Sequestration of auxin by the indole-3-acetic acid-amido synthetase GH3-1 in grape berry (Vitis vinifera L.) and the proposed role of auxin conjugation during ripening

Christine Böttcher*, Robert A. Keyzers, Paul K. Boss and Christopher Davies
CSIRO Plant Industry, PO Box 350, Glen Osmond, SA 5064, Australia
* To whom correspondence should be addressed: E-mail: christine.bottcher@csiro.au
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Abstract
In fleshy fruit, levels of indole-3-acetic acid (IAA), the most abundant auxin, decline towards the onset of ripening. The application of auxins to immature fruit can delay the ripening processes. However, the mechanisms by which the decrease in endogenous IAA concentrations and the maintenance of low auxin levels in maturing fruit are achieved remain elusive. The transcript of a GH3 gene (GH3-1), encoding for an IAA-amido synthetase which conjugates IAA to amino acids, was detected in grape berries (Vitis vinifera L.). GH3-1 expression increased at the onset of ripening (veraison), suggesting that it might be involved in the establishment and maintenance of low IAA concentrations in ripening berries. Furthermore, this grapevine GH3 gene, responded positively to the combined application of abscisic acid and sucrose and to ethylene, linking it to the control of ripening processes. Levels of IAA-aspartic acid (IAA-Asp), an in vitro product of recombinant GH3-1, rose after veraison and remained high during the following weeks of the ripening phase when levels of free IAA were low. A similar pattern of changes in free IAA and IAA-Asp levels was detected in developing tomatoes (Solanum lycopersicum Mill.), where low concentrations of IAA and an increase in IAA-Asp concentrations coincided with the onset of ripening in this climacteric fruit. Since IAA-Asp might be involved in IAA degradation, the GH3 catalysed formation of this conjugate at, and after, the onset of ripening could represent a common IAA inactivation mechanism in climacteric and non-climacteric fruit which enables ripening.

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

Introduction
Auxins make up a small group of plant hormones that are involved in the regulation of numerous aspects of plant growth and development. By far the most prominent and best studied auxin is indole-3-acetic acid (IAA), which has been linked to such diverse processes as elongation and division of cells, tropic responses, vascular development, response to biotic and abiotic stimuli, and the general organization of root and shoot architecture (Davies, 2004).

Much of what is known about IAA-regulated processes, including molecular mechanisms of action, perception, transport, and biosynthesis comes from studies of the model plant Arabidopsis (Arabidopsis thaliana L.) (Woodward and Bartel, 2005; Delker et al., 2008). As a consequence, there remains a lack of knowledge about the potential role of IAA in the development of fleshy fruit. Much of the research in this area has focused on tomato (Solanum lycopersicum Mill.), a climacteric fruit whose ripening is characterized by a sharp increase in ethylene levels and respiratory activity. The importance of auxins for fruit set and subsequent growth is well established in tomato and correlates well with IAA-levels peaking 8–14 d after flowering, which marks the end of cell division and the start of the cell expansion stage (Iwahory, 1967). Thereafter, auxin concentrations decline to low levels at the onset of ripening (Mapelli et al., 1978; Buta and Spaulding, 1994).

In grape berries (Vitis vinifera L.), developmental changes in auxin levels are still a matter of debate. Some studies suggest that IAA concentrations are high in the early
developmental stages of this non-climacteric fruit and then decline to remain low throughout the rest of berry development (Inaba et al., 1976; Cawthon and Morris, 1982; Zhang et al., 2003). However, IAA concentrations have also been claimed to be low and relatively constant throughout berry development (Symons et al., 2006). Common to all these reports is that, like in tomato, IAA levels are low at the onset of ripening, termed veraison by viticulturists and here defined as the last time point before the start of sugar accumulation.

