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Stress Metabolism in Green Coffee Beans (*Coffea arabica* L.): Expression of Dehydrins and Accumulation of GABA during Drying

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In order to produce tradeable standard green coffee, processed beans must be dried. The drying procedure affects the abundance of relevant aroma substances, e.g. carbohydrates. Using molecular tools, the corresponding metabolic basis is analyzed. A decrease in water potential of the still living coffee seeds induces massive drought stress responses. As a marker for these stress reactions, accumulation of a general stress metabolite, GABA (γ-aminobutyric acid), and associated gene expression of drought stress-associated dehydrins were monitored. The results of this study indicate that metabolism in drying coffee beans is quite complex since several events trigger accumulation of GABA. The first peak of GABA accumulation during drying is correlated with expression of isocitrate lyase and thus with ongoing germination processes in coffee seeds. Two subsequent peaks of GABA accumulation correspond to maxima of dehydrin gene expression and are thought to be induced directly by drought stress in the embryo and endosperm tissue, respectively. Apart from the significance for understanding basic seed physiology, metabolic changes in coffee seeds during processing provide valuable information for understanding the role and effect of the steps of green coffee processing on the quality of the resulting coffee.

Keywords: γ-Aminobutyric acid (GABA) • *Coffea arabica* • Dehydrins • Drought stress • Isocitrate lyase.

Abbreviations: ACN acetonitrile; ICL, isocitrate lyase; DMSO, dimethylsulfoxide; GABA, γ-aminobutyric acid; OPA, *o*-phthaldialdehyde; RT–PCR, reverse transcription–PCR; THF, tetrahydrofuran.

Introduction

Traditionally, green coffee is produced by either wet or dry processing of coffee cherries. Processing is required to remove the fruit flesh (exo- and mesocarp) and the parchment (endocarp) as well as to dry the coffee beans (seeds); morphological details are given in **Fig. 1**. In wet processing, coffee cherries are mechanically depulped—a process in which the major part of the fruit flesh is removed by squeezing. Considerable parts of the fruit flesh remain sticking on the parchment. These mucilaginous residues are degraded by fermentation; subsequently the resulting parchment coffee is dried. Before shipping, the dry parchment and the testa (seed coat, **Fig. 1**) are removed by hulling. In contrast, in dry processing, the entire coffee fruits are dried before all seed surrounding layers are removed in one husking step. The chemical composition of green coffee beans and the corresponding quality of the beverage produced by these two processes differ significantly (Bytof et al. 2005, Knopp et al. 2006). Recently, it has been shown that the variation in the drying procedure in the course of wet processing strongly affects the abundance of various sugars, representing important aroma precursors (Kleinwächter and Selmar 2010). Although sun drying is considered to yield the best coffee qualities, in this study the direct influence of sunlight could be excluded. Kleinwächter and Selmar (2010) noted that the main difference between continuous machine drying and diurnal sun drying is the lack of pauses during the night.

Differences in relevant aroma compounds in differentially processed coffees can be attributed to variations in the extent and time courses of metabolic processes, especially those related to germination that occur within the seeds during processing (Selmar et al. 2006, Bytof et al. 2007). Additionally, drought stress-induced reactions also appear to be important. The occurrence of corresponding stress-induced processes was deduced from the finding that the typical stress metabolite γ-aminobutyric acid (GABA) is accumulated in drying coffee beans (Bytof et al. 2005). This non-protein amino acid is thought to be accumulated in response to several stress conditions (Satya Narayan and Nair 1990, Bown and Shelp 1997). It is obvious that the strong decrease in water potential during the drying of coffee beans corresponds to classical drought stress. Based on these observations, GABA accumulation should represent a characteristic drought stress response (Bytof et al. 2005), but because GABA is also accumulated in a number of plants in the course of normal germination (Inatomi and Slaughter 1971, Kuo et al. 2004), the question arose as to whether the GABA accumulation that occurs in the course of

