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Biochemistry

Proteome analysis during pod, zygotic and somatic embryo maturation of *Theobroma cacao*

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ABSTRACT

Two dimensional electrophoresis and nano-LC–MS were performed in order to identify alterations in protein abundance that correlate with maturation of cacao zygotic and somatic embryos. The cacao pod proteome was also characterized during development. The recently published cacao genome sequence was used to create a predicted proteolytic fragment database. Several hundred protein spots were resolved on each tissue analysis, of which 72 variable spots were subjected to MS analysis, resulting in 49 identifications. The identified proteins represent an array of functional categories, including seed storage, stress response, photosynthesis and translation factors. The seed storage protein was strongly accumulated in cacao zygotic embryos compared to their somatic counterpart. However, sucrose treatment ($60\,g\,L^{-1}$) allows up-regulation of storage protein in SE. A high similarity in the profiles of acidic proteins was observed in mature zygotic and somatic embryos. Differential expression in both tissues was observed in proteins having high p*I*. Several proteins were detected exclusively in fruit tissues, including a chitinase and a 14-3-3 protein. We also identified a novel cacao protein related to known mabinlin type sweet storage proteins. Moreover, the specific presence of thaumatin-like protein, another sweet protein, was also detected in fruit tissue. We discuss our observed correlations between protein expression profiles, developmental stage and stress responses.

Introduction

Embryogenesis is a critical process in the life cycle of higher plants, providing the link between two sporophytic generations. Zygotic embryogenesis encompasses two phases: the early morphogenesis process, during which the embryo body plan is formed with shoot and root apical meristems; and the maturation phase, where the embryo accumulates storage compounds and acquires the capacity to withstand desiccation. Asexually derived embryos can be induced *in vitro* from a wide range of somatic and gametophytic donor tissues (Mordhorst et al., 1997). The transition of somatic cells into cells that are embryogenically competent is the first and crucial step in somatic embryogenesis.

In cacao, we recently showed that somatic embryos can be produced on a large scale using a temporary immersion bioreactor (TIB) system, which allows for an increase in embryo numbers and quality (Niemenak et al., 2008). However, embryo germination and plantlet development are still a bottleneck. In order for cacao somatic embryogenesis to reach its full potential, the somatic embryos produced must not only be viable but must exhibit adequate and predictable growth. It is desirable to alter the composition of the maturation medium in order to produce somatic embryos accumulating more lipid, starch and protein. In many species, the rate of conversion of somatic embryos into plantlets increases after exposure to exogenous abscisic acid (ABA) or by application of osmoticants, such as sucrose, sorbitol and polyethylene glycol (Krajňáková et al., 2009; Rai et al., 2009; Sghaier-Hammami et al., 2010). The general phenomena under the control of ABA include overall coordinated growth resulting in better proportioned embryos and better deposition of storage proteins and lipids (Joy et al., 1991; Gutmann et al., 1996; von Aderkas et al., 2002). The accumulation of sucrose inside tissue helps in maintaining cell viability during dehydration by stabilization of membranes (Olivier et al., 1998). In cacao, somatic embryo

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maturation in bioreactors is better achieved with transfer to a high sucrose containing media (Niemenak et al., 2008). The poor development potential observed in embryos grown on low sugar media suggests that essential processes required for their maturation have not been completed. The effect of different carbon sources on the induction of cacao somatic embryogenesis was achieved by Traore and Guiltinan (2006). These authors showed that, during embryo maturation and conversion, no significant difference was observed among glucose, fructose, maltose or sucrose for embryo weight, total shoot and root development.

Proteomics has become a powerful method to identify the physiological, biochemical and molecular changes associated with different developmental stages, treatments and environments. Several prior studies have used proteomic profiling to study embryogenesis and maturation of somatic embryos of *Picea glauca* (Lippert et al., 2005), *Vitis vinifera* (Marsoni et al., 2008), *Citrus sinensis* Osbeck (Pan et al., 2009), and *Phoenix dactylifera* (Sghaier-Hammami et al., 2010). The recent determination of the cacao genome sequence (Argout et al., 2011) greatly facilitates proteomics profiling of cacao tissues by providing a predicted proteome useful for rapid and automated identification of protein.