The ripening-delaying effects of applied auxins have been described in a range of climacteric and non-climacteric fruit (Vendrell, 1969; Frenkel and Dyck, 1973; Tingwa and Young, 1975; Given et al., 1988, Cohen, 1996; Davies et al., 1997). Numerous studies describe the effects for a range of grapevine cultivars in response to the application of natural or synthetic auxins to berries before veraison (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). In addition to the delay in the change of ripening-associated parameters, such as the accumulation of sugars and anthocyanins and a decrease in acidity and chlorophyll concentration, auxin treatments of berries also retard the usual ripening-associated increase in the levels of abscisic acid (ABA) (Davies et al., 1997). ABA is regarded as a positive regulator of grape berry ripening (Coome and Hale, 1973; Davies et al., 1997; Wheeler et al., 2009). The response of preveraison berries to auxin exposure is also reflected in changed gene expression patterns, for example, the suppression of anthocyanin biosynthesis genes (Davies et al., 1997; Ban et al., 2003).

Reduced levels of IAA in fruit entering the ripening stage could be achieved and maintained by changes in certain aspects of auxin metabolism. Examples of obvious targets are the various IAA-biosynthesis pathways or the numerous auxin inactivation mechanisms reported to occur in plants (Woodward and Bartel, 2005; Delker et al., 2008; Zhao, 2010). Of these, the conjugation of IAA to amino acids is of particular interest because it is catalysed by auxin-inducible GH3 proteins, providing a negative feedback loop to control auxin homeostasis (Staswick et al., 2005). Furthermore, various IAA-amino acid conjugates have been identified as endogenous compounds in a range of different plant species (Andersson and Sandberg, 1982; Cohen, 1982; Sitbon et al., 1993; Kowalczyk and Sandberg, 2001; Rampey et al., 2004; Matsuda et al., 2005; Ludwig-Müller et al., 2008; Staswick, 2009; Pencík et al., 2009). The first GH3 gene was isolated from soybean [Glycine max (L.) Merr.] by differential screening following auxin treatment (Hagen et al., 1984) and, since then, members of this ancient gene family have been found in mosses, gymnosperms, and a range of angiosperms (Terol et al., 2006). However, as they have only been studied in a small number of species, our understanding of the function of GH3 proteins in planta is just emerging. Nevertheless, it is becoming increasingly evident that their involvement in plant development is highly complex. In Arabidopsis GH3-5/1WES, one of eight IAA-amido synthetase genes (Staswick et al., 2005), is not only controlled by auxins, but also by salicylic acid and ABA and seems to be involved in light signal transduction pathways and stress responses (Park et al., 2007; Zhang et al., 2007). The overexpression mutant wesi-D and mutants overexpressing GH3-2/YDK1 (Takase et al., 2004) and GH3-6/DFL1 (Nakazawa et al., 2001), display a dwarf phenotype consistent with decreased levels of free IAA in these plants. Recently, the role in development of one of the rice (Oryza sativa L.) GH3 proteins (GH3-8) that potentially conjugate IAA, has been reported (Ding et al., 2008). Lines overexpressing GH3-8 show decreased IAA levels and increased IAA-Asp levels, enhanced resistance to the rice pathogen Xanthomonas oryzae pv. oryzae and, amongst other morphological abnormalities, retarded growth. The authors suggest that both developmental defects and increased resistance are due to the inhibition of IAA-inducible expansin expression, thereby preventing cell wall loosening and expansion. A dwarf-like phenotype with an increased number of tillers and enlarged leaf angles has also been described for rice plants overexpressing another rice GH3 gene (GH3.13), which has been linked to drought adaptation (Zhang et al., 2009). The activity of a GH3 protein has been associated with fruit ripening in the pungent pepper fruit (Capsicum chinense Jacq.) where it is highly expressed in the pericarp and placenta (Liu et al., 2005). Despite pepper being classified as a non-climacteric fruit (Watkins, 2002), this GH3 gene is ethylene-inducible (Liu et al., 2005). Moreover, tomato fruit overexpressing the pepper GH3 gene ripened earlier upon an ethylene stimulus (Liu et al., 2005).

