



Effects of somatic embryogenesis on gene expression of cloned coffee heterozygous hybrids

Kenia Carvalho de Oliveira¹ · Paula de Souza Guimarães¹ · Jaqueline Moraes Bazioli¹ · Juliana Camargo Martinati¹ · Mariana Martinis dos Santos¹ · Lillian Padilha² · Oliveiro Guerreiro-Filho¹ · Mirian Perez Maluf²

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Abstract

Key Message Our analyses suggest that no major genome organization occurred during SE process implying the non-occurrence of somaclonal variation. However, the genetic background determines the quality of the in vitro response.

Abstract Cloning of superior coffee plants by somatic embryogenesis can assist breeding programs on reducing the cost and time for launch of new cultivars. This study aimed to evaluate the efficiency of this methodology for cloning coffee trees with high heterozygosity, and to gather evidence that clonal progenies are faithful copies of mother plants. Selected plants IAC1 and IAC 2 from *Coffea arabica* breeding populations, resistant to leaf rust and leaf miner, respectively, were cloned via indirect somatic embryogenesis. Expression of selected genes involved in biological processes potentially affected by in vitro cultivation was evaluated by quantitative analysis. Genes encoding proteins associated with maintenance of DNA integrity and control of cell cycle presented predictable expression patterns along the clonal multiplication process. There were differences in the expression pattern of genes linked to in vitro cultivation-related stress, which were observed comparing either IAC 1 and IAC2 genotypes or clones and their corresponding mother plant. Those analyses suggest that the somatic embryogenesis does not lead to major genomic instability and clones are identical copies of mother plants, even with detected differences in the expression of genes that influence the response of in vitro cultivation.

Keywords *Coffea arabica* · In vitro cultivation · Differential expression · Somaclonal variation

Introduction

Coffee cultivation, processing, marketing, and transportation have long been considered as significant factors for socio-economic growth and development, as they provide millions of job opportunities around the world. However, the cost of coffee production is high due to several features, including the occurrence of biotic and abiotic stresses, and the operational costs of manual or mechanical harvest. In addition, the long cycle of the crop, the low genetic variability within the species, and the need for several years of production to evaluate adverse conditions throughout productivity cycle,

are among stimulus to use genetic improvement for developing novel productive and resistant cultivars.

Plant tissue culture techniques applied to coffee breeding are important tools for maintenance and evaluation of germplasm, genomic-assisted selection, identification of new genus, and, finally, multiplication of special coffee genotypes (Bertrand et al. 2011; Shahzad et al. 2017). Furthermore, breeding programs have available extremely promising agronomic F1 hybrid plants combining disease resistance and insect tolerance that are yet heterozygous, and, therefore, not suitable for seed multiplication. The exploitation of this heterozygosity in coffee may be possible with cloning processes such as somatic embryogenesis.

Somatic embryogenesis aims to regenerate identical plants from the parent plant, considering the concept of cellular totipotency (Pereira et al. 2007). The somatic embryogenesis in coffee occurs through two developmental pathways: direct somatic embryogenesis, in which somatic embryos originated directly from the mother tissues without the formation of intermediate stages of callus (Gaj 2004);

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✉ Mirian Perez Maluf
mirian.maluf@embrapa.br

¹ Coffee Center Agronomic Institute/IAC, Campinas, Brazil

² EMBRAPA and Coffee Center Agronomic Institute/IAC, Campinas, Brazil

and indirect, where somatic embryos originated from callus, a mass of cells with disorganized growth. The methodology of indirect somatic embryogenesis is the most promising in coffee, but still requires studies for adaptation to the reality of large-scale production (Donato et al. 2000; Menéndez-Yuffá et al. 2010).

Cloning of special Arabica coffee plants is a strategy that can shorten the time for launching new cultivars as well as exploit their hybrid vigor. However, the use of embryogenic cell suspensions has often been associated with a likelihood of genetic instability and somaclonal variation. This phenomenon results in the development of phenotypic changes associated with genomic re-arrangements, resulting in plants different from the parent plant. Thus, somaclonal variation has become a major concern in plant propagation as it may lead to loss of genetic fidelity. Somaclonal variation mostly occurs as a response to the stress imposed on the plant in culture conditions, in the form of DNA methylations, chromosome re-arrangements, and point mutations (Phillips et al. 1994). Therefore, a stringent quality check in terms of genetic similarity of the progeny and an efficient and feasible cloning protocol become mandatory for the analysis of these types of plants. Landey et al. (2013) performed a large-scale study using embryogenic suspensions and secondary embryogenesis for the multiplication of elite F1 hybrids of *C. arabica*. The molecular and phenotypical analyses indicated that the occurrence of somaclonal variation was very low, and possible genetic and epigenetic alterations occurred during somatic embryogenesis (Landey et al. 2013).

In this context, the objective of this work was to gather evidence that somatic embryogenic clones from two hybrid plants with high level of residual heterozygosity are faithful copies of their parent plants. Our strategy was to analyze the expression of genes potentially linked to the preservation of cell integrity and genetic material, as well as to stresses

involved in the in vitro multiplication process. Expression patterns for 20 selected genes suggest that no major genome organization occurred during the SE process, which implies the nonoccurrence of significant somaclonal variation. However, our analyses show that the genetic background determines the quality of the in vitro response, as each hybrid exhibited different expression profiles especially for genes linked to stress response.

Materials and methods

Plant material

Somatic embryogenesis (SE) cultivation stages from two *C. arabica* genotypes belonging to the Coffee Breeding Program of IAC were collected. The genotypes were IAC 1, an F₁ hybrid between Obatã and Icatu *C. arabica* cultivars, resistant to *Hemileia vastatrix* (leaf rust); and IAC 2, an F₃RC₄ line resistant to *Leucoptera coffeella* (leaf miner) and derived from a recombination between *C. arabica* L. and *C. racemosa* L. species.