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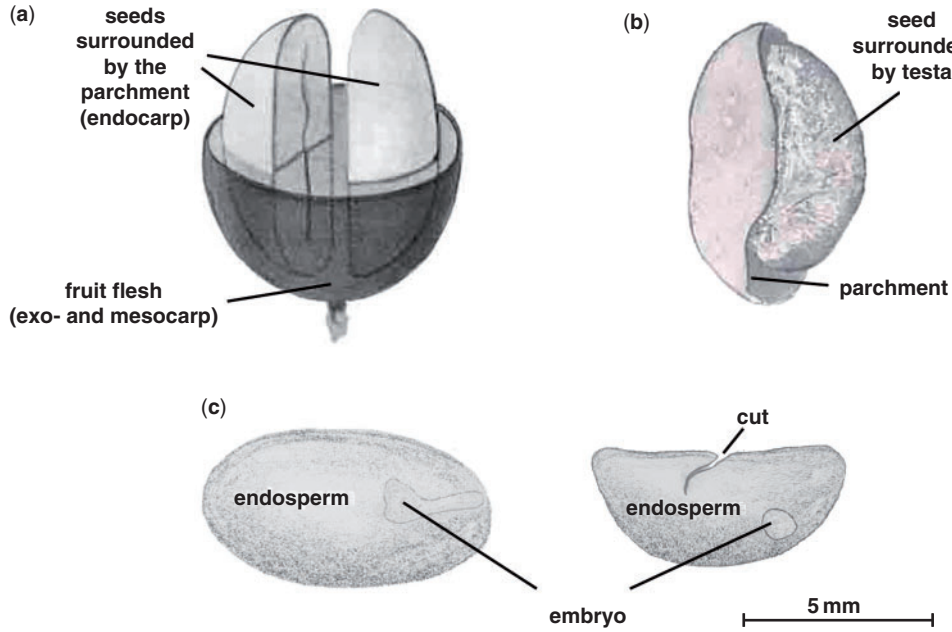

Fig. 1 Seed and fruit morphology of *Coffea arabica*. (a) Sectional image of a mature coffee fruit. Generally, in each coffee cherry, two seeds that are surrounded by the endocarpal parchment are embedded in the fruit flesh. (b) Sectional image of parchment coffee. After removing the entire fruit flesh by depulping and fermentation, the coffee seeds are still covered by the endocarp. When this corneous layer is removed, the testa surrounding the seeds becomes visible. During wet processing, both layers—the parchment and the seed coat—are removed by hulling. (c) External appearance of coffee seeds. On the left side, an endocarpal, exterior view of a coffee seed is shown. The hazy silhouette of the embryo can be recognized vaguely in the opaque endosperm. On the right side, a corresponding cross-section is displayed.

green coffee processing is a stress response or due to metabolic steps involved in the ongoing germination of the coffee beans. In this context, dehydrins provide additional markers for drought stress. Apart from their abundance in late embryogenesis, an increase in gene expression of dehydrins is strongly correlated with several types of stress conditions (Close 1997, Allagulova et al. 2003, Bouché and Fromm 2004). Due to their great hydrophilicity and thermostability, it is assumed that dehydrins are structure stabilizers with detergent- and chaperone-like properties (Borowski et al. 2002). Dehydrins are often associated with drought stress and dehydration (Nylander et al. 2001, Caruso et al. 2002, Rodriguez et al. 2005, Samarah et al. 2006). In this work, we considered gene expression of dehydrins as a suitable tool to monitor metabolic responses to putative drought stress. Additionally, gene expression of isocitrate lyase (ICL) was analyzed to describe germination processes. The alignment of these various metabolic events should contribute to the general understanding of the complex metabolism of coffee seeds during processing, thereby establishing the basis for deliberately modulating coffee quality by modifying drying conditions.

Results and Discussion

When fresh coffee seeds were dried continuously at 30°C and the residual water content declined to less than 43% a significant increase in the expression of dehydrins occurred (**Fig. 2**, left). Notably, when the drying was interrupted by pauses of 8 h, the enhancement of dehydrin mRNA was detectable earlier, although the drying process was delayed. A similar situation was also found for GABA accumulation (**Fig. 2**, left section). If we assume that drying of the seeds induces drought stress, which generally leads to typical metabolic stress responses such as GABA accumulation and dehydrin synthesis, these findings seem contradictory. Dehydration is much faster in seeds that are dried continuously compared with seeds resulting from an interrupted drying pattern. Consequently, drought stress-related responses should occur earlier in continuously dried seeds than in those dried with 8 h pauses. However, when seeds are dried at 40°C, gene expression of dehydrins, as well as the accumulation of GABA, appear nearly simultaneously in both drying regimes (**Fig. 2**, right section). Surprisingly, the pattern of GABA accumulation is bimodal.