Here the identification of a putative IAA-amido synthetase gene, GH3-1, is reported in grape berries. This gene displayed a developmental expression pattern and induction behaviour correlated with ripening-associated processes in the berry. The level of IAA-Asp, a product of the in vitro activity of GH3-1, was analysed and provided a possible explanation for the maintenance of low concentrations of free IAA during the ripening of grape berries. The analysis of IAA and IAA-Asp levels in tomato revealed a similar pattern to that found in grapes. This might be seen as further evidence that climacteric and non-climacteric fruit, as suggested by Adams-Phillips et al. (2004), may share some common ripening-associated processes.

Materials and methods

Plant material

For the analysis of developmental changes in gene expression and the quantitation of IAA and IAA conjugates, 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), at fortnightly intervals, 23 November 2004–16 March 2005) were collected from a commercial vineyard (Clare Valley, South Australia) 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 tissue frozen in liquid nitrogen and stored at –80 °C until used.

The localization of GH3-1 expression was carried out with Cabernet Sauvignon vegetative tissue and fruit (8 wpf) from a commercial vineyard in Waikerie, South Australia sampled during the 2007/2008 growing season. Young leaves, tendrils, and...
berries were sampled from 30 vines and immediately frozen in liquid nitrogen. Berries (100) were later separated into skin, flesh, and seed fractions. Roots were harvested from 30 potted Cabernet Sauvignon canes grown in the greenhouse (CSIRO PI, Adelaide, South Australia) and immediately frozen in liquid nitrogen.

For the *ex planta* berry induction experiment grape berries (*Vitis vinifera* L. cv. Shirraz) (Adelaide Hills, South Australia) 22 d (12 January 2009) and 12 d (22 January 2009) preveraison were sampled between 09.00–10.00 h and kept on ice until used.

Tomatoes (*Solanum lycopersicum*) Mill. var. Moneymaker) grown in the greenhouse (CSIRO PI, Adelaide, South Australia) were harvested at five standard ripening stages (Kader and Morris, 1976) [immature green (M1), mature green (M3), breaker to turning (TU), light red (R) and ripe red (RR)] and used to quantify IAA and IAA conjugates. For each stage, 2–4 fruit were deseeded, immediately frozen in liquid nitrogen, and stored at −80 °C.

**RNA extraction, cDNA synthesis, and qRT-PCR**

Total RNA was extracted from grapevine vegetative and reproductive 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, CA) 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: for *GH3-1* (CU459416), 5'-ATCTACGGAGCACAACAGTCC-3' and 5'-GGTGGCCAGTTGAG-3' and, for normalization of cDNA levels, *Actin2* (AM465189), 5'-GACCCCTTCGACGATATGA-3' and 5'-TGAGCAGAAGAAGACTGA-3'. Each PCR was performed in triplicate. To calculate the copy number of the *GH3-1* gene in each reaction, the purified *GH3-1* fragment used for the standard curve was 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**

The following chemicals were used for the induction assay: IAA (Sigma-Aldrich, St Louis, MO), 1-naphthalene acetic acid (NAA) (Gibco BRL Life Technologies, Grand Island, NJ), benzothiazole-2-oxacyclic acid (BTOA) (American Cyanamid Company, Prince-ton, NJ), ABA (AG Scientific, San Diego, CA), and Ethrel (Bayer CropScience, East Hawthorn, Vic).

