Supplementary Information

An investigation of zinc isotope fractionation in cacao (*Theobroma cacao* L.) and comparison of zinc and cadmium isotope compositions in hydroponic plant systems under high cadmium stress

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Table S1. Cacao clone names, accession numbers and donor organizations of the cacao germplasms used in this study.

Cacao Clones (Genotype) Name	**Accession number (ICQC, R)**	**Donor Collection**
B 5/7 [POU]	RUQ 522	ICG, T
Catie 1000	RUQ	CIRAD
CC 41	RUQ	ICG, T
CL 19/10	RUQ	ICG, T
GU 207/H	RUQ	CIRAD
GU 236/V	RUQ	CIRAD
IMC 27	RUQ	ICG, T
LP 1/41 [POU]	RUQ	ICG, T
Matina 1-7	RUQ	ICG, T
NA 702	RUQ	ICG, T
PNG 340	RUQ	CIRAD
Pound 12/A [POU]	RUQ	ICG, T
RB 46	RUQ	CATIE
RIM 179	RUQ	CATIE
SCA 9	RUQ	ICG, T
SPA 9 [COL]	RUQ	ICG, T
TARS 31	RUQ	USDA-TARS
TSA 654	RUQ	CEPLAC/CEPEC
U 70 [PER]	RUQ	UNAS

CATIE	Centro Agronómico Tropical de Investigión y Enseñanza (Costa Rica)
CEPLAC	Comissão Executiva do Plano da Lavoura Cacaueira (Brazil)
CIRAD	Centre de cooperation international en recherché agronomique pour le développement (France)
ICG, T	International Cocoa Genebank (Trinidad)
ICQC, R	International Cocoa Quarantine Centre, Reading (UK)
UNAS	Universidad Nacional Agraria de la Selva (Peru)
USDA-TARS	United States Department of Agriculture, Tropical Crops and Germplasm Research, Mayaguez (Puerto Rico)

Table S2. Composition of the half-strength Hoagland solution including additional $CdCl_2$.

Component	Species	Concentration	
		mg L^{-1}	**µmol L^{-1}**
Major nutrients			
KH_2PO_4	K^+	19.55	500
	PO_4^{2-}	47.49	500
	H^+	3.002	1500
KNO_3	K^+	97.75	2500
	NO_3^-	155.0	2500
$Ca(NO_3)_2 \cdot 4H_2O$	Ca_2^+	100.2	2500
	NO_3^-	310.0	5000
$MgSO_4 \cdot 7H_2O$	Mg^{2+}	24.31	1000
	SO_4^{2-}	96.07	1000
Micronutrients			
H_3BO_3	H_3BO_3	1.430	23.1
Fe-EDTA[a]	Fe^{3+}	2.383	42.7
	$EDTA^{4-}$	12.30	42.7
$MnCl_2 \cdot 4H_2O$	Mn^{2+}	0.253	4.6
	Cl^-	0.326	9.2
$ZnSO_4 \cdot 7H_2O$[a]	Zn^{2+}	0.025	0.38
	SO_4^{2-}	0.037	0.38
$CuSO_4 \cdot 5H_2O$	Cu^{2+}	0.010	0.2
	SO_4^{2-}	0.015	0.2
$Na_2MoO_4 \cdot 2H_2O$	Na^+	0.002	0.1
	MoO_4^{2-}	0.003	0.04
$CdCl_2$[b]	Cd^{2+}	2.248	20.0
	Cl-	1.418	20.0

[a]$C_{10}H_{12}FeN_2O_8$. [b]After 28 day growing period, $CdCl_2$ was introduced to the hydroponic solutions for an additional 14 days.

Table S3. Zinc concentrations ([Zn]; mg kg^{-1}) and stable isotope compositions (δ^{66}Zn) for quality control materials.