The occurrence of two maxima suggests that two distinct events are responsible for stress induction. The metabolic steps responsible for stress induction must be more complex than previously expected. In order to get a clearer picture of these processes, an enhanced sampling frequency was required. Accordingly, drying under laboratory conditions was performed. The resulting data confirmed the complex pattern of GABA accumulation and also revealed that the gene expression of dehydrins possesses two maxima (**Fig. 3**).

Although we earlier assumed that GABA accumulation is primarily related to drought stress, the results of more diligent

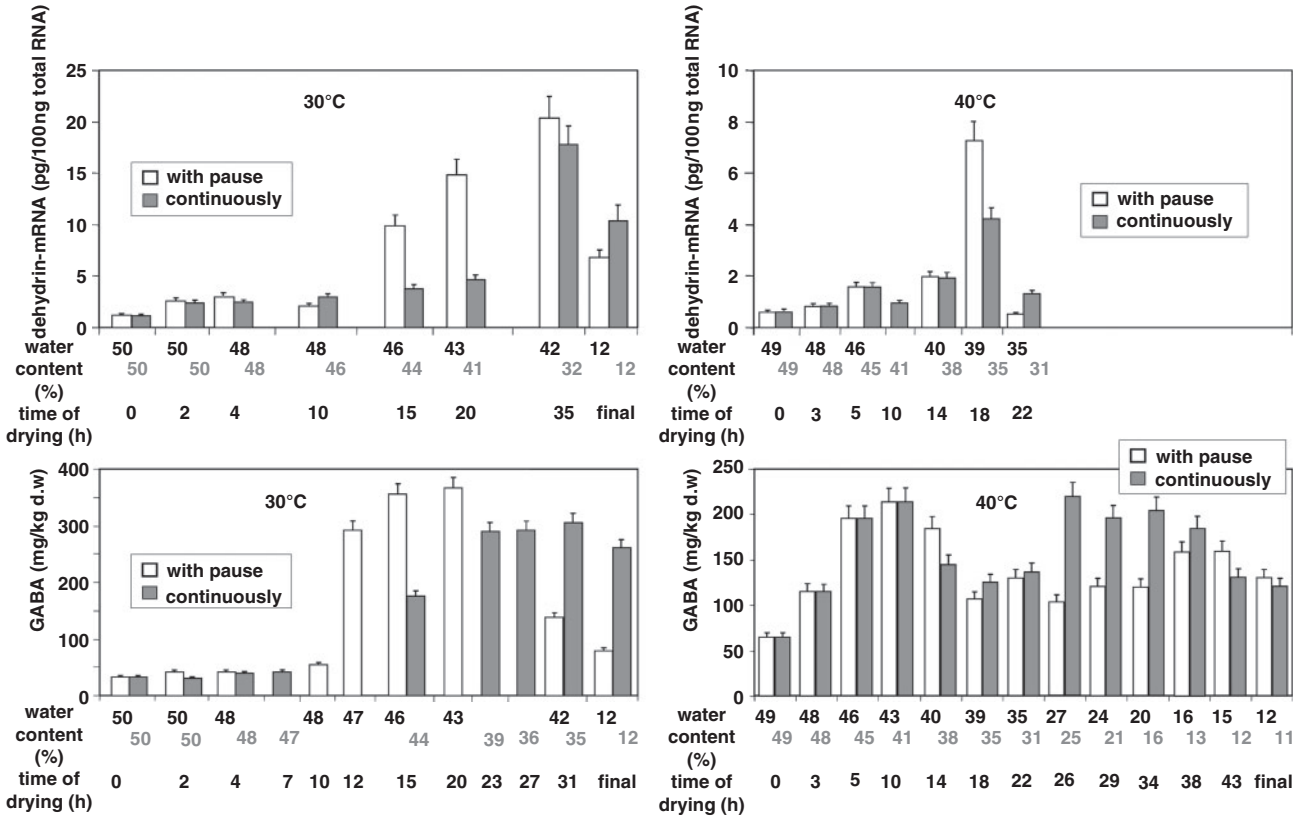

Fig. 2 Expression of dehydrins and accumulation of GABA during model drying modes at 30 and 40°C. Fully mature red coffee fruits of *C. arabica* were mechanically depulped and fermented prior to transportation. After transportation, the beans were dried in special model dryers either continuously or by applying a day–night cycle simulating a drying/pause rhythm of 14/10 h in the case of the 30°C drying. To accommodate the much faster drying rate at 40°C, the drying/pause rhythm was adapted to 8/4 h. Expression of dehydrins was determined by competitive RT–PCR; the average standard deviation—displayed as error bars—in general was <10%. The content of GABA was estimated by HPLC; the average standard deviation in general was <7 %. All values correspond to at least four analyses. The basic material for each quantification was obtained by grinding 150 coffee beans.