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) MeOH, 0.2% (v/v) acetic acid (IAA-Asp, *Rt*=7.8 min) or 60% (v/v) MeOH, 0.2% (v/v) acetic acid (IAA-Trp, *Rt*=8.4 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 *GH3-1* was amplified by PCR from a Cabernet Sauvignon berry cDNA template using gene-specific primers (5'-TATCATATGGCGGTGATCCGATTCTCTCCTC-3', 5'-ATA-GGGCCGCCGGCCGCGTGGCGGAGCTC-3') with additional *NdeI* and *NorI* sites in (bold). This product was cloned into the *NdeI* and *NorI* restriction sites of a pET-30b (+) vector (Novagen, San Diego, CA) to generate GH3-1-His as a C-terminal fusion protein. The construct and null vector were expressed in *Escherichia coli* (BL21 (DE3)) along with the pRIL plasmid (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The expressed fusion protein was purified with His GraviTrap columns (GE Healthcare, Little Chalfont, UK) and 10 μl of each fraction was analysed by SDS-PAGE (Laemmli, 1970), Western blot (Towbin et al., 1979), and immunodetection using a monoclonal antibody raised against poly-histidin (Sigma-Aldrich, St Louis, MO; 1:10000 dilution). Elution fractions containing GH3-1-His were desalted with PD-10 columns (GE Healthcare, Little Chalfont, UK) and the protein concentration of the eluate was determined using the Bio-Rad D_{2}C_{2} protein assay (Hercules, CA) with bovine serum albumin as protein standard.

Assays for IAA–amino acid conjugate formation and their detection by TLC were performed as described by Staswick et al. (2005) using 10 μg of protein in a total volume of 30 μl. Control reactions were without ATP, protein, and with heat inactivated protein, respectively. For further analysis of the reaction products samples were acidified to pH 1–2 and extracted three times with 200 μl of ethyl acetate. After evaporation of the solvent residues were dissolved in MeOH (30 μl) and subjected to LC-MS analysis.

**Chemical synthesis of IAA amino acid conjugates**

[Indole-3-D3]IAA was from Cambridge Isotope Laboratories (Andover, MA), IAA, l-tryptophan methyl ester hydrochloride and l-aspartic acid dimethyl ester hydrochloride were purchased from Sigma-Aldrich (St Louis, MO).

The synthesis of IAA–amino acid conjugates was adapted from Ilk et al. (1997) either using unlabelled IAA or [indole-3-D3]-labelled IAA as starting substrate. 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) or 60% (v/v) MeOH, 0.2% (v/v) acetic acid (IAA-Trp, *Rt*=8.4 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).

**LC-ESI-MS/MS analysis of IAA and conjugates**

For LC-MS quantification, IAA and conjugates were extracted from 100 mg of grape berry or tomato tissue, spiked with 500 pmol of [Indole-3-D3]IAA, [Indole-3-D3]IAA-Asp, and [Indole-3-D3]IAA-Trp as internal standards, as described by Kowalezyk and Sandberg (2001). After extraction and diethyl ether partitioning the aqueous phase was acidified to pH 1–2 and applied to a 50 mg Empore 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 quadrupole mass spectrometer). The sample (10 μl) was first separated on a Luna C18 column [75×4.6 mm, 5 μm (Phenomenex, Torrance, CA)] and held at 30 °C using the following solvent conditions: 0–7 min isocratic 60% (v/v) MeOH, linear gradient from 60% (v/v) to 95% (v/v) MeOH in 3 min, held for 5 min, from 95% (v/v) to 60% (v/v) in 3 min, held for 10 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 8.0 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 conjugates 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 conjugates, m/z 134 for labelled IAA and conjugates) and were used for quantitation.

For the analysis of conjugates produced by the in vitro reactions with recombinant GH3-1 10 µl of the dissolved residues were introduced into the ESI ion source (conditions as described above) using 90% (v/v) MeOH as solvent (flow rate 0.4 ml min⁻¹). The total ion current was monitored in MS2 scan mode (m/z 61–500, 0.5 scans s⁻¹) in positive ion mode.

**Results and discussion**

GH3-1 is a putative grapevine IAA-amido synthetase gene expressed in grapes

As supported by an increasing amount of data, the conjugation of IAA to amino acids, catalysed by GH3 proteins, is an important aspect in the control of auxin levels in plants (Woodward and Bartel, 2005; Delker et al., 2008; Wang et al., 2008), but how this regulatory system might affect major developmental changes like fruit-ripening is largely unexplored.

