; Cawthon and Morris, 1982; Zhang *et al.*, 2003; Deytieux-Belleau *et al.*, 2007; Böttcher *et al.*, 2010*a*), therefore the activity of these GH3 proteins in the early stages of berry development might contribute to the control of auxin homoeostasis. In contrast to *GH3-1,* which has a second peak of expression at veraison (Böttcher *et al.*, 2010*a*), transcript levels of all the other five group II *GH3* genes declined to be low at veraison and remained low for the rest of berry development.

When Shiraz berries at 22 d and 12 d prior to veraison were exposed to 0.5 μM IAA, NAA, or BTOA for 24 h in an *ex planta* induction experiment, *GH3-2* was the only gene

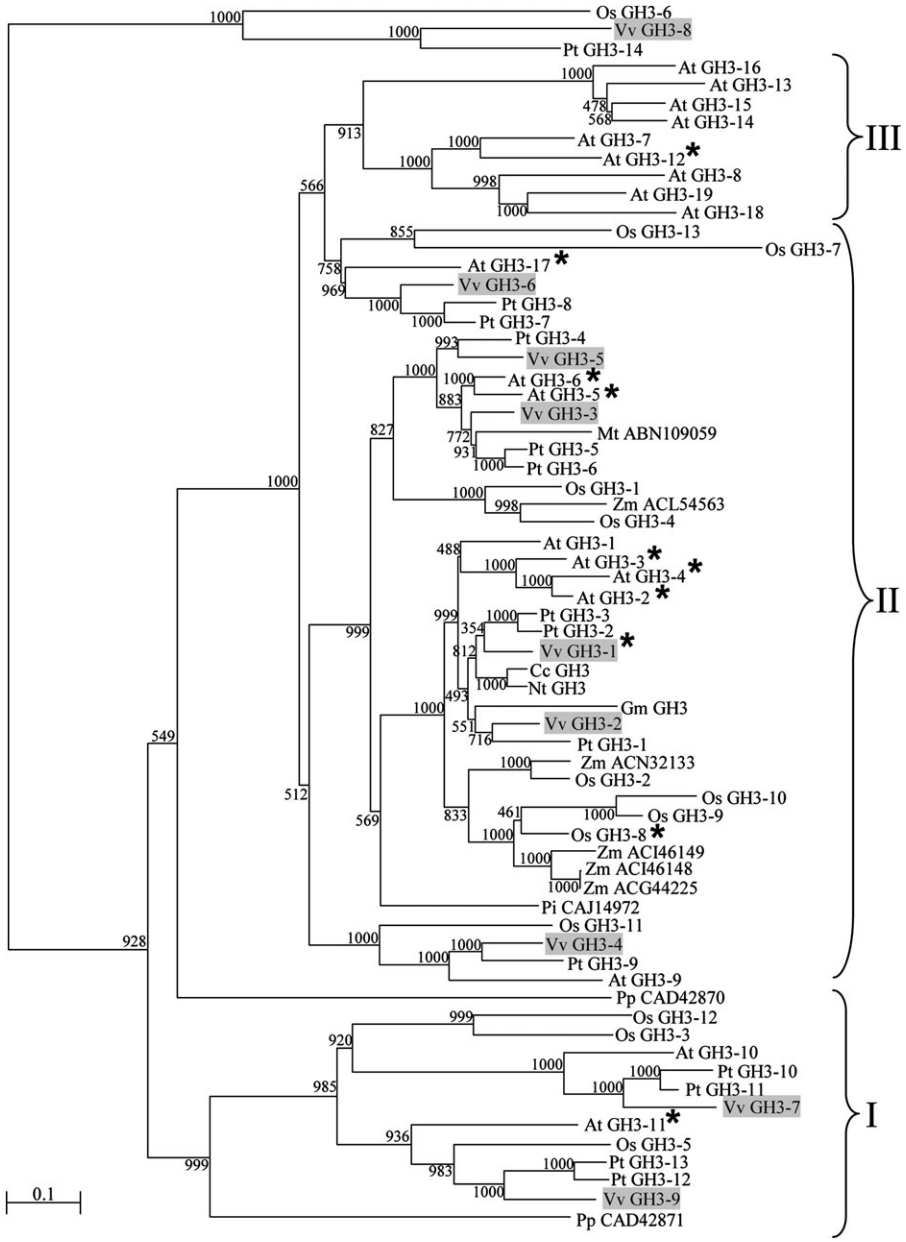

Fig. 2. Phylogenetic relationship of GH3 protein sequences from grapevine and other plants. The unrooted tree was generated with the PHYLIP program (Felsenstein, 1989) using the Neighbor–Joining method and a bootstrap test with 1000 iterations (bootstrap values are indicated at each node). The predicted grapevine proteins are highlighted with a shaded background. Asterisks indicate functionally characterized proteins and the scale bar indicates genetic distance based on branch length. I–III, Functional groups of GH3 proteins described for *Arabidopsis* (Staswick *et al.*, 2002, 2005); At, *Arabidopsis thaliana*; Cc, *Capsicum chinense*; Gm, *Glycine max*; Mt, *Medicago truncatula*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pi, *Pinus pinaster*; Pp, *Physcomitrella patens*; Pt, *Populus trichocarpa*; Vv, *Vitis vinifera*; Zm, *Zea mays*. Accession numbers of the protein sequences used in this analysis are provided in Supplementary Table S1 at *JXB* online.

out of the five tested that showed a significant induction of expression in response to all three auxin treatments (Fig. 3B). At both stages of berry development tested, NAA and BTOA were stronger inducers than IAA, the maximum being a 20-fold induction of *GH3-2* transcript levels 12 d preveraison by NAA. A similar but less pronounced response to the three auxins has also been reported for the closely related *GH3-1* (Böttcher *et al.*, 2010*a*).

The treatments with IAA and NAA also led to a significant but marginal increase of *GH3-3* expression in berries 22 d preveraison, whereas there was a decrease in transcript levels of this gene in response to all three auxins 12 d preveraison (Fig. 3B). Of the three other *GH3* genes tested, two (*GH3-5*, *GH3-6*) were not induced by auxin treatments and the expression of *GH3-6* was repressed in berries 12 d preveraison (Fig. 3B). The transcript accumulation of