drying and sampling indicate that the overall pattern of GABA accumulation is much more complex. The major difference in GABA accumulation and dehydrin gene expression, apart from GABA accumulation in freshly fermented, non-dried samples, discussed below, is due to the fact that GABA accumulation begins much earlier than dehydrin expression. As this accumulation occurs when water contents are still around 50%, it was assumed that this metabolic response, like GABA accumulation in fresh beans (see below), is not induced by decreasing water potential, but is due to germination processes that have previously been described to take place in coffee seeds during processing (Selmar et al. 2006, Bytof et al. 2007). Similar germination-related GABA accumulation has been observed in other plants, e.g. *Sinapis alba* (Vandewalle and Olsson 1983), *Castanea sativa* (Desmaison and Tixier 1986) and *Lens* sp. (Rozan et al. 2001). In order to differentiate drought stress-induced GABA accumulation from that putatively related to germination, the expression of ICL, a reliable germination marker, was analyzed in parallel. As predicted, gene expression of ICL was highest about 2 d before the maximum level of dehydrins (induced by drought stress) was detectable (**Fig. 4**). At this time point, GABA content increased in a similar manner. From this, it can be deduced that the first increase in GABA content is indeed related to the germination process. Subsequently, GABA accumulation seems to be associated with dehydrin gene expression and thus the initiation of drought stress.

As mentioned above, the expression of dehydrins also follows a bimodal pattern. One possible explanation might be due to a second phase of stress induction, possibly triggered by a significantly lower water content (i.e. a more pronounced level of drought stress) than that responsible for the first induction. Alternatively, the secondary expression of dehydrins could be induced differentially in different parts of the coffee seeds. As there are no data supporting incremental dehydrin expression in response to decreasing water potentials, the second possibility, i.e. spatial differentiation, appears more likely. In this context, the seed morphology has to be considered: the embryo is more or less entirely embedded in endosperm (**Fig. 1**). The drying process begins with a loss of

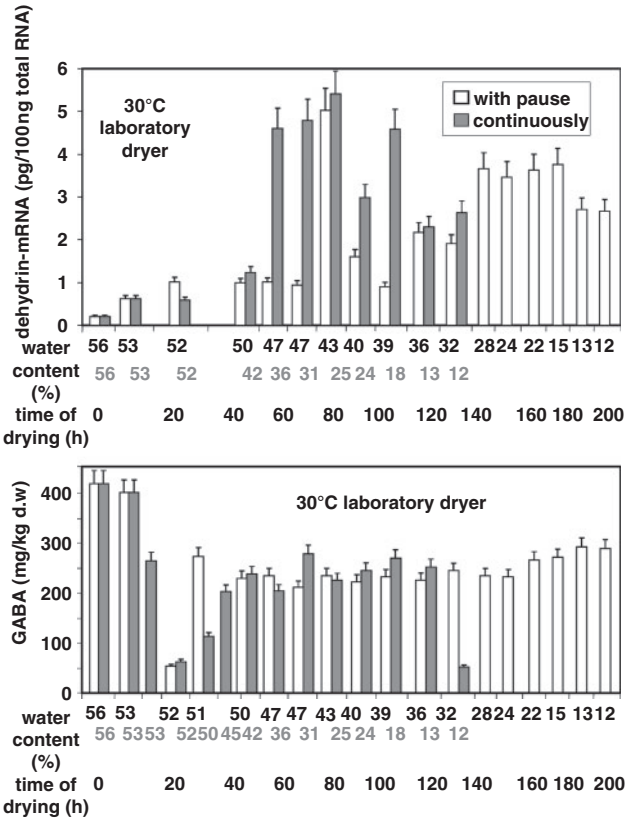

Fig. 3 Expression of dehydrins and accumulation of GABA during laboratory drying at 30°C. Fully mature red coffee fruits of *C. arabica* were mechanically depulped and fermented prior to transportation. After transportation, the beans were dried in regular laboratory dryers either continuously, or by applying a day–night rhythm of 14/10 h. Expression of dehydrins was determined by competitive RT–PCR; the average standard deviation—displayed as error bars—in general was <10%. The content of GABA was estimated by HPLC; the average standard deviation in general was <6%. All values correspond to at least four analyses. The basic material for each quantification was obtained by grinding 150 coffee beans.