			[Zn] ± SD mg kg^{-1}		δ^{66} Zn ± 2SD ‰	
Material	**Type**	**n**	**This study**	**Literature**	**This study**	**Literature**
London Zinc	Pure Zn solution	30	-	-	+0.10 ±0.06	+0.12 ± 0.06[a]
NIST SRM 1570a	Spinach leaf	19	79 ± 6	82 ± 4[b]	+0.41 ±0.06	-

All stable isotope compositions (δ^{66}) are reported relative to JMC-Lyon Zn. n represents the number of individual samples that were analysed. [a]The δ^{66}Zn ± 2SD (‰) is the mean value reported in Archer et al. (2017). [b] The Zn concentration data outlined in the original certificate. SD represents standard deviation. 2SD for individual samples represents the analytical precision of the Zn stable isotope data determined from multiple analyses of the Zn isotope standard (AA-ETH-Zn) that bracketed the sample runs.

Table S4. Summary of mass fractions (*f*), Zn concentrations ([Zn]; mg kg^{-1}) and Zn stable isotope compositions (δ^{66}Zn) for leaves, stems and roots determined for biological and analytical replicates.

Cacao Clones (Genotype)	Replicate	Leaf				Stem				Root				Total Plant
		f	[Zn] mg kg^{-1}	δ^{66}Zn ‰	2SD ‰	*f*	[Zn] mg kg^{-1}	δ^{66}Zn ‰	2SD ‰	*f*	[Zn] mg kg^{-1}	δ^{66}Zn ‰	2SD ‰	[Zn] mg kg^{-1}
CC 41[a]	1	0.66	24	−0.82	0.06	0.25	18	−0.19	0.06	0.10	16	0.58	0.06	21
	2	0.73	22	−0.75	0.02	0.18	19	−0.14	0.08	0.09	14	0.44	0.08	20
	3	0.77	35	−0.41	0.07	0.17	19	−0.20	0.08	0.06	11	0.50	0.08	27
Mean	-	**0.72**	**27**	**−0.66**	-	**0.20**	**19**	**-0.18**	-	**0.08**	**14**	**0.51**	-	**23**
1SD	-	**0.05**	**7**	-	-	**0.04**	**1**	-	-	**0.02**	**2**	-	-	**4**
2SD	-	-	-	**0.36**	-	-	-	**0.05**	-	-	-	**0.11**	-	-
GU 207/H[a]	1	0.68	27	−0.43	0.06	0.15	27	−0.44	0.06	0.17	18	0.33	0.03	25
	2	0.77	36	−0.43	0.03	0.16	32	−0.39	0.08	0.06	13	0.48	0.03	32
	3	0.73	33	−0.44	0.04	0.14	26	−0.42	0.07	0.12	18	0.38	0.08	29
Mean	-	**0.73**	**32**	**−0.43**	-	**0.15**	**28**	**−0.42**	-	**0.12**	**16**	**0.40**	-	**29**
1SD	-	**0.04**	**4**	-	-	**0.01**	**3**	-	-	**0.04**	**2**	-	-	**3**
2SD	-	-	-	**0.01**	-	-	-	**0.05**	-	-	-	**0.15**	-	-
Matina 1-7[b]	1	0.65	20	−0.68	0.09	0.27	23	−0.63	0.08	0.08	13	0.01	0.08	20
	1	0.55	19	−0.78	0.06	0.35	33	−0.63	0.04	0.09	16	−0.04	0.08	22
Mean	-	**0.60**	**20**	**−0.73**	-	**0.31**	**28**	**−0.63**	-	**0.09**	**15**	**−0.02**	-	**21**
1SD	-	**0.05**	**1**	-	-	**0.04**	**5**	-	-	**0.01**	**2**	-	-	**1**
2SD	-	-	-	**0.14**	-	-	-	**0**	-	-	-	**0.07**	-	-

[a] Biological replicates; single digests of leaf, stem and root samples from three separate plants of CC 41 and GU 207/H. [b] Analytical replicates; duplicate digests of leaf, stem and root samples from a single plant of Matina 1-7. SD represents the standard deviation; 1SD is used for *f* and Zn concentrations ([Zn]) and 2SD for Zn stable isotope compositions (δ^{66}Zn). 2SD for individual samples represents the analytical precision of the Zn stable isotope data determined from multiple analyses of the Zn isotope standard that bracketed the sample runs.

