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CHYE et al.

(54) **METHODS OF USING TRANSFORMED PLANTS EXPRESSING PLANT-DERIVED ACYL-COENZYME-A-BINDING PROTEINS IN PHYTOREMEDIATION**

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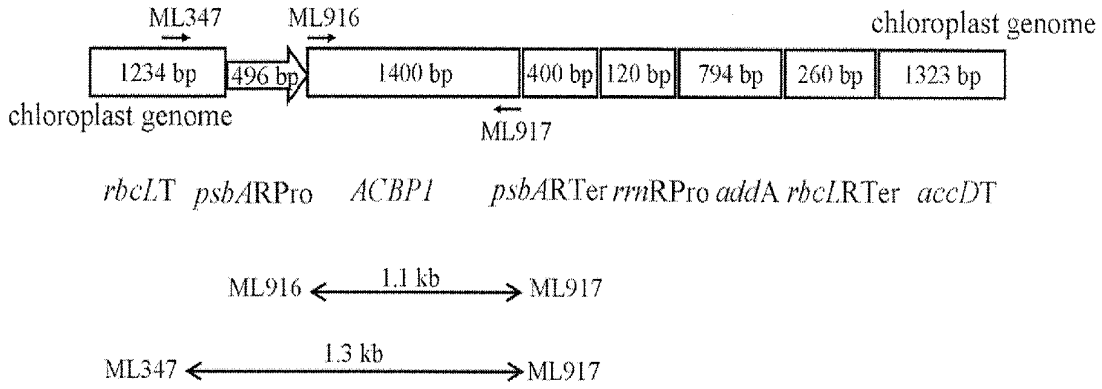
(52) **U.S. Cl.** **47/58.1SC**; 436/73; 435/252.33; 435/419; 800/298

(57) **ABSTRACT**

Methods of using genetically-transformed plants in the phytoremediation of lead are described. Unlike many organisms in which only 10-kDa ACBPs have been identified, there exists a family of six ACBPs in the model plant *Arabidopsis*. Other than a function in mediating the transfer of acyl-CoA esters in plant lipid metabolism, all six *Arabidopsis* ACBPs can bind the heavy metal lead and are therefore applicable for phytoremediation. These methods of phytoremediation will provide a cheap, simple and efficient method in the removal of contaminating lead from soil/water/environment by the growth of the ACBP-overexpressing genetically-transformed plants in the contaminated environment. There is also provided a method to remove lead from contaminated water.