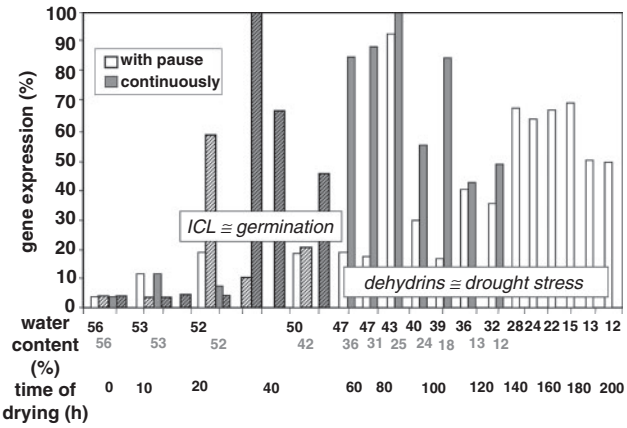

Fig. 4 Expression of isocitrate lyase (ICL) compared with the expression pattern of dehydrins during laboratory drying at 30°C. Stress responses in drying coffee beans. In order to correlate the pattern of dehydrin gene expression (shown in Fig. 3) with the germination process, the expression of ICL was determined as an example. Data for ICL are given in the hatched columns and those for dehydrins in open columns. Expression of ICL was determined by competitive RT–PCR; the average standard deviation—displayed as error bars—in general was <10%. As the absolute amounts of gene expression differ massively, for better alignment, data are presented as relative values. In the case of ICL, 100% corresponds to about 75 pg 100 ng^{-1} total RNA; in the case of dehydrins, 100% accounts for 5.5 pg 100 ng^{-1} total RNA. The basic material for each quantification was obtained by grinding 150 coffee beans.

water from the outer parts of the endosperm before the water potential of the interior cells decreases. Thus, the embryo is embedded in a relatively moist milieu for a prolonged time, and maintains a higher water potential than the outer endosperm cells. Consequently, when drought stress-related reactions are already induced in the outer parts of the endosperm, the embryo does not yet suffer from drought stress. Further experiments are needed to verify the assumption that induction of drought stress-related metabolic responses in the endosperm occurs before those in the embryo. A thorough separation of embryo and endosperm tissue is inevitable.

One other salient difference in the pattern of GABA accumulation in the course of drying was observed. In contrast to drying in standard dryers, drying in experimental laboratory dryers (**Figs. 3**, **4**) resulted in a massive accumulation of GABA in the fresh, fermented but not yet dried samples (**Fig. 2**). In this context, it has to be taken into consideration that the samples used for experimental drying in laboratory ovens had been transported directly as hand luggage on an airplane. Hence, the entire time span from depulping to the onset of drying was just about 32–36 h. In contrast, beans processed in the model dryer were shipped as air cargo. The corresponding time from depulping to the onset of drying was about 54–60 h. As massive accumulation of GABA in the non-dried beans decreases rapidly (within 18 h after the first sample was taken from the beans shipped as hand luggage), it could be assumed that this phase of GABA accumulation should not be detectable within the air cargo-shipped samples. In the latter case, the first sampling was made about 20 h later, at a point in time when the initial GABA accumulation had largely disappeared. This is supported by the finding that the initial GABA concentration in the air cargo-shipped samples varied significantly; in some trials the average content was <20 mg kg DW^{-1}, whereas in others it was >100 mg kg DW^{-1} (data not shown), probably due to variations in the time needed for transportation.