Table S5. Dry weights (g) of the organs from plants treated with 20 μmol L^{-1} $CdCl_2$.

Genotype	Leaf g	Stem g	Root g	Total Plant g
B 5/7 [POU]	0.554	0.246	0.092	0.892
Catie 1000	0.304	0.122	0.127	0.553
CC 41[a]	0.584	0.220	0.125	0.929
CL 19/10	1.361	0.289	0.159	1.809
GU 207/H[a]	0.393	0.094	0.115	0.602
GU 236/V	0.332	0.089	0.144	0.565
IMC 27	0.301	0.147	0.132	0.580
LP 1/41 [POU]	0.635	0.259	0.101	0.995
Matina 1-7	1.387	0.499	0.269	2.155
NA 702	1.181	0.341	0.198	1.720
PNG 340	0.940	0.551	0.206	1.697
Pound 12/A [POU]	0.525	0.139	0.121	0.785
RB 46	0.512	0.140	0.134	0.786
RIM 179	1.298	0.334	0.299	1.931
SCA 9	0.668	0.262	0.122	1.052
SPA 9 [COL]	0.535	0.218	0.127	0.880
TARS 31	0.534	0.181	0.280	0.995
TSA 654	0.616	0.153	0.138	0.907
U 70 [PER]	0.247	0.084	0.068	0.399
Mean	**0.679**	**0.230**	**0.156**	**1.065**
1SD	**0.370**	**0.131**	**0.065**	**0.527**

[a]Mean values for the single leaf, stem, and root aliquots of three separate plants of the CC 41 and GU 207/H genotypes (n = 3). SD represents the standard deviation.

Table S6. Summary of Zn concentration data (mg kg^{-1}) for cacao leaves from this study and the literature.

Reference	Country	Cacao leaf Zn concentrations (mg kg^{-1})
This Study	-	14 – 63
Gramlich et al. (2018)	Honduras	17 – 381
Barraza et al. (2021)	Ecuador	17 – 263
Gramlich et al. (2017)	Bolivia	36.8 – 48.8

Table S7. Summary of mean dry weights (g), mass fractions (*f*), Zn concentrations ([Zn]; mg kg^{-1}) and Zn stable isotope compositions (δ^{66} Zn) of leaves, stems and roots and total plant dry weights (g) for seedlings of the NA 702 genotype treated with 0, 5, and 20 µmol L^{-1} $CdCl_2$.

$CdCl_2$ µmol L^{-1}	n	Leaf					Stem					Root					Total Plant
		Dry weight g	*f*	[Zn] mg kg^{-1}	δ^{66}Zn ‰	2SD ‰	Dry weight g	*f*	[Zn] mg kg^{-1}	δ^{66}Zn ‰	2SD ‰	Dry weight g	*f*	[Zn] mg kg^{-1}	δ^{66}Zn ‰	2SD ‰	Dry weight g
0	3	1.22	0.63	15	−0.50	0.06	0.38	0.22	18	−0.50	0.06	0.23	0.16	20	0.21	0.04	1.83
5	2	1.19	0.69	15	−0.43	0.07	0.31	0.24	21	−0.52	0.05	0.20	0.07	10	0.23	0.07	1.70
20	1	1.18	0.71	16	−0.70	0.08	0.34	0.20	16	−0.28	0.07	0.20	0.09	12	0.40	0.06	1.72
Mean		**1.20**	**0.66**	**16**	**−0.51**	**-**	**0.35**	**0.22**	**18**	**−0.46**	**-**	**0.21**	**0.12**	**15**	**0.25**	**-**	**1.77**
1SD		**0.06**	**0.05**	**2**	**-**	**-**	**0.06**	**0.03**	**5**	**-**	**-**	**0.03**	**0.05**	**5**	**-**	**-**	**0.09**
2SD		**-**	**-**	**-**	**0.29**	**-**	**-**	**-**	**-**	**0.21**	**-**	**-**	**-**	**-**	**0.14**	**-**	**-**