In a berry development microarray study (data not shown) a GH3-like sequence (GH3-1) was identified that showed expression in grape berry tissue. The predicted open reading frame of 1797 bp encodes 598 amino acid residues and is represented by 19 ESTs from flowers and fruit in the NCBI database (http://www.ncbi.nlm.nih.gov). Interestingly, GH3-1 shares a high percentage of sequence similarity (93%) with the pungent pepper GH3 protein (CcGH3), which has been associated with ripening processes in the non-climacteric pepper fruit (Liu et al., 2005). Both protein sequences cluster into group II of GH3 proteins (Fig. 1) which consists of IAA-amido synthetases conjugating mainly IAA to a variety of amino acids (Staswick et al., 2005; Ding et al., 2008). CcGH3 was shown to be mainly expressed in fruit at, and after, the onset of ripening (Liu et al., 2005), at a time when the accumulation of IAA-amide conjugates has been reported for a number of different fruit species (Archbold and Dennis, 1984; Dunlap et al., 1996; Purgatto et al., 2002).

To investigate whether GH3-1 displayed a similar expression pattern, its mRNA levels were analysed throughout the course of development of Cabernet Sauvignon berries (Fig. 2A). The GH3-1 gene showed high transcript accumulation in flowers and young berries, when auxin levels have been reported to be high (Inaba et al., 1976; Cawthon and Morris, 1982; Zhang et al., 2003; Deytieux-Belleau et al., 2007), suggesting that the activity of the corresponding

![Fig. 1. Phylogenetic relation of grapevine GH3-1 to GH3 protein sequences from Arabidopsis and pepper. The sequences were aligned using ClustalW2 (http://www.ebi.ac.uk) and the Neighbor–Joining unrooted tree was generated with PHYLIP 3.6 (Felsenstein, 1989) using a bootstrap test with 1000 iterations (bootstrap values are indicated at each node). The predicted grapevine GH3-1 protein is highlighted with a shaded background. 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; Vv, Vitis vinifera.](https://academic.oup.com/jxb/article-abstract/61/13/3615/531360)
protein during these stages might contribute to the control of auxin homeostasis. A second peak of expression was observed at the onset of ripening and lasted until 12 weeks post-flowering (wpf) after which expression levels declined during the last weeks of berry ripening. The high expression of GH3-1 from veraison onwards is suggestive of a ripening-associated function for this gene.

To determine the distribution of GH3-1 expression within the berry, transcript levels were analysed in skin, flesh, and seed tissue (Fig. 2B) and found to be similarly high in skin and flesh and low in seed, suggesting skin and flesh as the main sites of GH3-1 activity. High transcript levels of GH3-1 were also detected in tendrils, which are considered to be sterile reproductive organs of grapevines (Carmona et al., 2008), whereas the expression levels in roots and leaves were comparatively low (Fig. 2B).

GH3-1 expression is regulated by auxins, ABA, and ethylene

A positive regulation by auxins is a common feature of group II GH3 genes (Staswick et al., 2005). Auxin responsiveness of GH3-1 is indicated by the occurrence of the core sequence (TGTCTC) of auxin response elements (AuxRE) (Liu et al., 1994; Ulmasov et al., 1995) in its promoter (http://www.dna.affrc.go.jp/PLACE/).