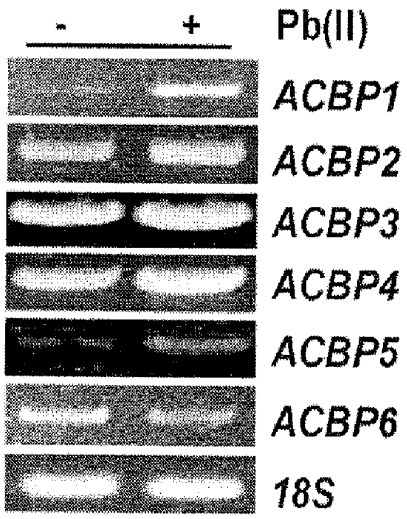

-
+
Pb(II)
ACBP1
ACBP2
ACBP3
ACBP4
ACBP5
ACBP6
18S

FIG. 1

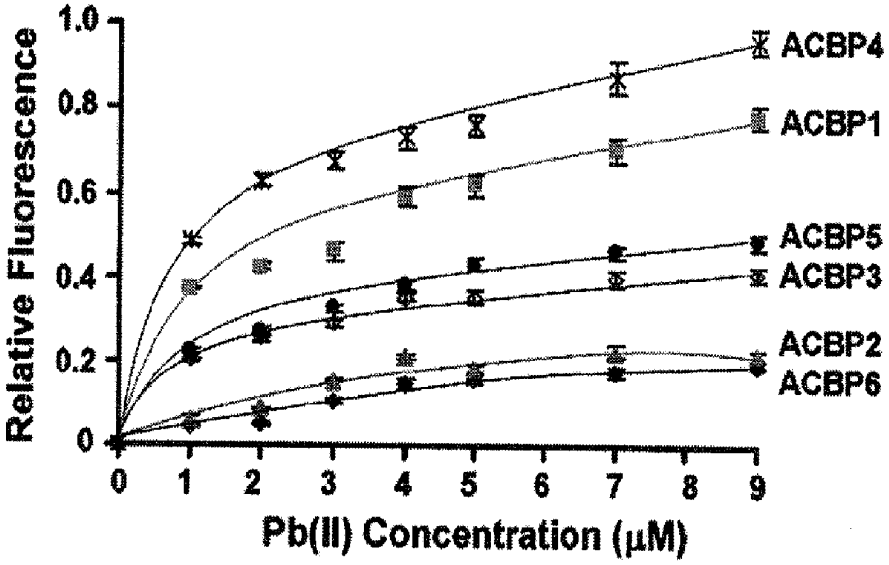

Relative Fluorescence
1.0
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9
Pb(II) Concentration (μM)
ACBP4
ACBP1
ACBP5
ACBP3
ACBP2
ACBP6

FIG. 2A

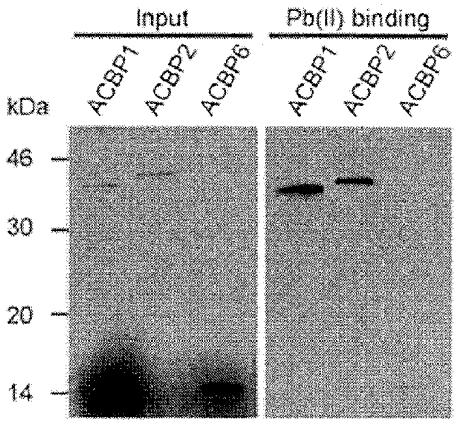

Input
Pb(II) binding
ACBP1
ACBP2
ACBP6
ACBP1
ACBP2
ACBP6
kDa
46
30
20
14

FIG. 2B

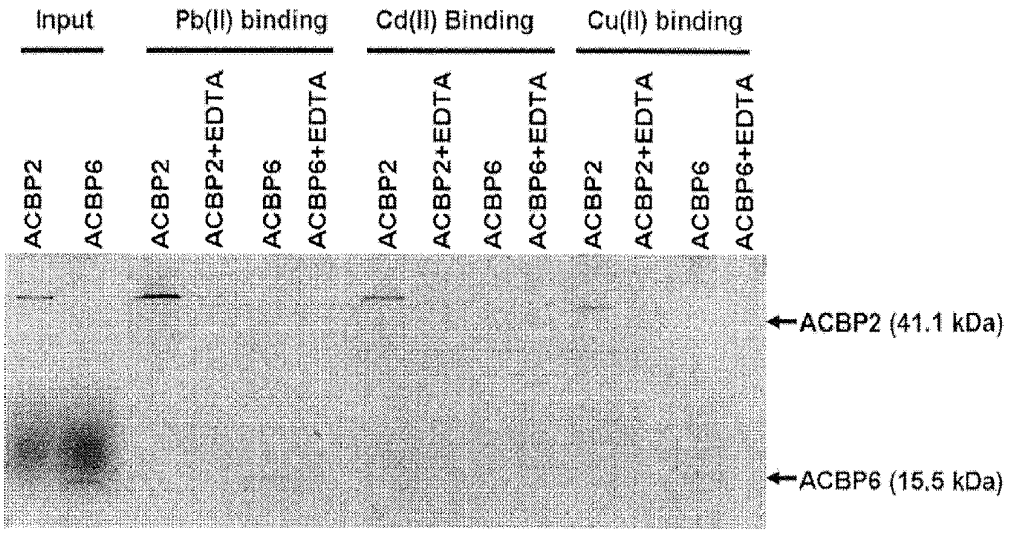

Input
Pb(II) binding
Cd(II) Binding
Cu(II) binding
ACBP2
ACBP6
ACBP2
ACBP2+EDTA
ACBP6
ACBP6+EDTA
ACBP2
ACBP2+EDTA
ACBP6
ACBP6+EDTA
ACBP2
ACBP2+EDTA
ACBP6
ACBP6+EDTA
ACBP2 (41.1 kDa)
ACBP6 (15.5 kDa)