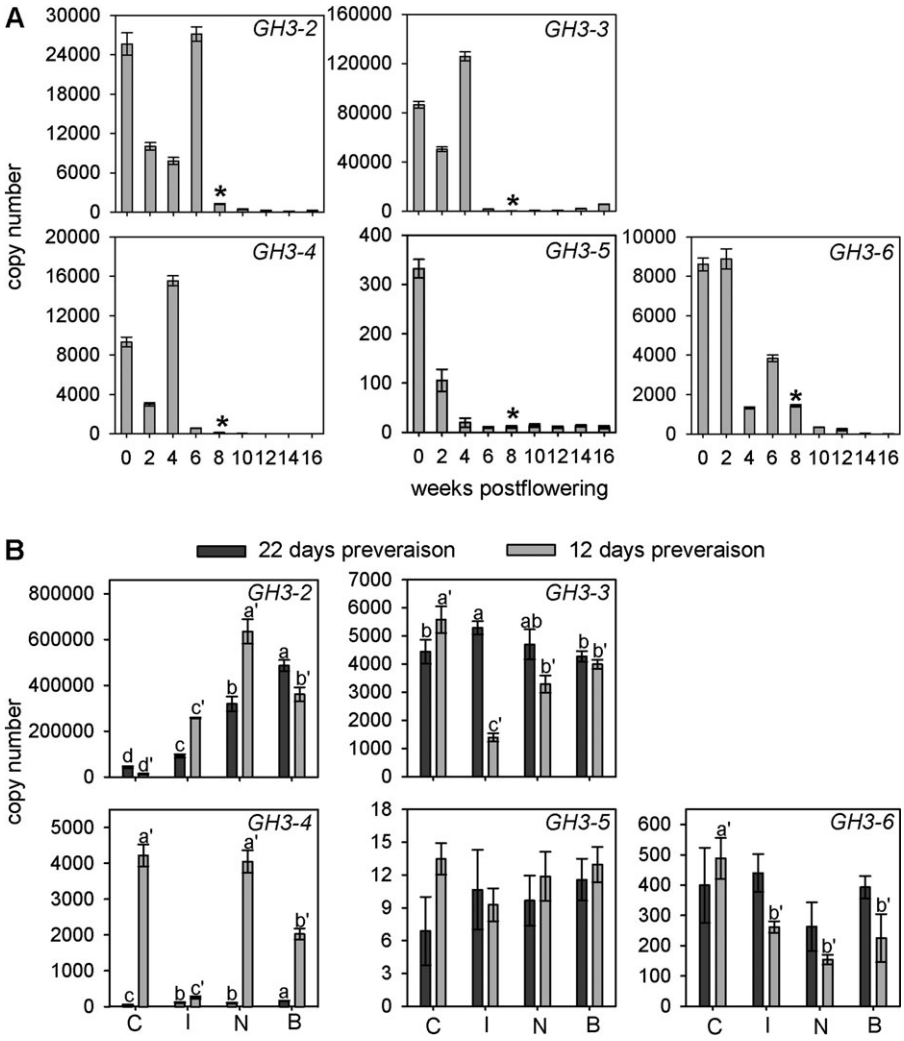

Fig. 3. Changes in mRNA levels of five group II *GH3* genes during grape berry development and in response to different auxins. (A) The expression of *GH3-2*–*GH3-6* in flower and berry tissue of field grown Cabernet Sauvignon plants was analysed by qRT-PCR at the indicated time points (0=anthesis). The asterisks indicate veraison and all data represent means ±STERR of n=3. (B) Expression patterns of *GH3-2*–*GH3-6* upon treatment with 0.5 μM of three auxins or auxin-like compounds in *ex planta* berry tissue analysed by qRT-PCR. For each treatment 20 berries (22 d and 12 d preveraison) were placed on 0.8% agar plates containing the indicated auxin concentrations and the plates were kept in the dark at room temperature for 24 h. Bars represent means ±STERR (n=3) and are denoted by a different letter if the means differ significantly (P <0.05) using one-way ANOVA followed by Duncan's *post hoc* test (a–d, 22 d preveraison; a'–d', 12 d preveraison).

GH3-4 was up-regulated 2–3-fold by IAA, NAA, and BTOA 22 d prior to veraison, when transcript levels were very low. In berries 12 d preveraison, the expression of *GH3-4* did not change in response to NAA and was significantly repressed by treatments with IAA and BTOA.

GH3-2 is an IAA-amido synthetase with the same amino acid preferences as GH3-1

Since *GH3-1* and *GH3-2* were the only group II genes displaying a clear induction in expression in response to the application of IAA, NAA, and BTOA, all further experiments focused on those two genes and the corresponding proteins. Like GH3-1 (Böttcher *et al.*, 2010*a*), the predicted coding sequence of GH3-2 contains all three sequence motifs involved in ATP/AMP binding (^{108}**SSG**TSAGER**K**^{117} (Motif I), ^{337}**YASSE**^{341} (Motif II), ^{413}YRV**GD**^{417} (Motif III)) that are characteristic of the acyl-adenylate/thioester-forming enzyme superfamily (Chang *et al.*, 1997). To get initial information about the *in vitro* function of GH3-2, the purified recombinant protein (Fig. 4A) was tested for its ability to conjugate IAA to 20 amino acids. The reaction mixtures were analysed by thin layer chromatography (TLC) (Fig. 4B) and products with a lower mobility than IAA were only observed in reactions containing Asp and Trp. These putative IAA conjugates had the same R_F as the corresponding standards (Fig. 4C) and were confirmed to be IAA–Asp and IAA–Trp using liquid chromatography-mass spectrometry (LC-MS) (data not shown). Due to the adenylation activity of GH3-2, the formation of the conjugates was

