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Genetic mapping of a caffeoyl-coenzyme A 3-O-methyltransferase gene in coffee trees. Impact on chlorogenic acid content

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Abstract Chlorogenic acids (CGA) are involved in the bitterness of coffee due to their decomposition in phenolic compounds during roasting. CGA mainly include caffeoyl-quinic acids (CQA), dicaffeoyl-quinic acids (diCQA) and feruloyl-quinic acids (FQA), while CQA and diCQA constitute CGA *sensu stricto* (CGA*s.s.*). In the two cultivated species *Coffea canephora* and *Coffea arabica*, CGA*s.s.* represents 88% and 95% of total CGA, respectively. Among all enzymes involved in CGA biosynthesis, caffeoyl-coenzyme A 3-O-methyltransferase (CCoAOMT) is not directly involved in the CGA*s.s.* pathway, but rather in an upstream branch leading to FQA through feruloyl-CoA. We describe how a partial cDNA corresponding to a CCoAOMT encoding gene was obtained and sequenced. Specific primers were designed and used for studying polymorphism and locating the corresponding gene on a genetic map obtained from an interspecific backcross between *Coffea liberica* var. Dewevrei and *Coffea pseudozanguebariae*. Offspring of this backcross were also evaluated for the chlorogenic acid content in their green beans. A 10% decrease was observed in backcross progenies that possess one *C. pseudozanguebariae* allele of the CCoAOMT gene. This suggests that CGA*s.s.* accumulation is dependent on the CCoAMT allele present and consequently on the activity of the encoded isoform, whereby CGA accumulation increases as the isoform activity decreases. Possible implications in coffee breeding are discussed.

Keywords Chlorogenic acids · Interspecific cross · *Coffea* · Gene mapping · CCoAOMT

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Introduction

Chlorogenic acids (CGA) in green coffee beans are major precursors of phenolic compounds in roasted beans. They are mainly responsible for coffee bitterness (Leloup et al. 1995). Chlorogenic acids are also involved in plant-pathogen interactions, increasing resistance to *Ceratocystis fimbriata* at high concentration, but stimulating pathogen growth at low concentration (Echandi and Fernandez 1962; Zuluaga et al. 1971). In tobacco, disease susceptibility to *Cercospora nicotianae* increases when their accumulation is inhibited (Maher et al. 1994). CGA content in green beans ranges from 0.14% (dry matter basis, dmb) in *Coffea rhamnifolia* Bridson to 11.3% (dmb) in *Coffea canephora* Pierre (Clifford and Jarvis 1988; Clifford et al. 1989; Anthony et al. 1993; Rakotomalala et al. 1993; Ky et al. 2001). CGA is a crucial parameter in breeding programs considering the high CGA content variations in the *Coffea* genus associated with their effects on organoleptic quality and disease resistance.

Sensu stricto CGA (CGA*s.s.*) includes only depsides of quinic acid with caffeic acid. They also commonly comprise other hydroxycinnamoyl conjugates such as ferulic or *p*-coumaric acid derivatives. In green coffee beans, three classes represent about 98% of the CGA content (Clifford and Staniforth 1977; Morishita et al. 1989): caffeoyl quinic acids (CQA), dicaffeoyl quinic acids (diCQA) and feruloyl quinic acids (FQA). Each has three isomers according to the position of the acylating residue (Clifford 1985a, b). The proportion of CQA ranges from 68% in *C. canephora* (Ky et al. 2001) to 100% in *C. rhamnifolia, Coffea farafanganensis* J.F. Ler. and *Coffea perrieri* Drake ex Jaarb. (Anthony et al. 1993). The relative diCQA content is maximum in *Coffea brevipes* Hiern (20% CGA), whereas the proportion of FQA in CGA reaches 13% in *C. canephora* (Anthony et al. 1993; Ky et al. 2001).