Berries at 22 d and 12 d prior to veraison were exposed to 0.5 μM IAA, the synthetic auxin NAA, and the synthetic auxin-like compound BTOA for 24 h in an ex planta induction experiment. The relatively long incubation period of 24 h was chosen because a prolonged induction by auxins has been reported for the pungent pepper fruit GH3 (Liu et al., 2005), contrasting with the otherwise rapid and transient response of GH3 gene expression to increased auxin levels in vegetative tissues (Hagen et al., 1984; Roux and Perrot-Rechenmann, 1997; Nakazawa et al., 2001; Goda et al., 2004; Jain et al., 2006; Park et al., 2007; Ding et al., 2008). GH3-1 expression was up-regulated by NAA and BTOA in 22 d preveraison berries and it was induced by all three compounds 12 d preveraison (Fig. 3A). The more pronounced effects in berries closer to veraison might be due to higher endogenous auxin levels in the younger berries masking the response to exogenous compounds (Inaba et al., 1976; Cawthon and Morris, 1982; Zhang et al., 2003). Treatment with NAA and BTOA led to the strongest induction with about 3.5-fold increases in expression levels 12 d preveraison. The stronger effect of synthetic auxins may be ascribed to their increased stability compared to IAA, which is highly unstable (Lee and Starratt, 1992; Petrounia et al., 1994), but could also be due to the inability of GH3-1 to conjugate these substrates leading to a continued induction. Arabidopsis GH3 proteins adenylate NAA less efficiently than IAA (Staswick et al., 2005) and it is possible that BTOA is not a substrate for these enzymes, as has been shown for other synthetic auxin-like compounds such as 2,4-dichlorophenoxacyclic acid (2,4-D) and dicamba (Staswick et al., 2005). This might explain why these compounds are such effective growth regulator herbicides and further underlines that GH3 proteins may play a major role in the control of auxin homeostasis.

While an auxin induction of GH3-1 in young berries is plausible, it is not a likely explanation for its increased expression at veraison (Fig. 2A). At this stage the IAA concentration is low (Inaba et al., 1976; Cawthon and Morris, 1982; Zhang et al., 2003) whereas ABA levels increase concomitant with sugar accumulation and colour development and reach their maximum about 2 weeks after veraison (Davies et al., 1997; Deytoux-Belleau et al., 2007; Wheeler et al., 2009). This developmental profile, in addition to numerous studies on the ripening-enhancing effects of ABA treatments (Hale and Coombe, 1974; Matsushima et al., 1989; Ban et al., 2003; Jeong et al., 2004; Cantin et al., 2007; Pepin and Fidelibus, 2008), has resulted in the general acceptance of ABA as a promoter of berry ripening. At 22 d and 12 d preveraison, GH3-1 showed moderate increases in expression in response to either ABA or sucrose treatments and the induction was amplified by a combined application of both (Fig. 3B), particularly in berries close to veraison (10-fold increase). This indicates that sugar and ABA may act together to increase GH3-1 transcript levels at veraison. Synergistic effects of ABA and sucrose on anthocyanin accumulation (Pirie and Mullins, 1976) and hexose transport (Atanassova et al., 2003) in grape berries have been described, suggesting that the induction of GH3-1 ex planta by the combination of ABA and sucrose may mimic the events during berry ripening. Furthermore,
a promoter scan (Higo et al., 1999) identified 10 elements (ABRE-like) potentially involved in ABA signalling 180–800 bp upstream of the translation start of GH3-1 (http://www.dna.affrc.go.jp/PLACE/).

Changes in ethylene concentration or perception around the time of veraison (Chervin et al., 2004) may be another possible trigger for increased GH3-1 transcript levels. The involvement of ethylene in grape berry ripening is a matter of ongoing debate (Davies and Böttcher, 2009), but it seems to be a less critical factor than in climacteric fruit such as tomato and banana (Musa paradisiaca L.) (Bower et al., 2002). The treatment of preveraison berries with the ethylene releasing compound ethephon (Warner and Leopold, 1969; Yang, 1969) led to a slight increase in the expression of GH3-1 in both developmental stages tested, which indicates a potential role for ethylene in the control of this gene in grapes. The reason why the ethylene response of GH3-1 seems less pronounced than that of the ripening-related pungent pepper GH3 (Liu et al., 2005) might be a different degree of involvement of ethylene in the ripening event of these two fruit species. Although generally considered to be non-climacteric (Watkins, 2002), pungent pepper has also been deemed climacteric by some research groups (Gross et al., 1986; Villavicencio et al., 1999) due to ethylene production and respiratory rates intermediate between climacteric and non-climacteric fruit.