Plants were in vitro cultivated by SBW, a commercial company (SBW 2018, <http://www.sbwbrasil.com.br/>). The SBW team developed the in vitro culture conditions and protocols for indirect somatic embryogenesis. Upon SE completion, developed embryos were transferred to proper substrate and kept at greenhouses conditions to grow into emblings. Samples corresponding to the SE stages embryogenic callus, multiplication, differentiation, maturation, germination, and embryo formation (Fig. 1) were collected for gene expression analyses. Table 1 summarizes the overall response of each genotype. On average, six petri dishes of each stage were sampled for each genotype. In addition, leaf samples from IAC 1 and IAC 2 mother plants and nursery emblings

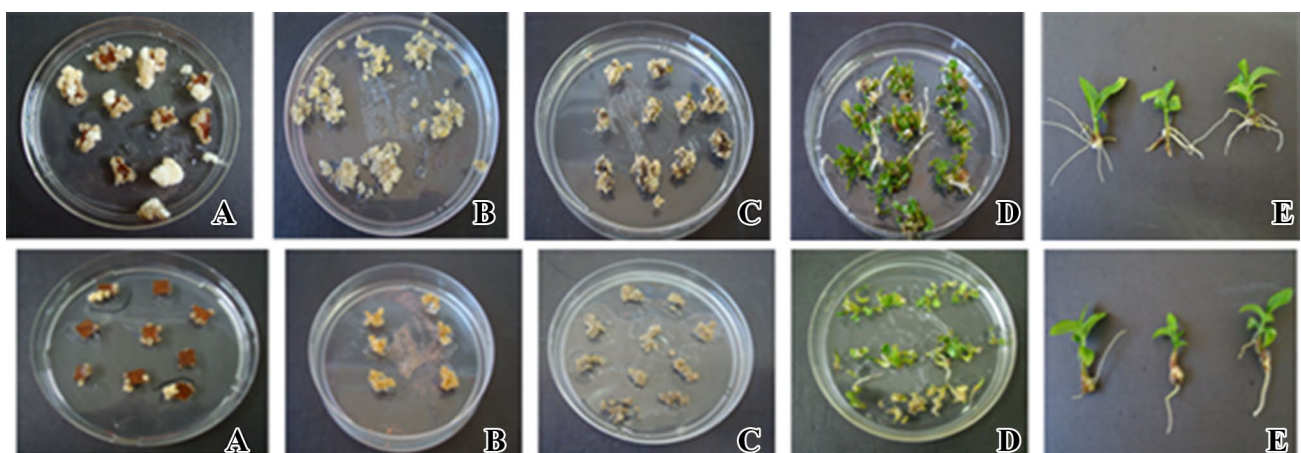


Fig. 1 Development stages during in vitro cultivation of IAC 1 (top) leaf-rust resistant; and IAC 2 (bottom) leaf-miner resistant. **a** callus; **b** multiplication; **c** differentiation; **d** maturation; **e** in vitro emblings

Table 1 Summary of phenotypic characteristics from coffee clones developed by indirect somatic embryogenesis

Parameters	Clones	
	IAC 1	IAC 2
Callus aspect	Friable, hyaline	Compact, oxidized
Earliness of development	Normal	Late
Synchronism of growth	High	Medium
Productivity	High	Medium

were also collected. The samples were frozen in liquid nitrogen (N_2), and stored in freezer $-80\text{ }^\circ\text{C}$ until needed.

Identification of coffee ESTs and gene-specific primers selection

Genes associated with major genomic re-arrangements, DNA integrity, cell maintenance and cycle regulation, and somatic embryogenesis responsiveness were evaluated here. Gene sequences were retrieved from the GenBank and used in directed Blast searches in Brazilian Coffee Genome Database (Altschul et al. 1990; Vieira et al. 2006). A list of selected genes, access number, and functional grouping is presented in Table 2. Homolog coffee ESTs were identified based on stringent similarity parameters such as $e\text{-value} < e^{-20}$, presence of protein-specific domains, and relative abundance on libraries from in vitro coffee cell cultures. To select gene-specific primers, ESTs sequences were clustered and re-aligned with corresponding genes, and highly conserved regions were identified using public databases (<http://www.ncbi.nlm.nih.gov/cdd>; <http://pfam.sanger.ac.uk/>). Coffee-based primer pairs were selected using Primer 3 (<http://frodo.wi.mit.edu/primer3/>).

Primer efficiency was established in DNA and cDNA samples according to defined parameters: single amplicon upon dissociation analysis, standard curve slope between -3.5 and -3.2 , and amplification efficiency between 0.93 and 1.05 .

Gene-expression analyses

Total RNA was extracted from $300\text{ }\mu\text{g}$ of frozen tissues using a Trizol-based protocol (Applied Biosystems). RNA quantification was performed by formaldehyde-agarose electrophoresis and absorbance at $220\text{--}340\text{ nm}$ using a Shimadzu UV spectrophotometer.

Gene-expression analysis was evaluated by quantitative RT-PCR (qPCR). A total of $2\text{ }\mu\text{g}$ RNA DNAase-free of each sample were used for cDNA synthesis using the commercial Kit SuperScript III First-Strand Synthesis SuperMix (Life Technologies). Quantitative RT-PCR was performed in an AB7300 System (Applied Biosystems) using the Sybr Green

Kit (Life Technologies), which include both SYBR green and passive reference ROX fluorescence. Reaction conditions are the same as described elsewhere, using 200 ng of cDNA (Maluf et al. 2009). To confirm the presence of single amplicons, all PCR products were analyzed through a dissociation curve, with temperature varying from 60 to $95\text{ }^\circ\text{C}$. qPCR results were analyzed with the sequence detection software SDS version 1.3.1 (Applied Biosystems), and transcript abundance was estimated using defined threshold value, baseline, and Ct parameters (Maluf et al. 2009). The GAPDH gene was used as the endogenous control (F-TTG AAGGGCGGTGCAAA; R- AACATGGGTGCATCCTTG CT). Relative expression quantification was calculated using average values of three replicates, for each stage, where each amplification was performed using a fresh cDNA pool. Relative expression was calculated using plant mother of each genotype as the calibrator sample.

Results

To understand the molecular basis of in vitro culture in coffee, we selected genes from different biological processes to analyze their expression profile by the qPCR technique (Table 2). Selected biological process included the maintenance of DNA integrity, control of cell division and cell cycle, response to oxidative stress derived from in vitro culture, and biotic and abiotic stresses.

Gene-expression analyses comprised the different stages of SE such as mother plant, embryogenic callus, multiplication and differentiation phase, maturation, and germination phase, in vitro and nursery emblings (Fig. 1). Table 3 exhibits the results of relative gene expression on each genotype. For these analyses, the calibrator stage was the original genotypes used for somatic embryogenesis, the mother plant. The expression values represent at least three independent estimates, for each gene and developmental stage. The analyses indicated that the SE process affected distinctly IAC1 and IAC2, as each genotype exhibited a different gene expression profile.