n represents the number of individual samples that were digested and analysed. SD represents standard deviation; 1SD is used for dry weight, *f* and Zn concentrations ([Zn]) and 2SD for Zn stable isotope compositions (δ^{66}Zn). 2SD for individual samples represents the analytical precision of the Zn stable isotope data determined from multiple analyses of the Zn isotope standard that bracketed the sample runs.

Table S8. Summary of $\Delta^{66}Zn$ for uptake, and Zn stable isotope compositions ($\delta^{66}Zn$) for root, shoot and leaf published for different plant species.

Reference	Plant Species	Zn concentrations in nutrient sources	$\Delta^{66}Zn$ represents	$\Delta^{66}Zn$ ‰[a]	Root $\delta^{66}Zn$ ‰	Shoot $\delta^{66}Zn$ ‰	Leaves $\delta^{66}Zn$ ‰
This Study	*Theobroma cacao* L.	0.38 µmol L^{-1}	Total plant–hydroponic solution	−1.21 to −0.73 and *−1.49 to −0.86*	−0.28 to +0.68	-	−1.11 to −0.43
Aucour et al. (2011)	*Arabidopsis halleri* and *Arabidopsis petraea*	10 and 250 µmol L^{-1}	Total plant–hydroponic solution	−0.36 to +0.00	+0.19 to +0.96	−0.54 to +0.16	-
Aucour et al. (2017)	*Typha latifolia*	2089 mg kg^{-1}[g]	Total plant–soil	−0.50[b]	+0.03[b]	-	−0.32[b]
Aucour et al. (2015)	*Phalaris arundinacea*	2129 mg kg^{-1}[g]	Total plant–soil	−0.60[b]	+0.24[b]	-	−0.19[b]
Deng et al. (2014)	*Thlaspi arvense*, *Alyssum murale and Noccaea caerulescens*	2 and 50 µmol L^{-1}	Total plant–hydroponic solution	−0.23 to +0.20[b]	+0.17 to +1.12	−0.59 to +0.23	-
Tang et al. (2016)	*Noccaea caerulescens* and *Thlaspi arvense*	0.02, 1, 5 and 50 µmol L^{-1}	Total plant–hydroponic solution	−0.26 to −0.06[b]	+0.14 to +0.82	−0.26 to +0.13	-
Houben et al. (2014)	*Agrostis capillaris* L.	9470 and 56600 mg kg^{-1}[g]	Total plant–soil leachate	−0.04 and +0.02[f]	+0.17 to +0.36[f]	−0.10 to −0.05[f]	-
Tang et al. (2012)	*Noccaea caerulescens and Silene vulgaris*	107, 115, 2220 and 2396 mg kg^{-1}[g]	Total plant–soil	−0.05 to +0.63[b]	+0.68 to +0.99[b]	-	+0.19 to +0.88[b]
Smolders et al. (2013)	*Lycopersicon esculentum* L. (tomato)	<0.03, 0.9 and 1.0 µmol L^{-1}	Total plant–hydroponic solution	−0.33 to −0.06	+0.16 to +0.33[b]	−0.36 to +0.24[b]	-
Arnold et al. (2015)	*Oryza sativa* L. (rice)	26 mg kg^{-1}[h]	Total plant above ground–soil leachate	−0.27 and −0.08[c]	-	+0.61 and +0.73[c]	-
Wiggenhauser et al. (2018)	*Triticum aestivum* L. (wheat)	0.4 and 10.9 mg kg^{-1}[h]	Total plant–soil extract	−0.33 and +0.13[d]	+0.56 and +0.63[d]	-	+0.10 to +0.72[e]