In coffee leaves, two biosynthesis pathways lead to 5-CQA production (Colonna 1986). Two precursors, i.e. *p*-coumaroyl quinic acid and caffeic acid, were highlighted by this author, with *p*-coumaroyl quinic acid being the

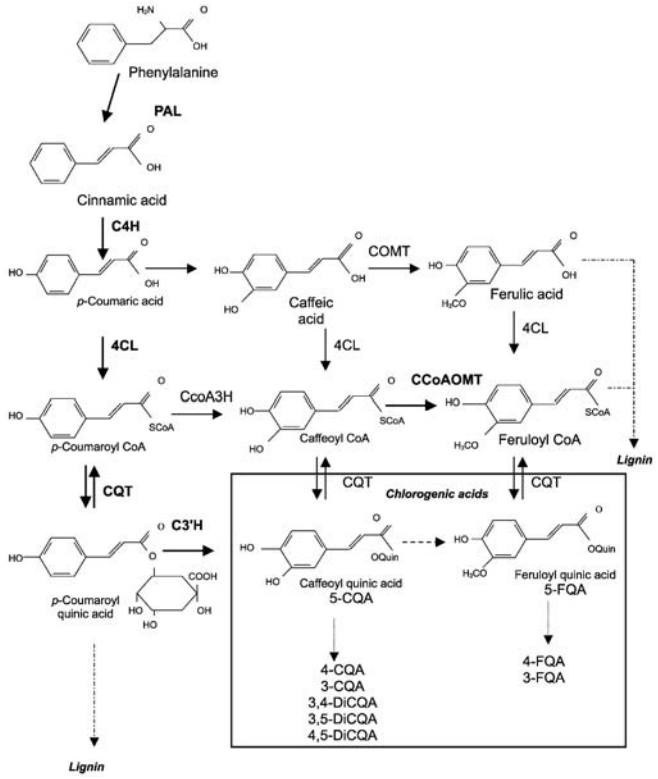

Fig. 1 Different theoretical ways of chlorogenic acid synthesis. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-(hydroxy)cinnamoyl-CoA ligase; CQT, hydroxycinnamoyl-CoA:D-quinate hydroxycinnamoyltransferase; COMT, caffeic-acid/5-hydroxyferulic acid *O*-methyltransferase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; C3′H, *p*-coumaroyl-shikimate/quinate 3′-hydroxylase; DiCQA: dicaffeoyl quinic acid

preferential way (Fig. 1). In *Arabidopsis thaliana* (Schoch et al. 2001) and *Daucus carota* (Kühnl et al. 1987), *p*-coumaroyl shikimate/quinate 3′-hydroxylase (C3′H) leads directly to 5-CQA production from *p*-coumaroyl quinic acid. The second way (caffeic acid) begins first with the formation of caffeoyl-CoA by the 4-(hydroxy)cinnamoyl-CoA ligase (Fig. 1). In *Coffea arabica* L., caffeoyl-CoA is then esterified by hydroxy-cinnamoyl-CoA:quinate hydroxycinnamoyl transferase (CQT) (Ulbrich and Zenk 1979). Two substrates, i.e. *p*-coumaroyl CoA and caffeoyl CoA, can be esterified by CQT, which is also reversible (Rhodes and Wooltorton 1976; Ulbrich and Zenk 1979). This reversibility leads to the formation of caffeoyl CoA from 5-CQA and is responsible for the production of feruloyl CoA via the CCoAOMT (Schoch et al. 2001).

Caffeoyl-Coenzyme A 3-*O*-methyltransferase (CCoAOMT) (EC 2.1.1.104) was initially considered as a methyltransferase involved in the formation of cell-wall ferulic esters, i.e. compounds involved in defense reactions (Pakusch et al. 1989; Schmitt et al. 1991). Its involvement in lignin biosynthesis has been suggested in *Zinnia* (Ye et al. 1994) as an alternative methylation pathway (Boudet et al. 1995). Recent studies on transgenic plants demonstrated the essential role of CCoAOMT in lignin biosynthesis for a number of herbaceous dicotyledonous species (Zhong et al. 1998) and some woody species (Zhong et al. 2000). In alfalfa, CCoAOMT seems to be preferentially involved in G lignin formation, while caffeic acid-3-O methyltransferase (COMT) is involved in S lignin formation (Inoue et al. 1998). As mentioned above, CCoAOMT could also be implicated in feruloylquinic acid biosynthesis. Its gene appears to be a candidate marker to explain chlorogenic variations in coffee green beans.