We identified three different expression patterns between IAC 1 and IAC 2. For most of the evaluated genes, expression differences between the genotypes result from variations on overall expression levels, as shown in Fig. 2, for DNAmet, EST1 and PPO. Genes that also exhibited this profile are Cell Div, EDS, H2A, IA, Oxired_NADH, AP2, PPT, RNA H, TIFY, and $\beta\text{-TUB}$. A second difference observed is a shift on which developmental stage occurs the higher gene-expression level. In this case, CELL, EMF-2, JAZ, LEA, and Wuschel transcripts accumulated at higher levels on different in vitro stages of IAC 1 and IAC 2 (Fig. 3 and Table 3). A third profile includes genes with different

Table 2 List of genes with corresponding accession number, biological process, and specific primers

Gene name	Blast	Biological role	Primers
<i>AP2/apetala-2</i>	AT4G36920	Plant organ morphogenesis	F: 5' GTTTCGGGCAGTCCATACTC 3' R: 5' CCTCCTCCTTACCCCTCTGT 3'
β -TUB/ β - Tubulin	AY081473	Nuclear division	F: 5' GCACGGAGCACACTTCAATA 3' R: 5' TGGTTGTTGAAATCAGGTGGT 3'
<i>CaDXMT/caffeine synthase</i>	AB084125	Secondary metabolism	F: 5' AAAGGGAGCATTTACTCTTCC AAAG 3' R: 5' AGCATGCATCCTGAGAAATGT GGTA 3'
<i>CELL/anaphase promoting complex</i>	XP_011098213	Cellular division	F: 5' AGAAGGCCCTGTCATGGTTT 3' R: 5' TGCTGAAATAGCGACGTGAC 3'
<i>Cell Div/cell division 48</i>	XP_021627792	Cellular division	F: 5' CTCTGCTTTGCGTGAGACAG 3' R: 5' GAACTTCTCCGGATGTTCCA 3'
<i>DNA Met/dna methyltransferase</i>	AM946610	DNA methylation	F: 5' GGTGGAATGGGATCCAGATA 3' R: 5' GTGAGGTTCTGCTCGTGTGA 3'
<i>EDS/enhanced disease susceptibility</i>	NP_001307178	Response to biotic stimulus	F: 5' TGAAGTGC GCAAGGTAGGT 3' R: 5' AGGTTCAACCAACCGCCTA 5'
<i>EMF-2/embryonic flower 2</i>	ACV87734	Regulation of reproductive development	F: 5' ACATTGCACCTCCA ACTGTG 3' R: 5' CTCTTCTGCAACAGCAAACG 3'
<i>EST1/telomerase activating</i>	AF135454	Telomere maintenance	F: 5' TTGTGGACCTTCAAGAGGTTG 3' R: 5' CCTCATGCTTTTGC GAAACT 3'
<i>GYF/protein19 analog</i>	OIT08406	DNA binding Response to abiotic stimulus	F: 5' TCAGACAAGAATCGGGAACC 3' R: 5' TGATTGTC ACTGGCATCCTC 3'
<i>H2A/histone 2</i>	LOC107004541	Chromosome organization	F: 5' CGTACGAAGCAGACTGCAAG 3' R: 5' CTTCTTCACTCCTCCGGTTG 3'
<i>IA/apoptose inhibitor</i>	XP_011098257	Regulation of apoptotic process	F: 5' AGTGCTACCTCTGGGCTCAA 3' R: 5' ACCCCAATAAAGGAAGGTG 3'
<i>LEA/late embryogenesis abundant</i>	AAY26119	Response to abiotic stimulus	F: 5' GTAGTCTTCCGCAAGTC 3' R: 5' TTTCTCTTCTTCCGCTCA 3'
<i>OXIRED_NADH/NADH oxidoreductase</i>	YP_817535	Oxidation/reduction process	F: 5' CGAATCGATTGCATTGAGG 3' R: 5' CGAGCATCATTAACCAACTCC 3'
<i>PPO/polyphenol oxidase</i>	BAA92317	Oxidation/reduction process	F: 5' CCCCTTAACAAGACCGTGAA 3' R: 5' CGGACACCACCAATCTCTCT 3'
<i>PPT/signal-peptide peptidase</i>	EOY04905	Protein metabolic process	F: 5' ACTTCCTTTGGTTGGCCATT 3' R: 5' GGAGAGCAGGTTGACCATGT 3'
<i>RNA H/RNA helicase</i>	XP_021644395	Nucleic acid binding	F: 5' AGGCATATTGCTCTGCCTTG 3' R: 5' GGCAATAAGCCAGCAAGTTC 3'
<i>SERK/Somatic embryogenic receptor kinase</i>	PHU30048	Regulation of cellular process	F: 5' TGTACTCGTCTTTGCGCTTG 3' R: 5' GATCTGCCAAGCTGACCTTC 3'
<i>JAZ/jasmonate zim domain protein</i>	ALI87031	Response to biotic stimulus Biological regulation	F: 5' GGAGCTCCAGCTCAGTTGAC 3' R: 5' TGCATTTCCAGCCAATAACA
<i>Wuschel</i>	EOY10442	Embryo development	F: 5' GACTCCCAAACAGAGCAAA 3' R: 5' CACCCACAGAACCGAATTTT 3'

patterns of transcript accumulation along SE, such as CS and SERK (Fig. 4).