All Zn stable isotope compositions ($\delta^{66}Zn$) are reported relative to JMC-Lyon Zn. [a] non-italicized values represent isotopic differences, whereas italicized values represent the isotope fractionation factor ($\varepsilon^{66}Zn$) for uptake. [b] mean values. [c] mean values for rice grown in anaerobic and aerobic soils, respectively. [d] mean values for wheat grown in soil from two different sites. [e] range of mean values for flag and senescent leaves for wheat. [f] mean values for *Agrostis capillaris* L. grown in soil from two different sites. [g] represents total Zn concentrations in soils. [h] represents total Zn concentration in soil leachates or extracts.

Note S1. Depletion of Zn from the hydroponic solutions.

Plants were grown in 3 L hydroponic systems containing 4 plants of the same genotype (see main text for further experimental details). The total Zn mass taken up by one plant from each system was calculated based on the Zn concentrations in each organ and their dry mass. For two of the 19 genotypes (CC 41 and GU 207/H), the total Zn mass was calculated for three plants and an average was taken. Given the good agreement between the total Zn masses for the replicates of CC 41 and GU 207/H, all plants in each system were assumed to take up the same mass of Zn, and therefore the total Zn mass of one plant was multiplied by four. This total mass of Zn that was removed from the hydroponic solution was then converted to a percentage of the total Zn mass available in the hydroponic systems (equation S1):

$$\text{Hydroponic Zn depletion (\%)} = \left(\frac{\text{Total Zn of 4 plants}}{\text{Total Zn in hydroponic substrate}}\right) \text{x } 100 \qquad \text{(S1)}$$

The percentages (21±8%, 1SD) were then used to calculate the initial and final Zn stable isotope compositions of the hydroponic solutions, and ultimately, the Zn stable isotope fractionation factor ε^{66}Zn during root uptake (see main text for details).

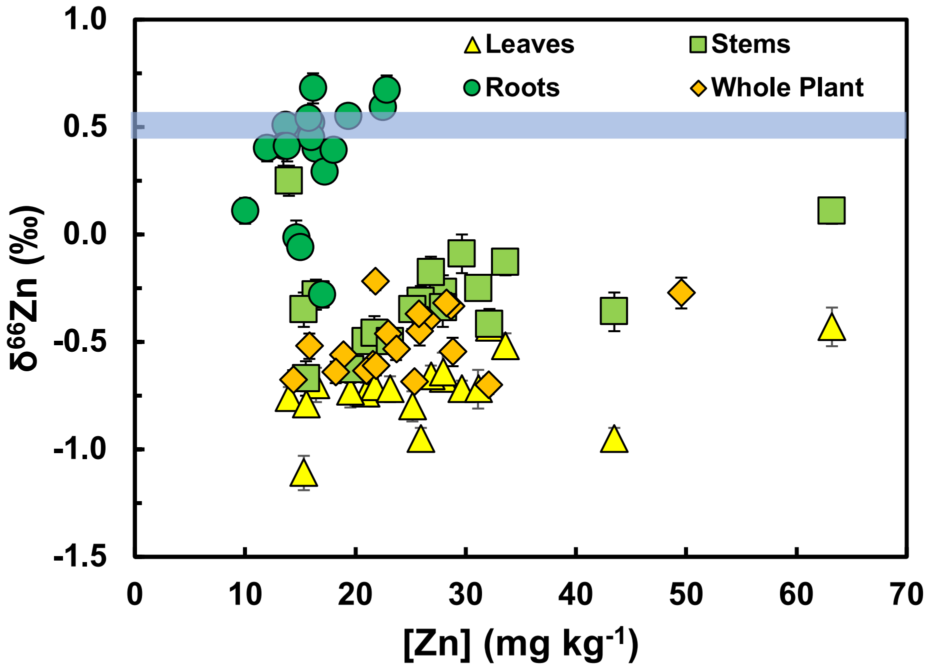

Figure S1. Zinc stable isotope compositions ($\delta^{66}Zn$) versus Zn concentrations ([Zn]; mg kg^{-1}) for leaves, stems, roots, and whole plants of the 19 cacao genotypes treated with 20 µmol L^{-1} $CdCl_2$. The error bars denote the 2SD precision determined for multiple analyses of the Zn isotope standard that bracketed the sample runs. The blue line denotes the $\delta^{66}Zn$ value of +0.51±0.06‰ for the $ZnSO_4$ that was added to the hydroponic solutions.