GH3-1 is an IAA–amido synthetase with preference for aspartic acid and tryptophan

The predicted protein sequence from an in silico translation of the GH3-1 coding sequence contains all three sequence motifs involved in ATP/AMP binding [\textsuperscript{107}SSGTA\textsuperscript{GER}K\textsuperscript{116} (Motif I), \textsuperscript{336}YASSE\textsuperscript{340} (Motif II), \textsuperscript{412}YRLGD\textsuperscript{416} (Motif III)] that are characteristic of the acyl-adenylation/thioester-forming enzyme superfamily (Chang et al., 1997). To examine whether GH3-1 can adenylate IAA in vitro, the purified recombinant protein (Fig. 4A) was tested with IAA in combination with each of 20 amino acids and the reaction mixtures were analysed for IAA conjugates with thin layer chromatography (TLC) (Fig. 4B). Products with a lower mobility than IAA were only observed in reactions containing Asp and Trp. These putative IAA conjugates had the same R\textsubscript{F} as the corresponding standards (Fig. 4C).

As expected and previously described for Arabidopsis GH3 proteins (Staswick et al., 2002, 2005) the synthesis of the IAA conjugates was dependent on ATP due to the adenylation activity of GH3-1. A heat treatment at 70 °C resulted in the inactivation of the enzyme (Fig. 4C).

The two reaction products, IAA-Asp and IAA-Trp, were also analysed by liquid chromatography–mass spectrometry (LC-MS) in the positive ion mode to confirm their identity. The protonated quasi-molecular ions ([M+H]\textsuperscript{+}) of the conjugates obtained from the reaction mixes were detected at m/z 291 (IAA-Asp) and m/z 362 (IAA-Trp), respectively (Fig. 4D) and matched those of the conjugate standards (data not shown).

Changes in IAA and conjugate levels are associated with the ripening of grape berries and tomatoes

The expression pattern (Fig. 2A, B) and hormonal response (Fig. 3A, B) of GH3-1 suggest an involvement of this gene in grape berry ripening, possibly realized through the conjugation of IAA to amino acids as indicated by the in vitro function of the encoded protein (Fig. 4A–D). Thus, the levels of IAA and the amino acid conjugates IAA–Asp and IAA–Trp in developing grape berries were analysed by LC-MS (Fig. 5). The concentration of free IAA was found to be high in flowers (800 pmol g\textsuperscript{-1} FW), after which it steadily declined to be \textasciitilde10-fold lower at the onset of ripening. It remained low throughout the rest of berry development and reached its minimum at harvest, 16 weeks after flowering (Fig. 5A). The observed decrease in IAA levels was in accordance with previous reports (Inaba et al., 1976; Cawthon and Morris, 1982; Zhang et al., 2003; Deytieux-Beliveau et al., 2007) and was not only due to a dilution effect caused by the increase in berry size, but was also caused by a decrease in the total amount of free IAA present, as observed by plotting IAA on a per berry basis (Fig. 5B). The concentration of the IAA–Asp conjugate was...
high in flowers, very low in berries up to the stage of veraison, and then increased to a maximum 12 weeks after flowering, after which it slowly declined (Fig. 5A). The sharp increase in IAA–Asp levels after veraison became even more apparent when calculated on a per berry basis (Fig. 5B). IAA–Trp was not detected in any of the analysed berry samples. A possible explanation for this might be the comparatively low concentration of tryptophan in relation to aspartic acid in grape berry tissue (C Davies, MR Thomas, P Corena, personal communication). Both amino acids reach their maximum levels 10–12 weeks after veraison, but the concentration of aspartic acid is generally about 5-fold higher than that of tryptophan, which might make aspartic acid the preferred substrate for conjugation.