The most relevant differences are those on expression of genes associated with differentiation and organogenesis, such as Wuschel, SERK, and EMF-2. For those genes, expression pattern on IAC 1 and IAC 2 may explain the observed phenotypic differences on the overall in vitro culture efficiency (Table 1). On IAC 1, Wuschel peaks during

maturation and germination phase, one stage after the expression peak observed on IAC 2, which is multiplication and differentiation. For EMF-2, a delayed peak also occurs for IAC 1, at multiplication and differentiation, when compared with the peak at embryogenic callus on IAC 2. The expression pattern for the gene SERK is inverted, as in IAC 1 expression levels increased in stages such as embryogenic callus and in vitro emblings, and in IAC 2, the expression

Table 3 Values for relative quantification of gene expression along SE process, considering Mother Plant as the calibrator stage

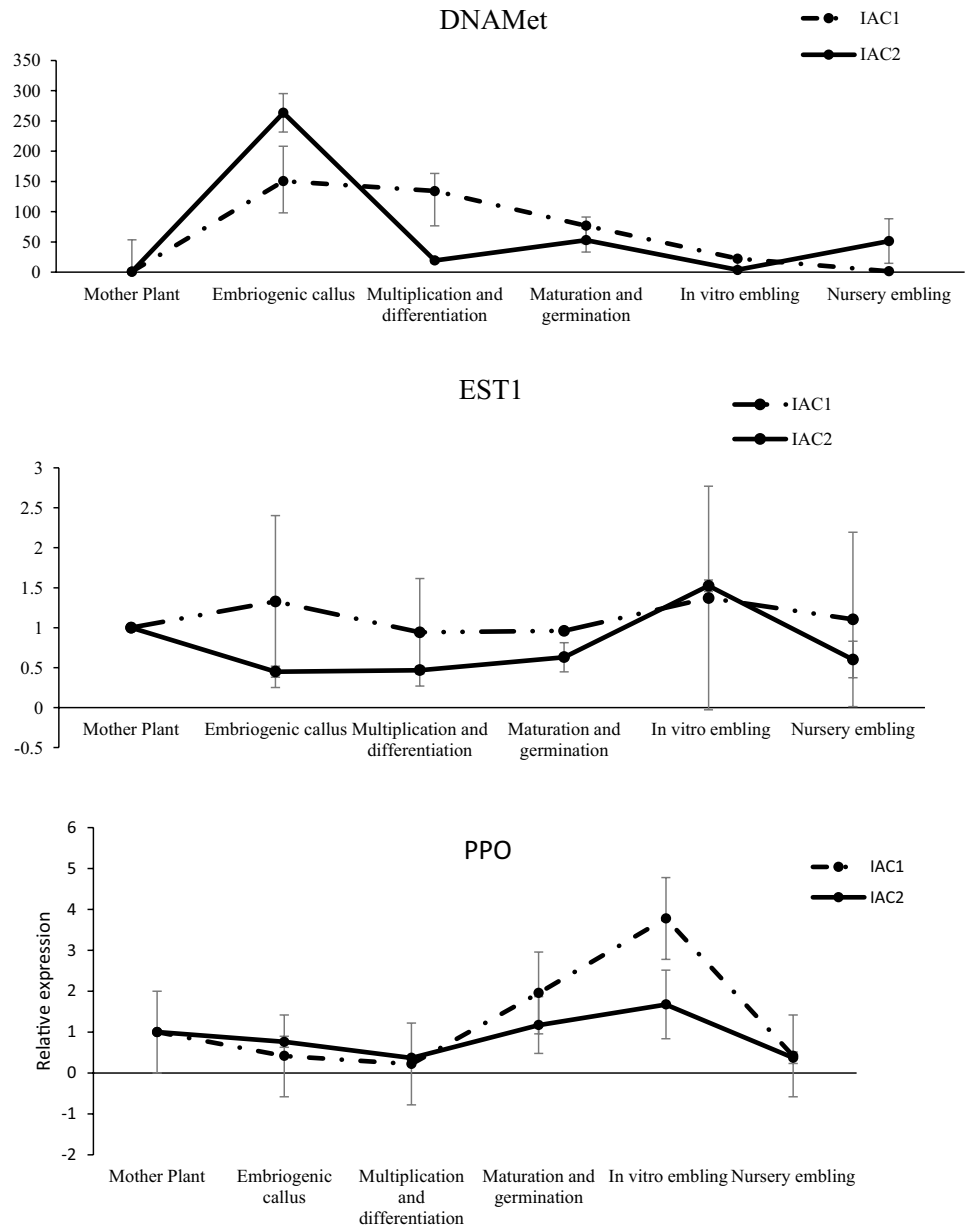
Gene ID	Genotype	Mother plant	Embryogenic callus	Multiplication and differentiation	Maturation and germination	In vitro embling	Nursery embling
AP2	IAC 1	1.00	0.058 ± 0.002	0.261 ± 0.19	0.103 ± 0.03	1.076 ± 0.09	1.094 ± 0.09
	IAC2	1.00	0.457 ± 0.385	0.194 ± 0.03	0.331 ± 0.04	1.695 ± 0.05	3.876 ± 0.84
CaDXMT	IAC 1	1.00	0.681 ± 0.28	230.09 ± 5.45	5.405 ± 0.75	1.328 ± 0.10	0.686 ± 0.13
	IAC2	1.00	0.118 ± 0.03	0.127 ± 0.02	0.412 ± 0.04	0.571 ± 0.01	0.002 ± 0.001
CELL	IAC 1	1.00	6.952 ± 1.44	5.946 ± 1.67	4.145 ± 2.42	2.023 ± 0.85	3.828 ± 1.52
	IAC2	1.00	8.519 ± 3.47	3.097 ± 1.57	2.077 ± 0.93	2.785 ± 1.49	4.267 ± 1.79
Cell Div	IAC 1	1.00	0.794 ± 0.08	0.551 ± 0.14	0.440 ± 0.13	0.808 ± 0.06	1.117 ± 0.17
	IAC2	1.00	1.288 ± 0.25	0.690 ± 0.12	0.337 ± 0.05	0.706 ± 0.18	0.971 ± 0.03
DNA Met	IAC 1	1.00	150.655 ± 52.57	134.196 ± 57.60	76.886 ± 29.24	22.508 ± 14.382	1.645 ± 0.47
	IAC2	1.00	263.550 ± 31.78	19.505 ± 3.61	52.998 ± 19.62	3.969 ± 1.13	51.434 ± 36.90
EDS	IAC 1	1.00	0.083 ± 0.03	0.161 ± 0.04	0.257 ± 0.10	0.414 ± 0.10	0.349 ± 0.001
	IAC2	1.00	0.422 ± 0.21	0.473 ± 0.10	0.240 ± 0.06	0.466 ± 0.13	0.277 ± 0.06
EMF-2	IAC 1	1.00	2.190 ± 0.36	1.364 ± 0.017	1.864 ± 1.26	2.059 ± 0.47	0.755 ± 0.08