The first backcross generation between *Coffea pseudozanguebariae* Bridson (PSE) and *Coffea liberica* Hiern var. Dewevrei De Wild et Dur. (DEW) was evaluated for chlorogenic acid content and a genetic map was drawn up using AFLP markers (Ky et al. 1999, 2000). Based on the same progeny, the genetic diversity of genes encoding some enzymes of the CGA pathway was studied by PCR using specific primers for each chosen gene. This paper presents the results obtained for a gene encoding CCoAOMT in *C. canephora*.

Materials and methods

Plant material

The plant material was maintained at the IRD Agricultural Station (Man, Côte d'Ivoire). The cross between *C. pseudozanguebariae*; genotype 8044 used as a female parent, and *C. liberica* var. Dewevrei, genotype 5851 used as a male parent, was made by hand-pollination under bags (Louarn 1992). Twenty five F1 hybrids were obtained and grown on the same plot at the station. Sixty two backcross hybrids on DEW (BCDEW) were obtained by open-pollination of 13 F1 hybrids (used as a female parent).

DNA purification

Total DNA was purified from DEW, PSE and 61 backcross offspring according to the previously described protocol of Ky et al. (2000).

DNA sequence analysis

Analysis of DNA sequences, including determination of ORFs and the derivation of predicted protein sequences, homology searches, multiple alignments, deduction of consensus sequences and primer design, were carried out using programs from the Lasergen package (Version 1.66; DNASTAR Incorporated, Madison, Wis., USA). Databank similarity searches were performed with the Blastx program maintained at NCBI[1].

Primer design

Sequences were aligned using the clustal method of the Megalign program from the DNASTAR package, with a gap penalty of 10 and a gap length penalty of 10. A multiple alignment was performed using 13 GenBank accessions for CCoAOMT mRNA sequences. (*Oryza sativa* 5091496 and 5257255, *Populus kitakamiensis* AB000408, *Populus tremuloides* U27116, *Mysembryanthemum crystallinum* AF053553, *Petrosilium crespum* M69184, *Medicago sativa* U20736, *Eucalyptus gunnii* Y12228,

[1] http://www.ncbi.nlm.nih.gov:80/BLAST/

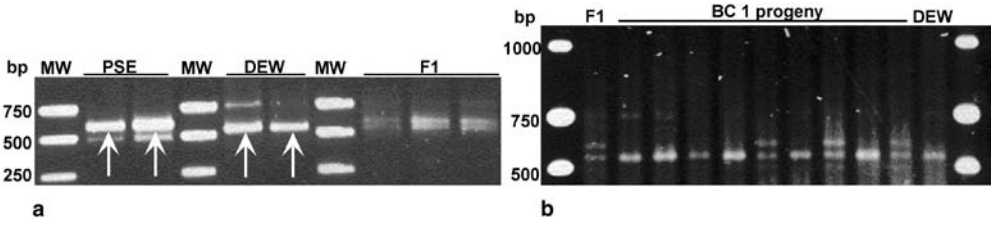

Fig. 2a, b CCoAOMT gene polymorphism between *C. pseudozanguebariae* (PSE) and *C. liberica* var. Dewevrei (DEW). **a** CCoAMT PCR pattern observed in the two parents *C. pseudozanguebariae* (PSE) and *C. liberica* var. Dewevrei (DEW), and in the F1 progeny (F1). *Arrows* indicate positive Southern-blot bands. **b** CCoAMT PCR pattern observed in some backcross progenies (BC1), the F1 and the DEW recurrent parent

Nicotiana tabacum Z56282, *Vitis vinifera* Z54233, *Zea mays* AJ242981 and AY104406, *Zinnia elegans* U13151). Two conserved domains, separated by approximately 320 base pairs (bp), were selected and primers were designed according to the consensus sequence of these domains. The forward primer (5′-GGTGTTTACACTGGCTACTCTCT-3′) is located between nucleotides 259 to 281 of the consensus sequence, and the reverse primer (5′-CCATAGGGTGTTGTCGTAGCC-3′) extends from nucleotide 559 to 579 on the same sequence.