The accumulation of IAA–amide conjugates combined with low levels of free IAA at, and after, the onset of ripening has previously been reported for the climacteric fruit banana (Purgatto et al., 2002) and muskmelon (Dunlap et al., 1996) as well as the non-climacteric strawberry (Archbold and Dennis, 1984). This might therefore represent a broadly conserved ripening-related process active in non-climacteric and climacteric fruit.

To investigate this further, levels of IAA, IAA–Asp, and IAA–Trp were analysed in tomato, the model species of climacteric fruit ripening. Again, IAA–Trp could not be detected, but remarkably, IAA and IAA–Asp levels in ripening tomatoes followed a very similar pattern to the one observed in grapes (Fig. 6A, B). High concentrations of both molecules were detected in immature green fruit and were found to be about 20- (IAA) and 80-fold (IAA-Asp) lower in mature green fruit. The concentration of IAA–Asp increased dramatically in fruit at the breaker/turning stage.

Fig. 4. In vitro activity of recombinant GH3-1. (A) The expression of recombinant GH3-1 protein was tested by separating 10 μl of His GraviTrap column elution fractions (E1-E5) 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 about 70 kDa corresponds to the His-tagged GH3-1 protein. (B) TLC analysis of GH3-1 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 Ehrmann’s reagent to detect indole compounds. (C) TLC analysis of GH3-1-catalysed IAA-Asp and IAA-Trp formation with indicated variations of the reaction mixture. (D) Reactions with Asp and Trp were analysed for their products by LC-MS in positive ion mode. The quasi-molecular ions ([M+H]+, [M+Na]+) are highlighted in bold letters.
and was even higher in light red and red ripe tomatoes. In contrast to grape berries the concentration of IAA–Asp was generally higher than that of free IAA and there was also a slight increase in free IAA concentrations at, and after, the initiation of ripening. A comparative study of grape and tomato EST databases has recently identified common ripening-associated transcription factors (Fei et al., 2004). This supports the possibility that certain aspects of the control of ripening may be conserved between climacteric and non-climacteric fruit.

The formation of the IAA–Asp conjugate suggests that the role of IAA–amino acid conjugation in grape berries and tomatoes may be more complex than merely resulting in the reduction of free IAA concentrations. IAA–Asp is a very poor substrate for a small family of Arabidopsis IAA-amino acid hydrolases (LeClere et al., 2002) which makes it unlikely that it serves as a storage form of IAA. It is possible that IAA-Asp is linked to IAA inactivation mechanisms, probably by oxidation of the bound IAA (Östlin et al., 1998; Staswick et al., 2005). In support of this it has been reported that IAA-Asp is converted into DiOxIAA–Asp in broad bean (Vicia faba L.) (Tsurumi and Wada, 1986) and to oxindole-3-acetyl-aspartic acid in populus (Populus tremula L.) (Plass et al., 1989). A different regulation of IAA-Asp degradation during fruit development, possibly co-ordinated with its GH3-catalysed formation, might contribute to the observed changes in levels of this IAA-amino acid conjugate.

IAA-Asp itself may represent a biologically active molecule with a yet to be identified role in fruit ripening. That IAA-amino acid conjugates might have more complex functions in plants than merely serving as IAA storage forms has recently been demonstrated by Staswick (2009), who showed that IAA-Trp is a negative regulator of auxin action in Arabidopsis.

In conclusion, the increasing levels of IAA-Asp in grapes and tomatoes might be linked to the low levels of active IAA that were observed at, and after, the onset of ripening and provide evidence for a possible mechanism for the maintenance of low auxin levels in ripening fruit. Since small changes in the ratio of free to conjugated IAA, as can be observed in Arabidopsis mutants overexpressing GH3 genes (Nakazawa et al., 2001; Takase et al., 2004; Park et al., 2007; Zhang et al., 2007), result in considerable changes in plant morphology, it seems a possible mechanism to control the profound alteration of metabolic processes that occur during fruit ripening. Future studies of grapevine GH3-1 mutants and the identification and characterization of tomato orthologues will be needed to confirm the in vivo function of IAA-amido synthetases in fruit.
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