	IAC2	1.00	2.540 ± 0.73	1.249 ± 0.87	0.760 ± 0.50	1.052 ± 0.80	0.690 ± 0.44
EST1	IAC 1	1.00	1.327 ± 1.07	0.943 ± 0.67	0.960 ± 0.00	1.370 ± 1.40	1.104 ± 1.09
	IAC2	1.00	0.450 ± 0.07	0.469 ± 0.05	0.632 ± 0.18	1.524 ± 0.07	0.602 ± 0.23
GFY	IAC 1	1.00	1.567 ± 0.06	1.728 ± 0.06	1.081 ± 0.33	0.872 ± 0.02	1.117 ± 0.22
	IAC2	1.00	3.545 ± 0.30	0.899 ± 0.31	0.807 ± 0.17	0.887 ± 0.38	1.008 ± 0.39
H2A	IAC 1	1.00	141.084 ± 53.57	88.219 ± 31.26	63.075 ± 24.29	24.948 ± 12.49	2.761 ± 0.59
	IAC2	1.00	37.860 ± 0.65	7.652 ± 1.22	5.432 ± 0.78	9.596 ± 5.35	1.571 ± 1.03
IA	IAC 1	1.00	1.629 ± 0.37	1.634 ± 0.35	1.379 ± 0.31	1.949 ± 0.65	2.274 ± 0.70
	IAC2	1.00	1.645 ± 0.31	0.982 ± 0.11	0.767 ± 0.08	1.069 ± 0.37	1.697 ± 0.34
LEA	IAC 1	1.00	31.902 ± 15.43	99.890 ± 93.76	2077.86 ± 25.46	2862.78 ± 527.83	7.383 ± 0.06
	IAC2	1.00	60.170 ± 5.21	112.54 ± 12.32	146.93 ± 21.07	4.11 ± 0.73	1.05 ± 0.19
OXIRED_NADH	IAC 1	1.00	0.038 ± 0.003	0.080 ± 0.009	0.090 ± 0.012	0.227 ± 0.037	0.414 ± 0.07
	IAC2	1.00	0.076 ± 0.010	0.068 ± 0.058	0.157 ± 0.134	0.425 ± 0.368	0.046 ± 0.08
PPO	IAC 1	1.00	0.416 ± 0.21	0.220 ± 0.09	1.957 ± 0.45	3.780 ± 1.78	0.419 ± 0.20
	IAC2	1.00	0.762 ± 0.13	0.368 ± 0.05	1.173 ± 0.70	1.677 ± 0.84	0.372 ± 0.14
PPT	IAC 1	1.00	2.472 ± 0.26	1.219 ± 0.07	0.931 ± 0.14	1.179 ± 0.32	1.743 ± 0.90
	IAC2	1.00	2.750 ± 0.48	0.793 ± 0.08	0.551 ± 0.04	1.146 ± 0.23	1.147 ± 0.19
RNA H	IAC 1	1.00	0.391 ± 0.07	0.848 ± 0.23	2.077 ± 0.52	3.394 ± 1.68	0.999 ± 0.44
	IAC2	1.00	0.567 ± 0.38	1.064 ± 0.05	0.447 ± 0.04	0.699 ± 0.08	0.849 ± 0.27
SERK	IAC 1	1.00	9.247 ± 3.49	2.376 ± 0.08	5.579 ± 0.13	25.747 ± 6.45	1.209 ± 0.19
	IAC2	1.00	4.025 ± 0.60	13.384 ± 4.05	12.119 ± 6.92	9.888 ± 3.61	1.175 ± 1.03
JAZ	IAC 1	1.00	1.173 ± 0.51	0.712 ± 0.02	1.533 ± 0.33	2.063 ± 0.40	0.801 ± 0.44
	IAC2	1.00	1.498 ± 0.87	1.011 ± 0.49	0.560 ± 0.28	0.831 ± 0.45	0.290 ± 0.14
Wuschel	IAC 1	1.00	16.95 ± 6.03	34.00 ± 12.53	392.90 ± 157.64	268.54 ± 121.69	0.00
	IAC2	0.00	1.00	26.57 ± 12.75	14.60 ± 4.83	2.271 ± 0.10	0.258 ± 0.17
β-TUB	IAC 1	1.00	0.195 ± 0.09	0.164 ± 0.06	1.005 ± 0.21	1.623 ± 0.93	1.600 ± 0.03
	IAC2	1.00	0.313 ± 0.13	0.104 ± 0.03	1.127 ± 0.34	2.073 ± 0.42	2.768 ± 0.69