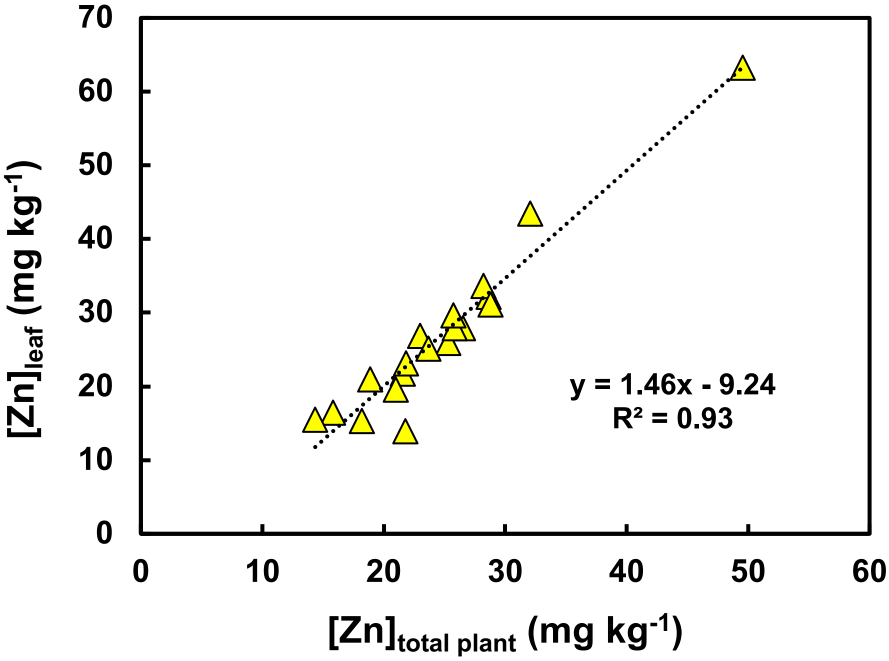

Figure S2. Leaf Zn concentrations ([Zn]; mg kg^{-1}) versus total plant Zn concentrations (mg kg^{-1}) for the 19 cacao genotypes.