PCR conditions, cloning and sequencing

PCR reactions were performed using the standard protocol recommended by the *Taq* DNA polymerase manufacturer (Promega, Madison Wis., USA) with an annealing temperature of 50 °C. The designed primers were used to amplify a fragment from a *C. canephora* fruit cDNA library. The resulting fragment was then purified using the EZNA gel extraction kit (Omega Bio-Tek, Doraville, Ga., USA) according to the manufacturer's recommendations. The purified fragment was cloned in a pCR4-TOPO plasmid using the "TOPO TA for sequencing" kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's recommendations. Cloned fragments were sequenced by MWG-Biotech (Ebersberg, Germany).

Southern-blot analysis

In case of multiple fragments amplified by PCR reactions, DNAs were electrophoresed on 1% agarose gel, transferred onto Hybond N or N+ membranes (Amersham, Piscataway, N.J., USA), and hybridization and washing were carried out as described by Sambrook et al. (1989). The DNA probe (323 bp) consisted of a purified amplified fragment from a *C. canephora* fruit cDNA library. The probe was randomly radiolabeled with nucleotides (Amersham) using the rediprime II kit (Amersham). After washing, the membranes were placed in a cassette on Kodak XAR-5 film with an intensifying screen at –80 °C to visualize the labeled DNA fragments.

Biochemical data and statistical analysis

Chlorogenic acid-content data are compiled on a separate data file containing all evaluations on backcross hybrids (morphological, biochemical, phenological, molecular data). Details on sample preparation, extraction and HPLC analysis are described in Ky et al. (1999). Quantitative effects of CCoAOMT on CGA were analysed using a one-way ANOVA with a fixed effect. The CCoCAOMT gene was then mapped using the MapDisto 1.2.0.3 software package (2002) Lorieux[2].

Results

Obtention of a partial cDNA sequence of CCoAOMT

PCR amplification with a *C. canephora* fruit cDNA library used as a template gave rise to one 323-bp band that was cloned after purification. Two different clones obtained independently, but from the same amplification reaction, were sequenced (accession number AF534905). These clones showed 100% homology between them and were 90.7% identical, at the amino-acid level, to a putative CCoAOMT from *Fragaria vesca* L. (accession # CAA04769, Blastx score = 210, expected value $2e^{-54}$) and 89.7% identical to a CCoAOMT from a *Medicago sativa* L. (accession # T09399, Blastx score = 207, expected value $2e^{-53}$). All similarities detected by the Blastx search were with CCoAOMT sequences from different species. This clone was then considered as being a partial cds of a *C. canephora* putative caffeoyl CoA 3-O-methyltransferase.

Highlighting two alleles specific to PSE and DEW of a CCoAOMT coding gene

PCR amplifications of total DNA extracted from DEW and PSE performed with the consensus primers revealed one or two bands for each species (Fig. 2). After confirmation of the band identities by Southern blotting using the amplified fragment from the cDNA library as a probe, DEW was found to have a slightly shorter amplified fragment than that obtained from PSE (around 600 and 650 bp, respectively). As expected, F1 hybrids had both bands. The size difference between the observed amplified fragments suggests that at least one intron is located between the two regions corresponding to the designed primers. The total length of this (these) intron(s) is about 275 bp for DEW and 325 bp for PSE.

Among 61 backcross hybrids, 31 showed the F1 hybrid pattern and 30 exhibited the DEW pattern. The observed segregation complied with the 1:1 ratio expected for mono-locus Mendelian segregation.