levels are lower at those stages. These differences on genes important for in vitro culture may explain the relative higher efficiency of IAC 1 to produce healthy emblings.

Besides genes related to organogenesis, we evaluated other biological aspects potentially related to somaclonal variations between mother and clonal plants. Among

these, DNA methylation is an important issue during in vitro culture due to its association with gene silencing. The maximum expression level of a DNAm gene occurs during the embryogenic phase for both IAC 1 and IAC 2. However, transcription levels remain high at the

Fig. 2 Relative quantification of gene expression along SE process considering Mother Plant as the calibrator stage. Examples of profiles displaying differences on transcript levels



multiplication and differentiation stage on IAC 1, and drop at this stage on IAC 2 (Fig. 2).

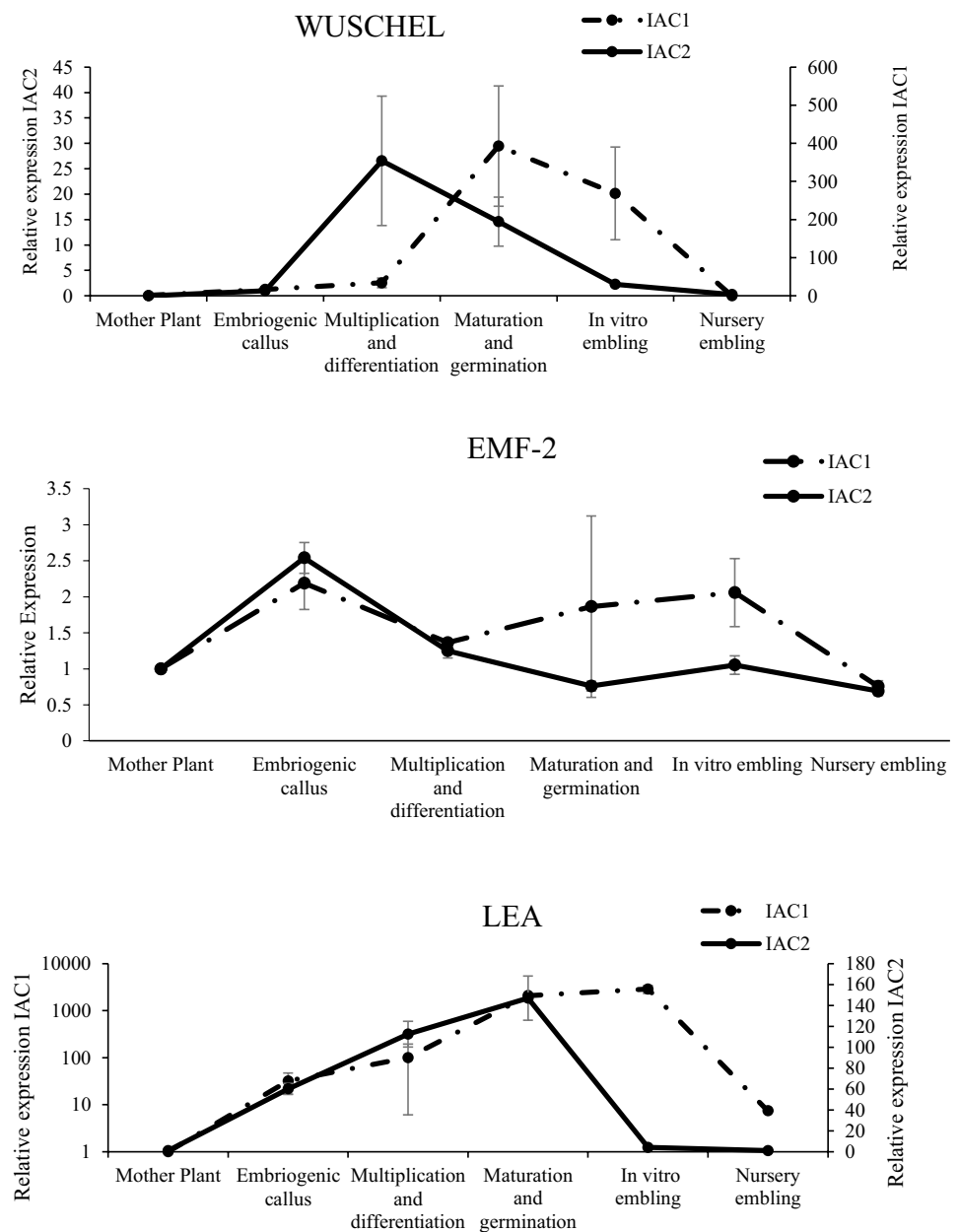
Due to the potential stress caused by SE, we also evaluated genes responsive to desiccation (LEA) and from secondary metabolism (PPO and CS). LEA expression increases along in vitro culture in both genotypes, but, at IAC 1, the levels are higher up to the nursery embling stage, a step forward compared to IAC 2 (Fig. 3). These observed levels on IAC 1 nursery emblings are also higher than the levels on their mother plants.

Among all evaluated genes, CS is the one with the most divergent profile between IAC 1 and IAC 2 (Fig. 4). Not only the overall levels are much higher in IAC 1, but also the expression profile is completely different: transcript levels

on IAC 1 are low, with an increase at the multiplication and differentiation stage, and decrease rapidly on the following stages; at IAC 2, transcript levels are very low and decrease along in vitro cultivation. Although caffeine is the major compound in coffee plants, its role on plant physiology is not clear, and therefore, those observed differences may be or not relevant at these stages.

To assess possible effects of SE on expected agronomic traits, we also compared gene-expression levels on mother plants and emblings. Transcript levels are expected to be steady at similar environments, in both mother and clonal plants. As clonal plants were not available at the time, we checked the embling expression. For almost all evaluated genes, embling transcript levels are close to those

Fig. 3 Relative quantification of gene expression along SE process considering the Mother Plant as the calibrator stage for each genotype. Examples of profiles displaying differences on peaking stage