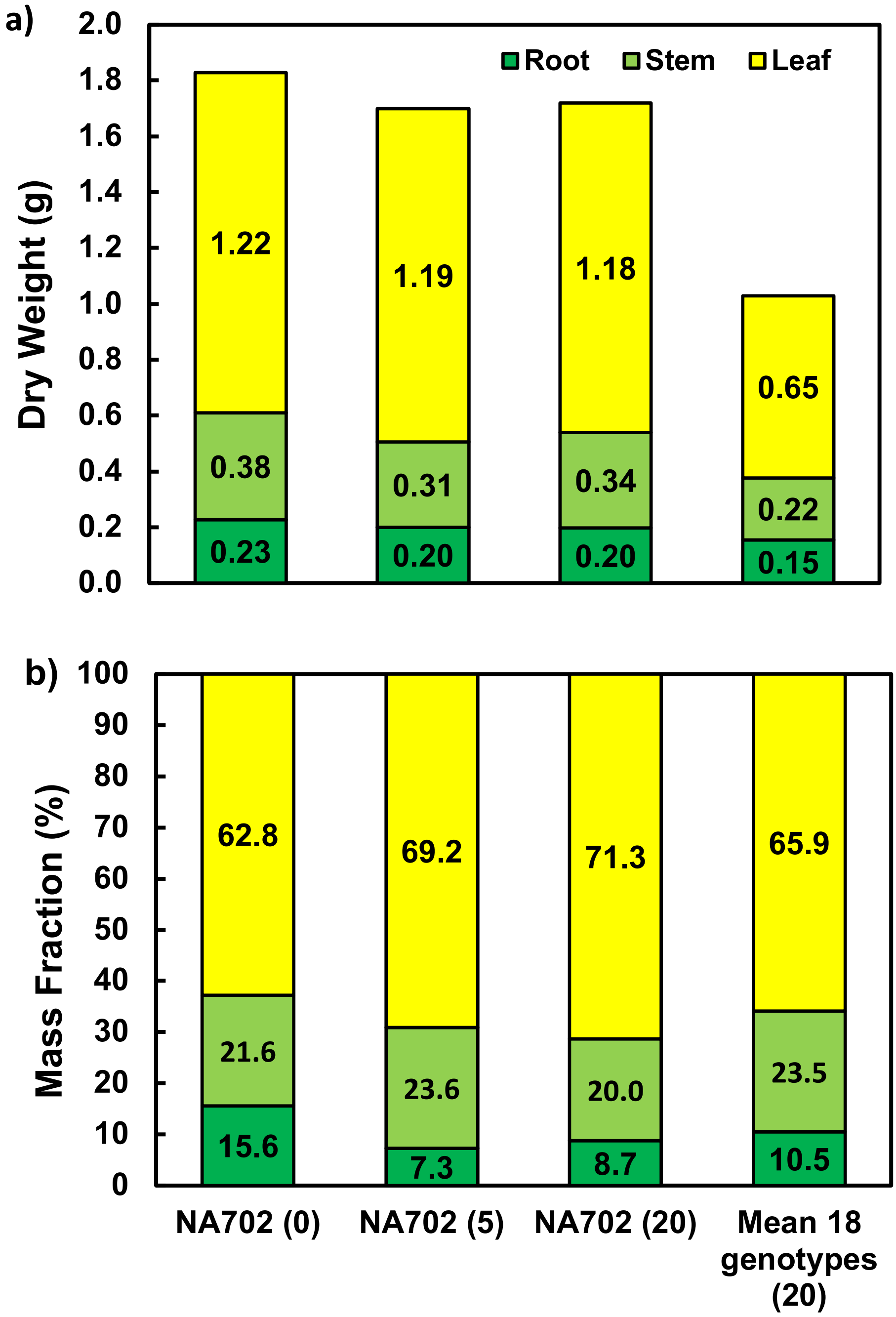

Figure S3. (a) Dry weights (g) and (b) mass fraction (%) for organs of NA 702 plants cultured with 0, 5 and 20 μmol L^{-1} $CdCl_2$ and mean results for the other 18 cacao genotypes cultured with 20 μmol L^{-1} $CdCl_2$. The notations (0), (5), (20) represent NA 702 plants treated with 0, 5, and 20 μmol L^{-1} $CdCl_2$, respectively.