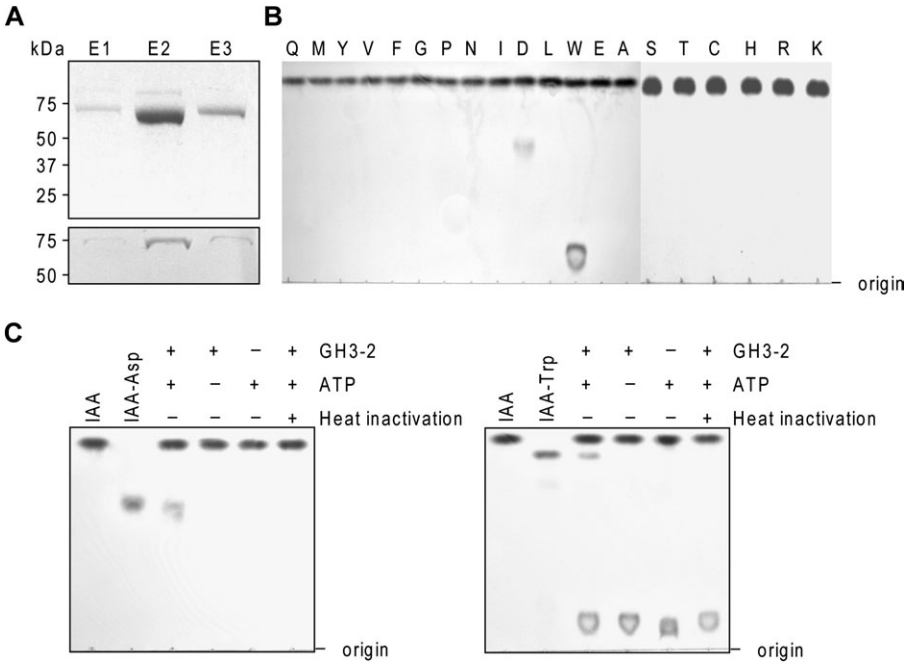

Fig. 4. *In vitro* activity of recombinant GH3-2. (A) The expression of recombinant GH3-2 protein was tested by separating 10 µl of His GraviTrap column elution fractions (E1-E3) on a 4–12% polyacrylamide gel followed by Coomassie Brilliant Blue staining (upper panel) or immunodetection using a monoclonal antibody raised against poly-histidine (lower panel). The band with the size of approximately 70 kDa corresponds to the His-tagged GH3-2 protein. (B) TLC analysis of GH3-2 enzyme reactions with 20 amino acids (single letter code). The spot near the origin for the reactions with Trp represents the unbound amino acid. Plates were stained with Ehmann's reagent to detect indole compounds. (C) TLC analysis of GH3-2-catalysed IAA–Asp and IAA–Trp formation with indicated variations of the reaction mixture.

dependent on ATP. An incubation at 70 °C resulted in a complete loss of activity of the enzyme (Fig. 4C).

The high degree of similarity between the protein sequences of GH3-2 and GH3-1 seems to translate into identical substrate specificities since IAA–Asp and IAA–Trp have also been reported as the sole reaction products of GH3-1 (Böttcher *et al.*, 2010*a*). To date a similarly strict requirement for just one or two amino acids has only been demonstrated for the *Arabidopsis* group III protein GH3-12, which, in conjunction with 4-aminobenzoate as the acyl substrate, only conjugated glutamic acid (Okrent *et al.*, 2009).

Recombinant GH3-1 and GH3-2 are highly selective for their amino acid and acyl substrates

To analyse the specificities of GH3-1 and GH3-2 for their amino acid and acyl substrates, kinetic parameters for both enzymes were determined by quantifying the accumulation of conjugates using LC-MS/MS. Suitable protein amounts and reaction times for the formation of IAA–Asp and IAA–Trp were identified by adding increasing amounts of protein (0–1 µg) to standard reaction mixtures and stopping the reactions at various times (0–20 min). For both enzymes and substrates it was found that between 0.25–0.75 µg protein and for up to 15 min the reactions were linear and proportional to the amount of added protein (data not shown). For the determination of initial velocities under varied experimental conditions, all reactions were initiated by the addition of 0.5 µg of either GH3-1 or GH3-2 and stopped immediately after the addition of enzyme (0 min) as well as after 2, 5, and 10 min. The initial velocity data (nmol min^{-1} mg^{-1} protein) obtained for each substrate concentration series was fit to the Michaelis–Menten equation, exemplified by the formation of IAA–Asp with varying IAA concentrations (Fig. 5). Table 3 shows the kinetic parameters obtained in that way for the conjugation of IAA with Asp (IAA, ATP, and Asp varied) and with Trp (Trp varied). GH3-1 and GH3-2 showed very similar trends in their substrate affinities (K_m) and turnover rates (k_{cat}), having higher affinities for IAA than for ATP or the amino acids. With K_m^{IAA}10.4 µM (GH3-1), K_m^{IAA}28.4 µM (GH3-2) and corresponding turnover rates of 14.3 min^{-1} (GH3-1) and 32.6 min^{-1} (GH3-2) the grapevine enzymes had 4–30-fold higher affinities and catalytic efficiencies (k_{cat}/K_m) than have been reported for acyl substrates of rice (Chen *et al.*, 2009, 2010) and *Arabidopsis* (Okrent *et al.*, 2009) GH3 proteins. The grapevine GH3s conjugated Asp 100-fold (GH3-1) and 80-fold (GH3-2) more efficiently than Trp, making Asp the preferred amino acid substrate. In addition the physiological concentrations of Trp in grapes are about 100-fold lower than the K_m^{Trp} values determined for GH3-1 and GH3-2, whereas Asp concentrations are about 10-fold higher than the respective K_m^{Asp} values (Table 3; C Davies, MR. Thomas, P Corena, personal communication). This is in agreement