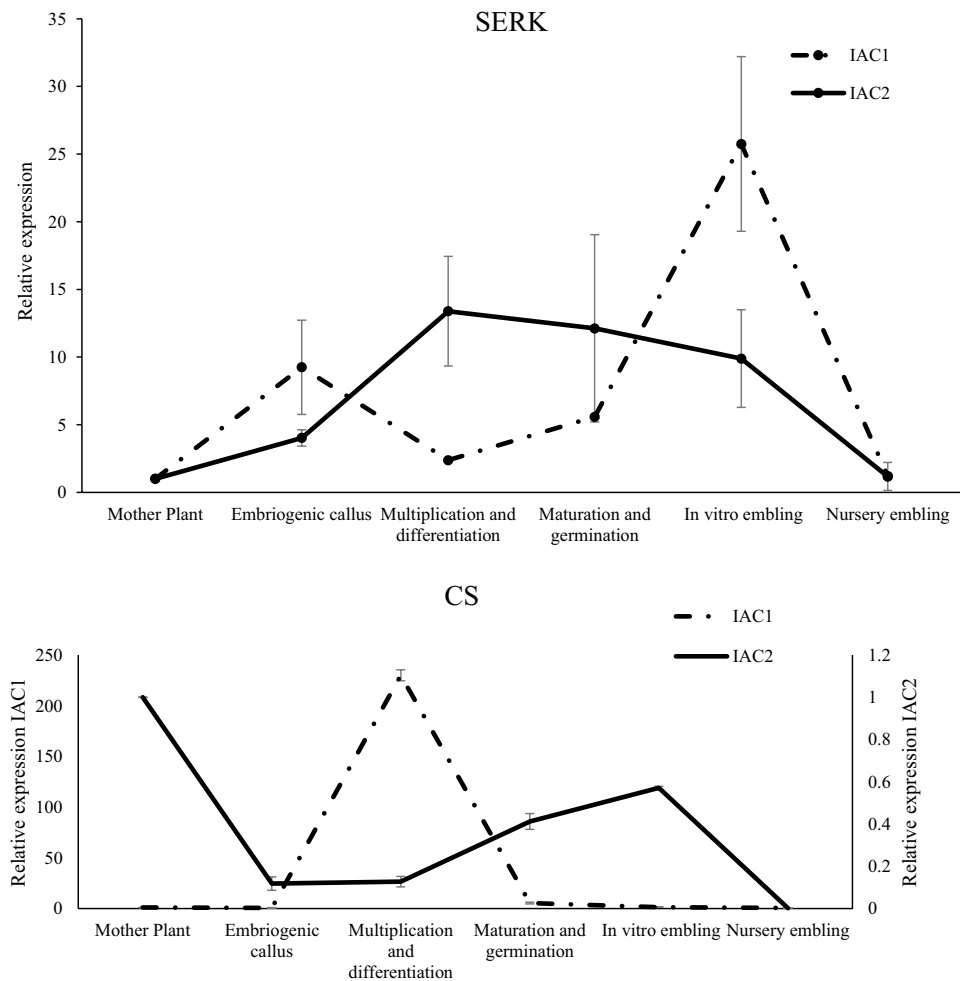


observed on mother plants, suggesting that physiological responses may be stable in clonal plants (Table 3). The major exception is for DNAm_{et} expression on IAC 2 emblings, which exhibits higher transcript levels than mother plants (Table 3, Fig. 2). This pattern could explain the low regeneration efficiency observed for IAC 2. Other interesting observation was the regular levels for EST1 in both genotypes (Table 3, Fig. 2). Once EST1 acts on the activation of a telomerase enzyme active during senescence, this observation indicates that clonal plants are evolving as expected.

Discussion

The occurrence of somaclonal variation is a major concern when choosing any tissue culture strategy to multiply desired genotypes. There are several advantages to select this multiplication method for perennial and long-life cycle species such as coffee, including the possibility to explore promising hybrids still segregating for non-target characteristics. However, a high level of somaclonal variation could ruin a potential genotype. In coffee, studies

Fig. 4 Relative quantification of gene expression along SE process considering Mother Plant as the calibrator stage for each genotype. Examples of genes displaying incomparable profiles



using molecular markers to compare *C. arabica* hybrids and their corresponding emblings indicated very low rates of somaclonal variation (Landey et al. 2013). Although this observation is an encouragement to use in vitro culture, the evaluation of emblings is important to assess the uniformity of the observed response on other genetic backgrounds.

The somatic embryogenesis involves steps of calluses tissue formation and differentiation, re-organization of competent cells to re-differentiate into specific cells, and, finally, the development of clone identical to the mother plant. Here, we selected candidate genes from different biological processes for gene expression analysis aiming to understand the molecular basis of somatic embryogenesis on coffee. Selected pathways include those related to DNA integrity maintenance, cell division and cell cycle control, organ development and plant regeneration, and response to oxidative stress derived from in vitro cultivation. For those gene expression analyses, we assessed all stages of somatic embryogenesis, and also mother plants and regenerated emblings.

DNA integrity

To assess the overall genome integrity and functionality, we selected two genes: DNA met and H2A. The involvement of these genes on epigenetic modifications of genomes makes them ideal targets to uncover occurrences of somaclonal variations. In a study evaluating epigenetic alterations on *C. canephora* somatic embryogenic tissues, Nic-Can et al. (2013) proposed a model where global DNA methylation and histone modifications control epigenetic changes that, in their turn, control the whole process. The study indicated that embryogenesis-related genes LEC, BBM1, and WOX4 are targets for this epigenetic control.

DNA methyltransferases are responsible for methylation of the 5' position of the pyrimidine ring from cytosines, which may cause gene silencing (Bowler et al. 2004; Fuchs et al. 2006). The observed increase in DNAm_{et} transcripts level at early development stages of IAC 1 and IAC 2, indicates a high activity of DNA methylation. In addition, the decrease on DNAm_{et} expression at the final stages of in vitro culture suggests a low level of DNA methylation.

The high levels observed on embryogenic callus are interesting, as high methylation levels may silence a large amount of genes, leaving active at this stage only those genes necessary for cell division and undifferentiated growth. In addition, the high transcript levels observed on IAC 2 emblings may suggest that gene silencing is still undergoing in these young tissues, affecting the other genes than the ones required to maintain cell identity and function. This observation could explain the delay on embryo development from IAC 2 callus compared to IAC 1 (Table 1).

Histones are proteins responsible for chromatin conformation, which possess several biological functions, such as DNA packaging and cell division, besides being responsible for the epigenetic control of gene expression (Bender 2004). Here, the H2A expression profile (Table 3) is comparable to the one observed for DNAm_{et} (Fig. 2), on both genotypes. This co-linearity of expression shows that both methylation and chromatin condensation are coordinated for gene regulation during the early stages of embryo development. As the expression for both genes decreases at the final stages of cultivation, reaching levels similar to the ones observed on the mother plant, probable epigenetics modifications might be reverted on later developmental stages.

Embryo development and regeneration

In vitro culture involves two contradictory aspects: first, an undifferentiated growth and maintenance of stem cells; at the end, a regeneration of a fully functional plant identical to the mother. Here, we selected embryogenesis-related genes, SERK and Wuschel, to characterize the first aspect, and the genes AP2 and EMF-2 for the second aspect.

The expression profile of genes CELL and CELL DIV (Table 3) implies an intense activity of cell multiplication during the embryogenic callus and multiplication stages, as expected in any SE process. In addition, low β -TUB transcript levels at these stages suggest that a disorganized growth, which followed by an increase of these transcripts during maturation stages, proposes cell re-organization into specialized tissues. These results demonstrate the progress of a regular SE in both genotypes.

The SERK gene is a traditional marker for somatic embryogenesis, and its expression indicates a totipotent cell state (Talapatra et al. 2014). In coffee, expression analyses of an orthologous SERK gene on embryogenic cells indicated that it might be a reliable marker for embryogenic potential of genotypes (Silva et al. 2014). In our study, SERK expression occurs mostly during in vitro culture, and both mother plants and emblings have very reduced transcript levels (Fig. 4), indicating a shift from a totipotent to a differentiate cell state. The expression pattern, however, varies between the cultivars, and may reflect the different potential for SE of each genotype.

Wuschel is a member of a homeobox gene family associated with stem cell and embryogenic status maintenance (Taiz and Zeiger 2009). In somatic embryos of *C. canephora*, WOX-4, a Wuschel-related gene, has an expression profile similar to the ones observed in this study (Nic-Can et al. 2013); there is no transcript accumulation in mother plants and emblings, the higher expression level occurs during the callus stages, and the levels decrease as the embryogenic tissue differentiates (Fig. 3). These results confirm the role of Wuschel expression as an activator of callus formation and maintenance (Arroyo-Herrera et al. 2008). In our study, we observed differences on both the accumulation profile and relative amount of Wuschel transcripts between genotypes. Transcript levels are significantly lower on IAC 2 embryogenic tissues and peak at an early stage compared to transcript profile on IAC 1 (Fig. 3). This profile may indicate a deficiency of IAC 2 on callus maintenance and differentiation, which explain its overall lateness and lower productivity.