[2] http://www.mpl.ird.fr/mapdisto/

Table 1 Effect of CCoAOMT on chlorogenic acid contents in BCDEW hybrids. Columns DD and DP give observed means of homozygote (DEW/DEW) and heterozygote (DEW/PSE) hybrids for CCoAOMT alleles, respectively. Column PP gives expected means of homozygote hybrids (PSE/PSE) under the codominance hypothesis (computed only when the ANOVA results were significant)

Item	DD	DP	PP	F	*p*
CGA	6.04	5.49	4.94	6.53	0.014
CGA*s.s.*	5.64	5.04	4.40	8.16	0.006
CQA	4.73	4.20	3.67	7.15	0.010
diCQA	0.91	0.83	–	2.52	0.118
FQA	0.40	0.46	–	0.96	0.331
4- & 5-CQA	4.41	3.92	3.43	6.86	0.012
3-CQA	0.31	0.28	–	3.31	0.075
3,5-diCQA	0.20	0.18	–	3.41	0.071
3,4-diCQA	0.22	0.21	–	0.74	0.393
4,5-diCQA	0.49	0.44	–	2.29	0.137
5-FQA	0.30	0.35	–	0.82	0.370
4-FQA	0.08	0.09	–	0.32	0.577
3-FQA	0.02	0.02	–	2.34	0.134

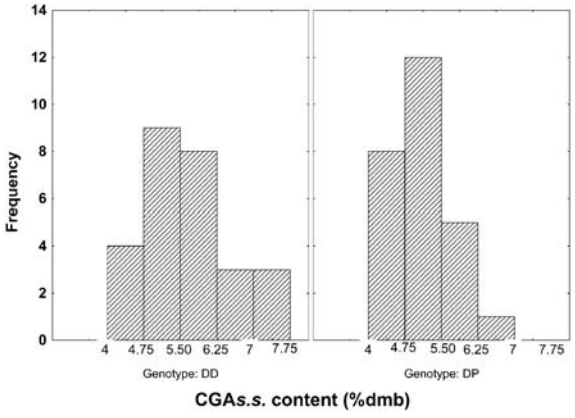

Fig. 3 Distribution of CGA*s.s.* for DD and DP genotypes in BCDEW progeny

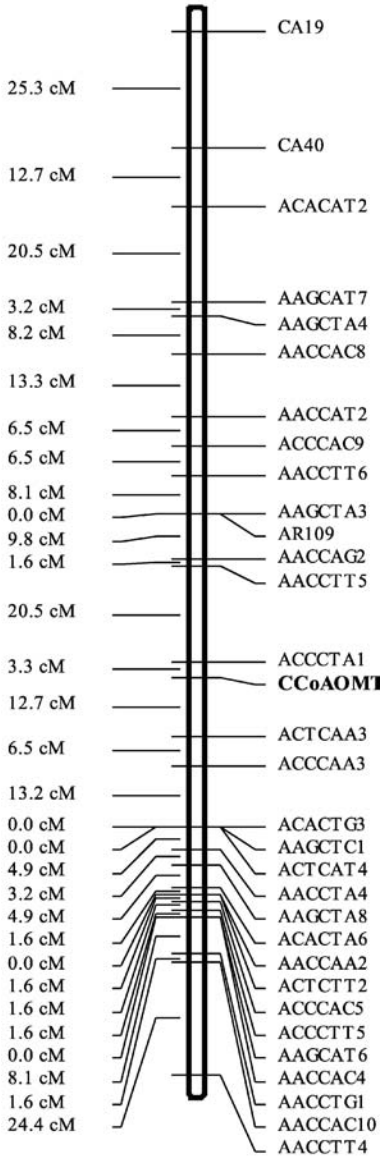

Fig. 4 Linkage group A showing the location of the CCoAOMT gene at 3.3 cM of the AFLP marker ACCCTT1

Effects of alleles on chlorogenic acids and CCoAOMT gene mapping

Among the backcross progeny, DEW-DEW (DD) and DEW-PSE (DP) genotypes differed significantly with respect to CGA content (Table 1). Nevertheless, when CGA was split into CGA*s.s.* and FQA, the effects on CGA appeared to be only on CGA*s.s.* without any difference in FQA content.