As fresh, unprocessed coffee seeds do not contain a significant concentration of GABA (Bytof et al. 2005), the high GABA concentration prior to the drying procedure must be due to massive synthesis. Synthesis putatively should be initiated during the course of green coffee processing and may be due to strongly decreasing oxygen content within the fermenting green coffee. Whereas fermentation normally takes place in processing tanks at the coffee farms, in the air-transported specimen it probably took place in a similar manner in plastic

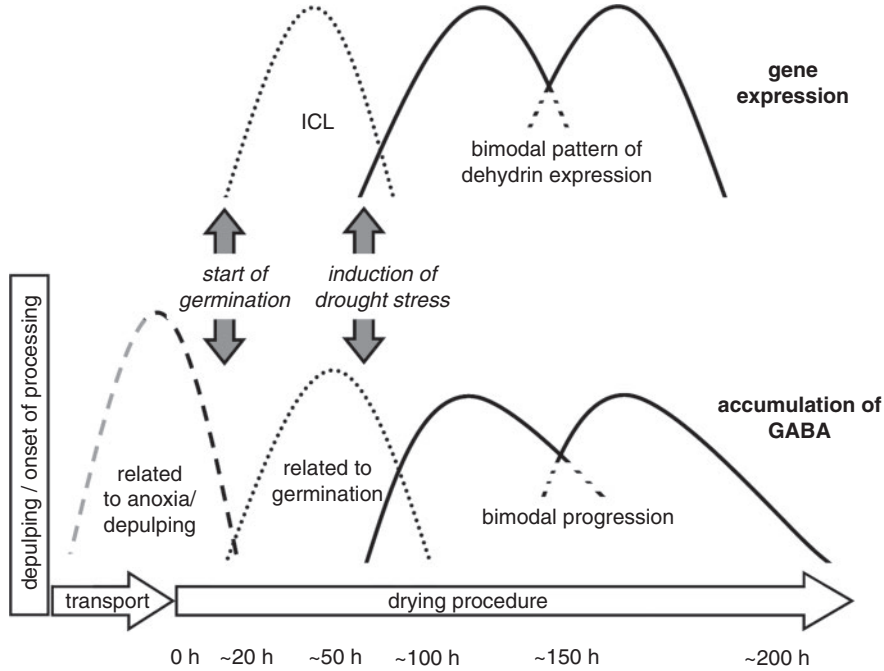

Fig. 5 Scheme of the temporal succession of the relevant metabolic events. With respect to GABA accumulation there are numerous phases of metabolic activity that are thought to be triggered by different events. The first peak of GABA accumulation during drying is correlated with expression of isocitrate lyase and, thus, with germination processes in the coffee seeds. Subsequent peaks of GABA accumulation are correlated with corresponding maxima of dehydrin expression and are thought to be induced directly by drought stress in endosperm tissue and embryo, respectively. GABA accumulation in fresh green coffee prior to the drying procedure is attributed to the strong decrease in oxygen concentration during fermentation and perhaps to bruising resulting from the mechanical depulping process and demucilation procedure. The small arrows point to the time span from depulping of the seeds to the onset of drying. In the case of laboratory drying, this corresponds to about 35 h, whereas the model drying—due to the longer traveling time as air cargo—could not be started before 50 h after depulping.

barrels during transport. Due to similar fermentation conditions, fermentation in these samples should also cause the massive oxygen decrease thought to be responsible for the first phase of GABA accumulation. Corresponding anoxia-induced GABA accumulation has been reported for radish leaves (Streeter and Thompson 1972) and for rice roots (Reggiani et al. 1988, Reggiani et al. 2000).

The mechanical stress the beans suffer during depulping and demucilation may provide an alternative possibility for the elicitation of the observed GABA accumulation during the course of the fermentative processing phase. Although covered by the flexible endocarp (parchment layer), the seeds may be injured by squeezing and shearing forces during the course of depulping and mechanical demucilation. The resulting damage could represent injuries such as those that occur in *Phragmites* shoots as a result of a complex die-back syndrome that elicits massive GABA accumulation (Koppitz et al. 2004). When elucidating the path of GABA metabolism, we should be aware that stress-induced GABA accumulation represents a complex metabolic syndrome, in which numerous factors, e.g. drought stress, anoxia and injuries, can induce and modulate GABA synthesis and accumulation. As these different factors may occur in parallel or successively and are differentially expressed in different tissues, a complex, nearly non-assessable metabolic situation may result. A synopsis of the various events impacting on the dehydrin expression and its correlation with the GABA accumulation and ICL expression is presented as sketch in **Fig. 5**. According to Goldberg et al. (1989), the initiation of ICL expression determines the onset of germination. The subsequent events until radicle emergence are thought to be germination related (Bewley 1997). A detailed outline on the succession of metabolic events of seed development, germination and seedling development is given by Bewley and Black (1994).