The genetic origin may also account for differences on SERK and Wuschel expression values and overall embryogenesis response. IAC 1 and IAC 2 are hybrids of *C. arabica* crossed with *C. canephora* and *C. racemosa*, respectively. *C. canephora* is a very robust species, resistant to several pathogens, highly productive, and submitted to intense breeding (Alkimin et al. 2018; Ferrão et al. 2017;). On the other hand, *C. racemosa* is resistant to leaf miner, drought tolerant, but, with a poor cup quality, its use in breeding programs is limited (Guerreiro-Filho 2006; Guerreiro-Filho et al. 1999). In this context, *C. canephora* genotypes are genetically more uniform compared to *C. racemosa* ones, and, therefore, may result on hybrids more physiologically balanced. In addition, as *C. arabica* is an allotetraploid species, resulting from a natural hybridization between *C. eugenioides* and *C. canephora*, crosses with this last species are genetically compatible and, thus, more stable.

Differentiation of embryogenic tissues onto reproductive organs results from a genetic control, which include AP2 and EMF-2 expression. Both genes regulate organ morphogenesis and reproductive identity (Cang-Hsien et al. 1995). Our results show decrease of AP2 expression during SE, and re-activation in later stages, when reproductive organs develop. As observed with Wuschel, AP2 expression increases intensively on IAC 2 emblings, suggesting an unbalanced gene control at this stage. The gene EMF-2 has a similar behavior in both IAC 1 and IAC 2, with increasing expression on embryogenic callus and multiplication and differentiation phase, indicating that reproductive tissues may be under development. Results from both genes are in accordance with a healthy path towards plant regeneration.

Response to stress

In the same way to any in vitro cultivation, SE represents a source of stress to cells due to the continuous control of growth under specific conditions of nutrition, space, and differentiation. A strong response to these stresses may result in poor recovery of healthy plants. Here, we considered the expression patterns of LEA, OXIREN, PPO, EST, and IA to measure the strength of stress response.

LEA-related genes are a family of proteins associated with desiccation processes, such as these occurring during seeds and embryo development. Dussert et al. (2018) carried out an integrative study through genomic and microarray analyses of LEA accumulation during *C. arabica* seed development, a physiological process comparable to embryogenic cultivation. Although those authors could not explain how the LEAs contribute to the desiccation tolerance required on these processes, they reported a dramatic transcription switch characterized by the up-regulation of genes encoding LEA proteins with the acquisition of desiccation tolerance in embryos and endosperms of coffee seeds. In our study, LEA transcripts accumulate increasingly during the cultivation demonstrating the desiccation stress undergone by cultured cells (Fig. 3, Table 3). However, the decreasing on transcript levels at embling stages implies a full cell recovery. An interesting observation is that IAC 1 mother plant presented more than 30 times relative amount of LEA transcripts than IAC 2 at SE maturation and germination stage, and transcript accumulation extended until the nursery embling stage. We expected this behavior on IAC 2, which genetic background has *C. racemosa*, a species known for its drought tolerance and, therefore, likely associated with high expression of genes related to desiccation response. On the other hand, the LEA expression profile could also indicate an excessive stress response of IAC 1 emblings. In accordance to a normal stress response, expression of OXIREN, an oxidative stress-related gene, is low during the whole SE process (Table 3).

The PPO encodes polyphenol oxidase, an enzyme involved in several stress responses, and, therefore, represents an important marker for this. Here, PPO transcript levels increase during the in vitro embling stage, indicating a significant stress during acclimation period. However, the decreasing of transcript levels in the following stage indicates a recovery of their regular condition.

Another important aspect related to stress is the tissues and organs' overall survive condition. Here, we assess this aspect by evaluating EST and IA gene expression pattern. The EST transcript levels, a gene associated with telomeres integrity and cell aging, remain stable and at similar levels as those on mother plants (Fig. 2, Table 3), suggesting that in vitro culture has no impact on tissue age.

Similar results were observed for IA expression, a gene related to apoptosis (Table 3). Together, those results represent a positive indication which the SE does not lead to either intense stress or precocious senescence of coffee tissues.

Finally, the maintenance of desired traits, such as the defense response to biotic stress of IAC 1 and IAC2 and overall quality, is also an important aspect to evaluate on clonal coffee plants. Here, we considered expression pattern of EDS, JAZ, and PPT as markers to assess the potential defense response of clonal plants, and CS as marker of caffeine synthesis, the most known coffee component. Transcript accumulation on clonal plants of IAC 1 and IAC 2 is similar to mother plants for PPT, but lower for EDS and JAZ (Table 3). These genes are responsive to biotic stimulus and play important roles during stress and JA-mediated response (Devoto and Turner 2003). The observed differences suggest that the emblings are not as stressed as their mother plants, probably because they were growing under protected conditions. Further assays are under analysis to evaluate the response to biotic stress. The CS accumulation patterns are very irregular. Neither clonal emblings exhibit similar levels to mother plants, nor the expression pattern is consistent during SE (Fig. 4; Table 3). Once caffeine synthesis occurs on coffee young tissues, these results are intriguing, and an evaluation of caffeine levels on older clonal plants will confirm if these observations result from somaclonal variation. Therefore, a continuous evaluation of clonal plants is important to understand the effect of somatic embryogenesis on agronomic traits.

Based on the analyses here, we concluded that, despite the observed variations in gene expression during the process of indirect somatic embryogenesis in coffee, evaluated plants and clones showed the same pattern of expression for most of the studied genes. Those patterns suggest a genomic stability of clone plants and validating this methodology for coffee multiplication.

Author contribution statement OGF, LP, and MPM conceived the work. KCO, PSG, JMB, JCM, and MMS performed the analyses. PSG, JMB, and MPM wrote the manuscript. OGF and LP revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest We declare no potential interest conflicts associated with this study.

Human and animal rights There is no involvement of humans and animals. We consent access to our original data upon request.

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