The CGA*s.s.* content was 5.64% dmb and 5.04% dmb in the (DD) and (DP) genotypes, respectively, which led us to expect a 4.40% dmb level in (PP) genotypes under the codominance hypothesis for allelic effects. Figure 3 clearly shows that mean differences between the (DP) and (DD) genotypes did not result from a distribution shift because the minimum did not change between the two distributions. The mean decrease was actually due to a variance decrease without any decrease in the minimum value.

The CCoAOMT gene was located on linkage group A of the BCDEW genetic map (Ky et al. 2000) at 3.3 cM of the ACCCTA1 AFLP marker and 12.7 cM of the ACTCAA3 AFLP marker (Fig. 4).

Discussion

Four main results were obtained: (1) a partial sequence of a putative CCoAOMT cDNA; (2) evidence of a sequence difference between PSE and DEW, certainly due to a putative intron; (3) localisation of the corresponding gene on linkage group A; and (4) a relationship between the presence of the PSE allele in the hybrid and the chlorogenic acid content in their green beans.

Identification of the obtained sequence

Two PCR-amplified DNA fragments obtained from the CAN fruit cDNA library were found to be perfectly homologous, suggesting that they originated from the same cDNA. A Blastx search performed on non-redundant protein databases indicated that the obtained sequences were very similar to CCoAMT encoding mRNA (up to 90.7% homology). PCR amplifications of total DNA purified from DEW and PSE gave longer fragments than those amplified from the fruit cDNA library, indicating the presence of at least one intron. Descriptions of the genomic sequences of putative CCoAMT genes indicated that intron numbers, lengths and locations are highly variable in the plant kingdom, e.g. one intron in *Citrus natsudaida* (107 bp), two in *O. sativa* L. (89 and 112 bp) and four in *Petroselinum crispum* (Mill.) Nym. (108, 264, 127 and 160 bp). The amplified fragment from the CAN cDNA library covers the region containing one intron of *C. natsudaida*, two introns of *O.sativa* and introns #3 and # 4 of *P. crispum*. As the fragments issued from DEW and PSE, total DNA amplification was not cloned and sequenced, and we could not determine whether or not they contained one or more introns; but the relatively high conservation of all available CCoAMT mRNA and amino-acid sequences strongly suggested that at least one intron was present in these sequences.

Hypotheses on the effect of the CCoAOMT gene on chlorogenic acid content

The PSE allele induced a mean 11% decrease in CGA*s.s.* content in green beans of BCDEW hybrids. Two hypotheses could be put forward to explain this decrease: (1) a linkage desequilibrium; or (2) a CCoAOMT gene impact.

In the first case, the gene encoding CCoAOMT would not have any effect on CGA*s.s.* content, but would be closely linked to another gene involved in CGA*s.s.* biosynthesis. The PSE allele effect should thus decrease in successive backcross generations due to recombination events between both genes.

In the second case, the gene encoding CCoAOMT would at least partly control the CGA*s.s.* content in green beans. In contrast with the previous hypothesis, the PSE allele effect should be constant in successive backcross generations, with regards to the mean DEW allele effect. Based on this second hypothesis, we could try to understand how the CCoCAOMT gene could act on CGA*s.s.* content.

Effect of the PSE allele consisted of a decrease in CGA*s.s.* content in green beans. This CGA group includes six quinic esters of caffeic acid (5-, 4-, 3-CQA and 4,5-, 3,4-, 3,5-diCQA), all derived from a pathway starting with 5-CQA (Fig. 1). In this figure, two pathways lead to 5-CQA biosynthesis: esterification of quinic acid with caffeoyl CoA by CQT (Stöckigt and Zenk 1974; Colonna 1986), and *p*-Coumaroyl quinic acid hydroxylation by C3′H (*p*-Coumaroyl-shikimate/quinate 3′hydroxylase) (Colonna 1986; Kühnl et al. 1987; Schoch et al. 2001). Higher CCoAOMT activity would decrease its substrate content, i.e. caffeoyl CoA, and consequently CGA*s.s.* content through the reversible CQT.