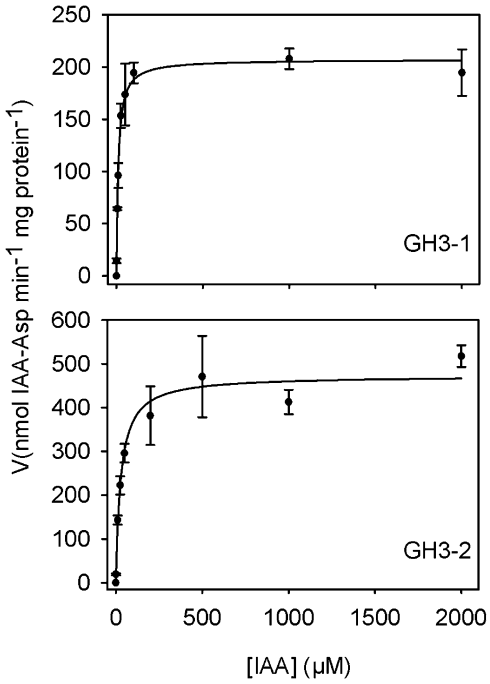

Fig. 5. Example of Michaelis–Menten kinetics obtained for the formation of IAA–Asp by GH3-1 and GH3-2. Enzyme assays were performed using standard conditions (0.5 μg recombinant GH3-1 or GH3-2 per sample) with IAA as the varying substrate. Reactions were stopped and extracted after 0, 2, 5, and 10 min and IAA–Asp formation was quantified by LC-MS. Initial velocities were plotted versus the various concentrations of IAA and the data was fitted to the Michaelis–Menten equation using non-linear regression (SigmaPlot 11.0). All data represent means ±STERR of n=3.

with the previous finding that IAA-Asp accumulated in considerable amounts in grapes whereas IAA–Trp could not be detected in berries of various developmental stages (Böttcher *et al.*, 2010*a*).

For GH3-1 and GH3-2 a change of the acyl substrate from IAA to NAA or BTOA with Asp as the amino acid substrate led to dramatic reductions in affinities, turnover rates, and resulting efficiencies (Table 4). With NAA as the auxin, substrate efficiencies were 50-fold (GH3-1) and 21-fold (GH3-2) lower when compared with IAA (Table 3) and the formation of the BTOA–Asp conjugate was either not detected (GH3-1) or was 47 000-fold less efficient than the formation of IAA–Asp (GH3-2).

It has been suggested before that the naphthalene ring of NAA might not be a perfect steric fit for the active site of GH3 proteins (Chen *et al.*, 2010) which possibly resulted in the decrease in catalytic efficiencies observed for GH3-1 and GH3-2. BTOA, with a sulphur atom in the indole ring and an oxyacetic acid side chain is structurally even less closely related to IAA than NAA which could explain why it was such a poor substrate for the grapevine GH3 proteins. Assuming that NAA and BTOA applied to preveraison berries cannot be metabolized by alternative pathways their inefficient conjugation by GH3-1 and GH3-2 (Table 4) could lead to the berries being exposed to high levels of free

Table 3. Steady-state kinetic parameters for GH3-1 and GH3-2

Enzyme	Substrate	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
GH3-1	IAA	10.4 (1.2)	207.3 (4.8)	14.3	22848.2
	ATP	42.7 (8.3)	193.4 (4.6)	13.3	5200.4
	Asp	73.4 (10.8)	184.2 (4.4)	12.7	2884.6
	Trp	5044.1 (1243.7)	119.4 (10.0)	8.2	27.2
GH3-2	IAA	28.4 (4.7)	473.1 (15.7)	32.6	19147.6
	ATP	72.0 (19.7)	414.8 (24.9)	28.6	6622.0
	Asp	134.3 (20.7)	481.1 (13.4)	33.2	4114.5
	Trp	9125.5 (1075.3)	404.3 (18.6)	27.9	50.9