Based on the complex metabolic conditions mentioned above, detailed studies on the formation of aroma-relevant substances must be performed in order to elucidate how the drying procedure affects coffee quality and how to apply this knowledge in practice.

Materials and Methods

Sample processing

Green coffee samples were produced under carefully controlled processing conditions at the facilities of EDE Consulting America, San José, Costa Rica. For these sample preparations, only sound and fully mature red coffee fruits of *Coffea arabica*

were mechanically depulped using a Pinhalense drum pulper, followed by mechanical demucilation. Fermentation was performed during transportation by air in small plastic barrels (5 liters). The entire process from the depulping to the arrival of the beans in the laboratory in Braunschweig took either about 55 h when shipping was performed as cargo, or 36 h when the green coffee was transported as hand luggage; the temperature was about 20°C. Thus, these conditions correspond closely to standard wet processing. Immediately after arrival, blank samples of each batch (about 150 beans per lot) were shock frozen in liquid nitrogen. The major part of the samples were dried down to 11% water under controlled parameters in special model dryers or laboratory ovens. The conditions of experimental drying varied with respect to two parameters. On the one hand, temperatures of either 30 or 40°C were used. On the other hand, the beans were dried either continuously or by applying a day–night rhythm, e.g. after 14 h of drying, the oven was shut down for 10 h in order to mimic classical sun drying, traditionally applied in coffee-producing countries. In the course of the introduced pauses, the temperature decreased to about 20°C.

At appropriate intervals, samples (150 beans) were taken and shock frozen. Additional drying experiments were performed in standard laboratory drying ovens at 30°C in order to provide better sampling frequencies, necessary to monitor the fast metabolic changes. Frozen samples were ground in liquid nitrogen and stored at −80°C for further analyses.

RNA extraction

Aliquots (200 mg) of each sample were extracted using 2 ml of Trifast solution (peqLab, Erlangen, Germany) according to the corresponding instruction leaflet. DNA was removed by DNase I treatment. The complete DNA degradation was checked by PCR. The quality of the RNA was verified by electrophoretic analysis.

Design of dehydrin primers and the internal standard

An alignment of an expressed sequence tag (EST) sequence of a dehydration-like protein (Fernandez et al. 2004) from *C. arabica* and a cDNA sequence of a dehydrin from *Coffea canephora* (DH3; Hinniger et al. 2006) revealed that the sequences are 94% identical. Consequently, the cDNA sequence of the dehydrin 3 (DQ333960.1) from *C. canephora* was chosen as the basis for our expression studies. Based on this known dehydrin sequence, the forward primer 3dh27 (5′-tgctttacgtacatccatcagc-3′) and the reverse primer 3dh660R (5′-aatccttggccctcaccttcag-3′) were designed. The resulting 633 bp PCR product was amplified from chromosomal DNA, sequenced and analyzed for restriction sites.

For reliable competitive reverse transcription–PCR (RT–PCR)-based quantification, the RNA standard has to differ from the transcript in size without losing the corresponding primer-binding sites. Therefore, a 289 bp fragment was cut out of the central region of the sequence. The remaining parts were religated and cloned into the pDrive-vector (Qiagen, Hilden, Germany) that contains a T7 RNA polymerase promotor site in front of the dehydrin standard sequence.

To produce the artificial RNA standard, the vector, containing the standard sequence, was linearized with *Xba*I, purified by gel extraction and then in vitro transcribed with a T7 RNA polymerase. Subsequently, the sample was digested with DNase I to remove the contaminating plasmid DNA. The sample was checked by a PCR to ensure that all DNA had been removed. Finally, the RNA standard was stabilized with a poly(A) tail and quantified by gel electrophoresis. As a basis for this quantification, the response was compared with different portions of an RNA ladder (MBI-Fermentas, St.Leon-Rot, Germany). The resulting non-degraded RNA standard was diluted with diethylpyrocarbonate (DEPC)-treated water and aliquoted. The aliquots were mixed with RNase inhibitor (MBI-Fermentas, St.Leon-Rot, Germany) and stored at −70°C until they were used in the reverse transcription reation.

The use of a specific reverse transcription primer requires a slightly modified reverse primer for the PCR to amplify the cDNA. Consequently, PCR was performed with 10 µM of the forward primer 3dh27 (5′-tgctttacgtacatccatcagc-3′) and 10 µM of the reverse primer 3dh619R (5′-aatccttggccctcaccttcag-3′).