Contrasting with its previous effect, the PSE allele of CCoCAOMT had no impact on feruloyl quinic acids. Higher CCoAOMT activity should also increase the level of feruloyl CoA, which is a FQA precursor (Fig. 1). The absence of the PSE allele effect on FQA content in green beans is surprising. The PSE allele may code for a CCoAOMT that is only involved in the formation of feruloyl CoA for lignification, or FQA synthesis may be regulated by the remaining lignin pathway. Other explanations could be: (1) CQT is not involved in the FQA biosynthesis when feruloyl CoA is used as precursor; and (2) the CCoCAOMT effect on FQA was diluted among other FQA biosynthesis controls. Indeed, we noted that the FQA content in green beans was: (1) highly variable within DEW (0.24–0.82%) and within PSE (0.04–0.13%), (2) environmental effects and genotype × environment interactions represented 41% of this within-species variation, and (3) there is no difference in mean and range when comparing DEW and BCDEW (Ky et al. 1999).

Impact of the PSE allele of the CCoAOMT gene on the hybrid phenotype in terms of CGA*s.s.* content

To understand the PSE allele effect, it is important to note that the minimum values in the two groups of hybrids (DD) and (DP) were similar (Fig. 3). In addition, this minimum corresponded to that expected in F1 hybrids (4.01% dmb) under the additive hypothesis (CGA*s.s.* content is 1.44 and 7.51% dmb in PSE and DEW, respectively; Ky et al. 1999). In BCDEW, some hybrids have an F1 value irrespective of the genotype (DP) or (DD) for the CCoAOMT gene. The first means that other genes would control CGA*s.s.* content. Secondly, hybrids with (DD) for the CCoAOMT gene, but with F1 *CGAs.s.* content, should be (DP) for at least one of these other genes. Thirdly, this heterozygosity would have a limiting epistatic effect on CGA*s.s.* content.

The effect of the PSE allele concerned the maximum value of the distribution, which was higher in hybrids with (DD) for the CCoAOMT gene (Fig. 3). Such hybrids showed a CGA*s.s.* content close to that of DEW (7.51% dmb; Ky et al. 1999). They would be (DD) for all genes controlling CGA*s.s.* content. Hybrids with (DP) for the CCoAOMT gene and showing the highest value for this group would be (DD) for all other genes controlling CGA*s.s.* content. Consequently, under the codominance hypothesis, it would be possible to estimate the CGA*s.s.* content of hybrids whose PSE CCoAOMT gene in DEW is fully introgressed [such a hybrid would be (DD) for all genes, but (PP) for the CCoAOMT gene]. Its CGA*s.s.* content would thus be about 6.2% dmb.

Prospects for coffee breeding

On the basis of our results, interspecific CCoAOMT gene polymorphism could lead to a decrease in the CGA*s.s.* content in green beans of *C. canephora* or *C. liberica* var.

Dewevrei when the PSE allele is introgressed at the homozygote level (PP). A decrease of about 22% could be expected. Nevertheless, we are still far from the CGA*s.s.* content of *C. pseudozanguebariae* green beans. Other genes that have strong limiting effects and probably located upstream of the 5-CQA biosynthesis should therefore be sought. This could concern CQT, C3′H, 4CL, C4H and PAL (see Fig. 1). The same process will be carried out for all of these enzymes: (1) seeking database evidence of consensus zones in the gene; (2) detecting interspecific polymorphism between DEW, or CAN, and PSE; (3) mapping the loci; (4) evaluating Mendelian inheritance; and (5) assessing the impact on CGA content.

The second point will concern the hypothesis on the effect of the PSE allele on CGA*s.s.* content. Is the PSE CCoAOMT allele physiologically responsible for the decrease in the CGA*s.s.* content, or is it a marker of another gene (CQT, C3′H, etc.) involved in determining the CGA*s.s.* content? The response could be obtained through the second backcross generation. The lowering of the effect would mostly likely be proportional to the genetic distance (expressed in cM) between the CCoAOMT and another gene on linkage group A. Consequently, the use of the marker in assisted selection in further introgressed backcross generations would last longer when the genetic distance is shorter.

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