Table 4. Kinetic analysis of GH3-1 and GH3-2 with synthetic auxinic compounds

Enzyme	Substrate	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
GH3-1	NAA	190.3 (26.2)	75.2 (3.1)	5.2	454.5
	BTOA	n/a	n/a	n/a	n/a
GH3-2	NAA	554.3 (92.2)	435.9 (27.4)	30.1	903.9
	BTOA	565.2 (160)	0.2 (0.02)	0.01	0.41

auxinic compounds for extended periods which, in turn, could be the reason for the observed delay in ripening (Fig. 1). The fact that BTOA is less acceptable as a GH3 substrate than NAA, which itself is a worse substrate than IAA, matches the degree of berry ripening delay reported in this study (Fig. 1; Table 1). Although the presented data suggest that NAA and BTOA are poor substrates for the analysed grapevine GH3 proteins, it has to be considered that changing the acyl substrate might influence the affinity for the amino acid substrate as has recently been demonstrated for the group III *Arabidopsis* GH3-12 (Okrent *et al.*, 2009).

In IAA-treated berries changes in IAA and IAA–Asp levels are associated with the expression of GH3-2

If a delay in berry ripening after treatment with NAA and BTOA was caused by a lack of GH3-catalysed conjugation of these compounds, the opposite would have to be true for exogenous IAA which had no effect on the timing of ripening. It was therefore determined if the application of IAA to preveraison berries (Fig. 1) led to the formation of IAA–Asp. One day after berries were sprayed with IAA a 7.5-fold increase in IAA levels was measured (Fig. 6A) which was matched by a 200-fold increase in IAA–Asp levels (Fig. 6B). The discrepancy between the elevation of IAA and IAA–Asp levels is probably due to the fact that conjugation and therefore accumulation of the IAA–Asp conjugate started in the 24 h period before the first sampling. The concentration of IAA was back to Control levels 2 d after the application,