Attempts to characterize the cacao proteome profile, especially during embryogenesis, are scarce. Information on cacao proteomics has focused on protein degradation during fermentation (Lerceteau et al., 1999; Kratzer et al., 2009) and method development (Pirovani et al., 2008; Micheli et al., 2010; Bertazzo et al., 2011). A detailed cacao pod husk proteome was conducted by Awang et al. (2010) using MALDI-TOF/TOF MS on two clones, LAF17 and ICS39. The authors found that the majority of proteins expressed in 3-month-old cacao pod husk are involved in metabolism and energy. Recently, our groups compared the proteomes of *Theobroma cacao* somatic embryos with their zygotic counterparts at an early developmental stage and found about 1000 protein spots per fraction using two-dimensional isoelectric focusing/SDS. Many of the identified proteins are involved in genetic information processing, carbohydrate metabolism and stress response (Noah et al., 2013).

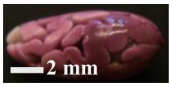
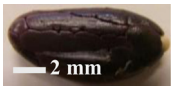
The objectives of this study were to: (a) compare the protein profiles of mature somatic embryos derived from 30 g L^{-1} and 60 g L^{-1} sucrose in order to identify alterations in protein spots that correlate with maturation potential; (b) compare somatic embryos with zygotic embryos at different development stages in order to define the zygotic counterpart to the mature somatic embryos; and (c) study the zygotic embryo and cacao pod development to understand their protein status. Proteins were analyzed by 2D PAGE and nano-LC–MS. A comparison of protein profiles between somatic and zygotic embryogenesis was conducted in order to identify key similarities and differences in the two embryogenic pathways. Zygotic/somatic embryos' specific accumulation of protein provide information necessary for improving cacao somatic embryos maturation, a challenging step for *in vitro* mass propagation of cacao. Therefore, investigating and identifying differences in the late embryo development of the zygotic and somatic embryos should provide information to potentially increase somatic embryos' conversion into plantlets.

Results

Number of spots detected in Coomassie-stained 2-DE gels

Analysis of the embryo and pod protein profiles by 2-D PAGE revealed both qualitative and quantitative changes in protein profiles during development. In zygotic embryos, 692 ± 92, 715 ± 75, and 710 ± 77 protein spots were reproducibly resolved at 16, 18 and 20 WAP (weeks after pollination), respectively. Torpedo somatic embryos grown on 30 g L^{-1} sucrose exhibited 653 ± 35 protein spots. The number of detectable proteins was 845 ± 112 after two weeks and 884 ± 44 four weeks after transfer into media containing 60 g L^{-1} sucrose. During cacao development, pod husks displayed 960 ± 165 proteins spots at their early maturation phase (14 WAP). At 16 WAP, 18 WAP and 20 WAP 903 ± 232, 846 ± 93 and 1104 ± 29 proteins spots were detected in pod husks, respectively (Table 1, Supplemental data 1, 2 and 3).

Table 1
Number of spots detected in Coomassie-stained 2-D gels. Results are from three independent biological replicate gels. ZE16WAP, ZE18WAP and ZE20WAP are zygotic embryos analyzed at 16, 18 and 20 weeks after pollination respectively. SE_ED30_4W: somatic embryos cultivated in ED medium supplemented with 30 g l^{-1} sucrose for 4 weeks. SE_ED60_2W and SE_ED60_4W: somatic embryos cultivated in ED medium supplemented with 60 g l^{-1} sucrose for 2 and 4 weeks respectively. Pod14WAP, Pod 16WAP, Pod18WAP and Pod 20WAP are orherwise like ZE.

	Total number of spots
ZE16WAP 	692 ± 92
ZE18WAP 	715 ± 75
ZE20WAP 	710 ± 77
SE_ED30_4W 	653 ± 35
SE_ED60_2W 	845 ± 112
SE_ED60_4W 	884 ± 44
Pod14WAP 	960 ± 165
Pod16WAP 	903 ± 232
Pod18WAP 	846 ± 93
Pod20WAP 	1104 ± 29

Protein identification and differential expression analysis

Proteins were excised from gels, subjected to proteolytic digestion and then analyzed by mass spectrometry to precisely measure the molecular weights of cleavage fragments. The cleavage profiles were compared to a predicted cacao proteome fragment database to identify likely protein identities. The number of identified proteins in the 2D gels is summarized in Table 2. In several cases, multiple proteins were identified in a single spot. In other cases, the same protein was identified in more than one closely migrating spot, which is most likely due to the different isoforms or post-translational modifications of these proteins. 2D PAGE analyses