Primers and internal standard for isocitrate lyase

ICL expression studies were performed according to Bytof et al. (2007), using as ICL-forward primer 3′-gggattgggacctgcctagaacc-5′ and as ICL-reverse primer 3′-agaggaaaggaaccatgaggttga-5′, which results in a 424 bp PCR product. As internal standard, an ICL RNA fragment elongated by the introduction of a 942 bp fragment from pBR322 was used, yielding a PCR product of 1,129 bp.

Reverse transcription reaction of dehydrins

The reverse transcription reaction was performed with 100 ng of total RNA, using the RevertAid™ H Minus First Strand cDNA Synthesis kit (MBI-Fermentas, St.Leon-Rot, Germany). Instead of the oligo(dT) primers, specific dehydrin reverse transcription primers (3dh659R: 5′-agcttcctccttggat-3′) were used (0.5 µl of 10 µM). Dimethylsulfoxide (DMSO; 20%) was added to the reverse transcription reaction to increase the yield of cDNA. In order to quantify the transcript concentration reliably, the internal RNA standard was added to the reverse transcription assays at six different concentrations (25, 10, 5, 1, 0.5 and 0.1 pg) for the dehydrin analyses. The samples were incubated for 1 h at 37°C followed by an inactivation time of 10 min at 70°C.

Reverse transcription reation of ICL

The reverse transcription reaction was performed with 100 ng of total RNA, using the RevertAid™ H Minus First Strand cDNA Synthesis kit (MBI-Fermentas, St.Leon-Rot, Germany). For the corresponding reverse transcription reaction of ICL, the ICL-RT primer (3′-ccatgaggttgacacact-5′) was applied instead of an oligo(dT) primer. In order to quantify the reverse

transcription reaction, four different concentrations of ICL standard (50, 25, 10 and 1 pg) were added. The reverse transcription reaction was performed at 50°C followed by an inactivation time of 10 min at 70°C.

PCR

PCR was performed using the cDNA generated by the reverse transcription reaction described above. For the PCR, 0.5 µl of the reverse transcription incubation was applied as template. As the polymerase, a Hotstart Taq enzyme (HssT-taq plus, Qiagen, Hilden, Germany) was used. Cycling conditions for dehydrin expression studies were as follows: denaturation, 5 min at 95°C, 30 cycles; 95°C for 30 s, 63°C for 30 s, 72°C for 60 s; final extension, 72°C for 10 min. PCR products were analyzed by agarose electrophoresis (1.5%) and stained with ethidium bromide. For each sample, eight reverse transcription reactions with four different amounts of standard and 16 PCRs were performed. In the case of ICL, the annealing temperature was 63°C, and 29 cycles were performed. DMSO (3%) was added in the PCR to optimize the amplification specificity.

For evaluation of the gels, bands with nearly the same intensity between transcript and standard were taken and plotted in chromatograms by the Scion Image Analyzer program. The displayed square pixel of the integrated peak for the standard correlates linearly with the concentration applied. In this manner, the level of gene expression of all RNA samples could be calculated per 100 ng of total RNA.

Extraction and determination of GABA

GABA was quantified by HPLC according to Bytof et al. (2005). Frozen plant material was ground in liquid nitrogen and aliquoted. To each sample (500 mg) 0.8 µmol norvaline was added as internal standard before extraction. The frozen powders then were repeatedly extracted with sulfosalicylic acid (4%, w/v). The extracts were adjusted to 100 ml, centrifuged and filtered. The amino acids were derivatized with *o*-phthaldialdehyde (OPA) prior to HPLC analysis. The OPA derivatization procedure was performed according to Kirchhoff et al. (1989); however, a Spark Holland (Emmen, The Netherlands) Midas autosampler was used for derivatization and sample injection. The amino acid derivatives were separated on a C18 column [Nucleosil 100, 5 µm, Macherey & Nagel (Düren, Germany), 250×4.0 mm] using a binary gradient [A: 5% MeOH, 5% acetonitrile (ACN), 2% tetrahydrofuran (THF), 88% 50 mM sodium acetate buffer, pH 6.2; B: 40% MeOH, 40% ACN, 20% sodium acetate buffer] at a flow rate of 1.3 ml min^{-1}. The derivatives were detected by means of an RF-551 Shimadzu (Duisburg, Germany) fluorescence detector (λ_{ex} = 334 nm; λ_{em} = 425 nm) and quantified by external calibration.

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