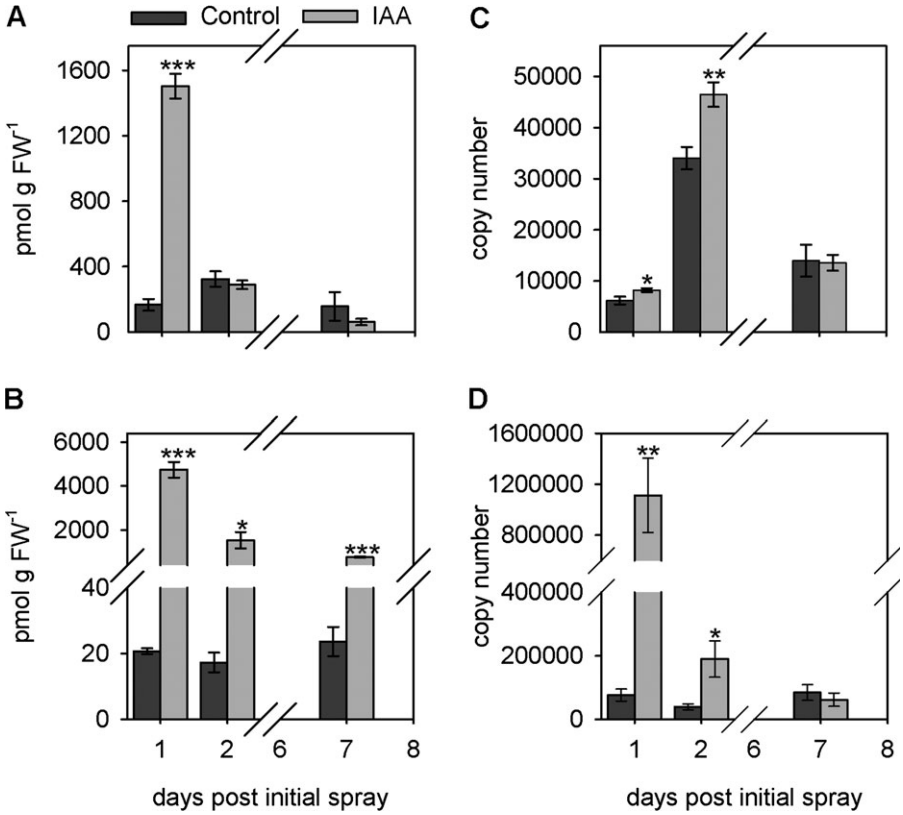

Fig. 6. Changes in levels of IAA and IAA–Asp and expression of *GH3-1* and *GH3-2* in preveraison berries in response to IAA application. (A) IAA and (B) the IAA-amino acid conjugate IAA–Asp were quantified by LC-MS/MS in tissue of preveraison Shiraz berries 1, 2, and 7 d after treatment with a Control solution (0.1% (v/v) Chemwet 1000) or 80 mg l^{-1} IAA in 0.1% (v/v) Chemwet 1000. FW, fresh weight. (C) The expression of *GH3-1* and (D) *GH3-2* in the same berry samples was analysed by qRT-PCR. All data represent means ±STERR (n=3). Asterisks indicate significant differences of the mean values of IAA-treated samples from the mean values of Control samples as determined with Student's *t* test (*P <0.05, **P <0.01, ***P <0.001).

whereas IAA-Asp levels decreased more slowly and were still elevated 1 week after the treatment. The reduction of the IAA–Asp levels is probably due to oxidation of the bound IAA (Östin *et al.*, 1998; Staswick *et al.*, 2005), a process that links this conjugate to the irreversible inactivation of IAA.

In planta activities of GH3 enzymes in Arabidopsis (Staswick and Tiryaki, 2004; Staswick *et al.*, 2005; Okrent *et al.*, 2009) and rice (Ding *et al.*, 2008) appear consistent with *in vitro* findings and therefore the increased levels of IAA–Asp in IAA-treated berries (Fig. 6B) could be an indication of GH3-1/GH3-2 activity. To investigate this possibility further, the gene expression of *GH3-1* (Fig. 6C) and *GH3-2* (Fig. 6D) was analysed in the same berry tissues. Transcript levels of *GH3-1* were similar in Control fruit and IAA-treated berries with *GH3-1* expression transiently increasing 3.5–4-fold 2 d after the applications (Fig. 6C). This indicates that the treatment itself induced *GH3-1* expression, possibly as a response to wounding. By contrast, transcript levels of *GH3-2* were increased 12-fold 1 d after the IAA treatment followed by a decrease back to Control levels 1 week after the auxin application. From these result