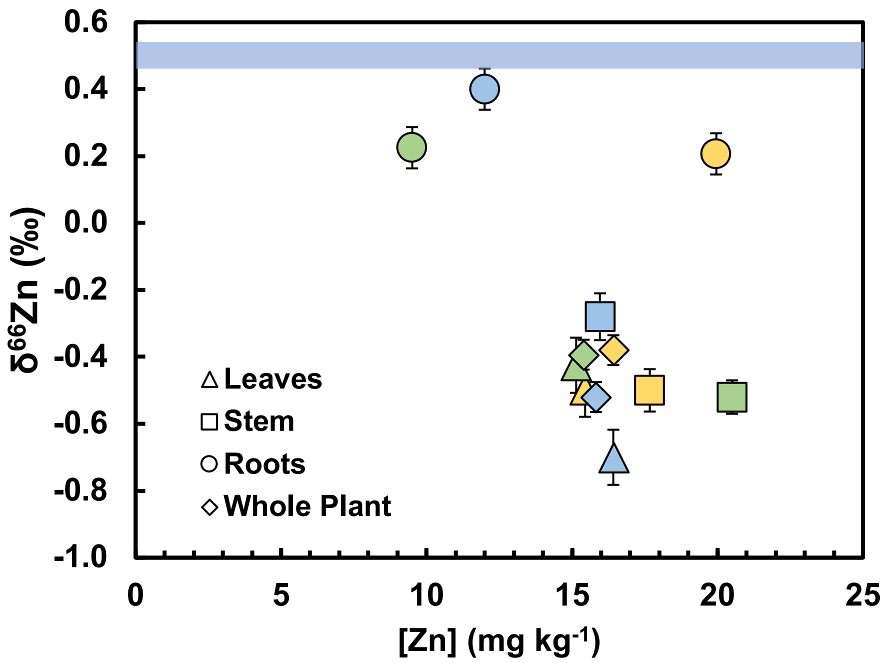

Figure S4. Mean Zn stable isotope compositions ($\delta^{66}Zn$) for leaves, stems and roots versus mean Zn concentrations ([Zn]; mg kg^{-1}) of the NA702 seedlings treated with 0 µmol L^{-1} (yellow), 5 µmol L^{-1} (green) and 20 µmol L^{-1} (blue) $CdCl_2$. The error bars denote the 2SD precision determined for multiple analyses of the Zn isotope standard that bracketed the sample runs. The blue line denotes the $\delta^{66}Zn$ value of +0.51±0.06‰ for the $ZnSO_4$ that was added to the hydroponic solutions.

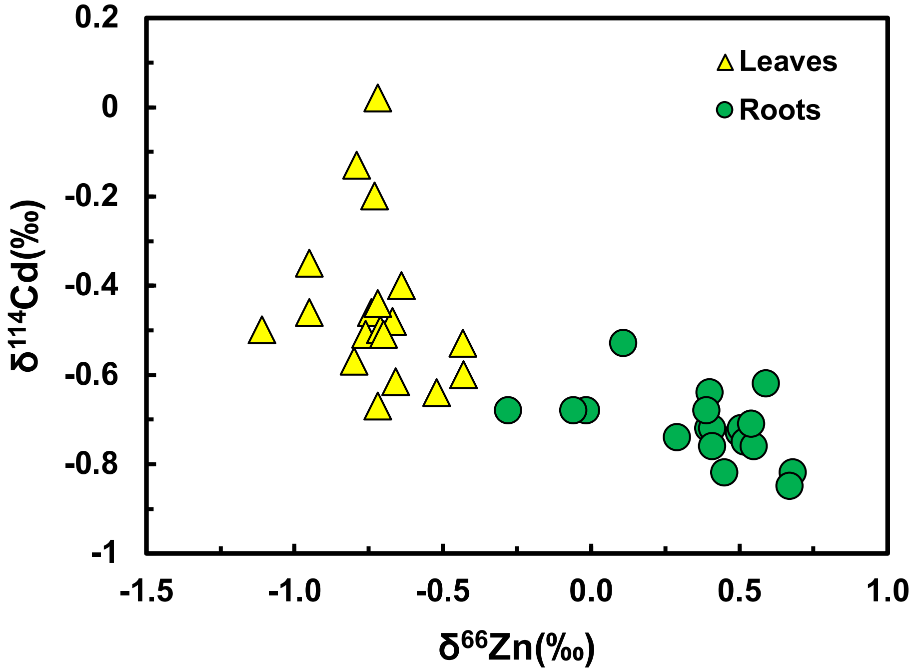

Figure S5. Cadmium stable isotope compositions ($\delta^{114}Cd$) versus Zn stable isotope composition ($\delta^{66}Zn$) for roots and leaves of the 19 cacao genotypes treated with 20 µmol L^{-1} $CdCl_2$. The Cd isotope data is from Moore et al. (2020).

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