it can be deduced that GH3-2 might be linked with the accumulation of IAA–Asp and therefore the decrease of free IAA levels in IAA-treated preveraison berries. An involvement of GH3-1 in the conjugation of applied IAA was not supported by the presented data, but it cannot be excluded that GH3-1 might have participated in the IAA–Asp formation in the time period between treatment and the first sampling after 24 h. It also has to be considered that, depending on the basal levels of GH3 proteins available, the transcriptional induction of *GH3* genes might not be requisite for the initial conjugation of IAA.

In conclusion, acyl substrate preferences of an auxin-inducible IAA-amido synthetase expressed in preveraison berries might be responsible for the variations in ripening delay caused by the application of different auxinic compounds.

Studies on a variety of plants have previously shown that the effect of auxinic compounds is highly dependent on the plant species, the plant organ, the compound and its concentration, as well as the physiological process assayed (Ferro *et al.*, 2006). It will be interesting to investigate whether these differences in auxin response can be correlated with expression patterns and specificities of GH3 proteins.

The ubiquitious distribution of *GH3* genes in the plant kingdom, as well as numerous studies on their function in *Arabidopsis*, rice, and the moss *Physcomitrella patens* have established GH3-catalysed auxin conjugation as an

important factor in plant growth, organ development, and response to light and stress (Woodward and Bartel, 2005; Ludwig-Müller, 2011). The findings presented in this paper further support the addition of fruit ripening (Liu *et al.*, 2005; Böttcher *et al.*, 2010*a*) to the list of GH3-regulated processes.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. NCBI, GenBank or Swiss-Prot accession numbers of the protein sequences used for the phylogenetic analysis.

Acknowledgements

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