FIG. 2C

```
ACBP6  1   MGLKEEFEEHAEKVNT- --------LTELPSN EDLLILYGLYKQAKF GPVDTSRPGMFSMKE 52
ACBP1  94  --.D.A.SAATAF.AAA ASD-----RLSQKVSN ELQLQ......I.TE ..CTAPQ.SALK.TA 149
ACBP2  104 --.D.A.SAATLF.TTA AAD-----RLSQKVPS DVQQQ......I.TE ..CTAPQ.SALK.TA 160
ACBP3  231 --.EKA.AAA---.NLL EES----GKAEEIGA EAKME.F..H.I.TE .SCREAQ.MAVMISA 283
ACBP4  12  --YP.R.YAAASY.GLD GSDSSAKNVISKFPD DTALL..A..QQ.TV ..CNTPK.SAWRPV. 71
ACBP5  22  --YP.R.YAAASY.GLD GSQSSVKQLSSKFSN DTSLL..T.HQQ.TL ..CSIPK.SAWNPV. 72
Con            F AA  V                      L  L   AT  G C    P A
Com          LDEAFSAAASFVGLA ASDSS KRLSSKVSN DTQLQLYGLYKIATE GPCTAPQPSALKMTA
             YP R Y  T Y   D G         Q FP  E   L    HQQ          K   W PVE

ACBP6  53  RAKWDAWKAVEGKSS EEAMNDYITKVKQLL EVAASKAST        92
ACBP1  150 ....Q..QKLGAMPP ....EK..DL.T..Y P-              180
ACBP2  161 ....Q..QKLGAMPP ....EK..EI.T..Y P-              190
ACBP3  284 ....N..QKLGNM.Q ....EQ.LAL.SKEI PG              315
ACBP4  72  QS..KS.QGLGTMP. I...RLFVKILEEDD PG              103
ACBP5  73  QS..KS.QGLGTMP. I...RLFVKILEEAD PG              104
Con          KW  WQ IG M    EAM           P
Com        RAKWQAWQKLGAMPP EEAMEKYIKIVTQLY PG
           QS  KS  G  T  S I   RLFV LLEE D
```

FIG. 2D

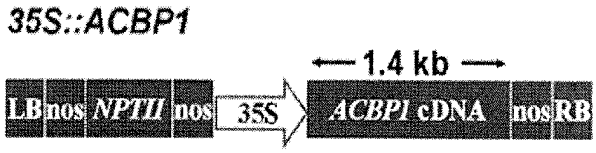


FIG. 3A

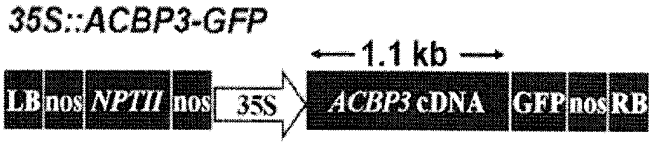


FIG. 3B

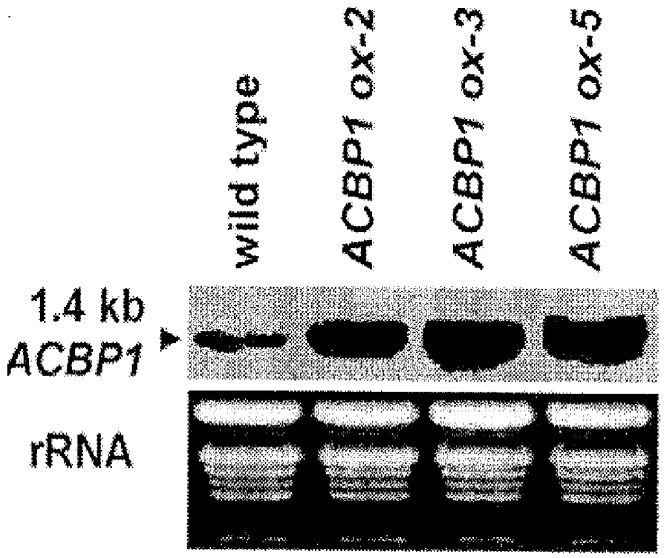

wild type
ACBP1 ox-2
ACBP1 ox-3
ACBP1 ox-5
1.4 kb
ACBP1
rRNA

FIG. 4A

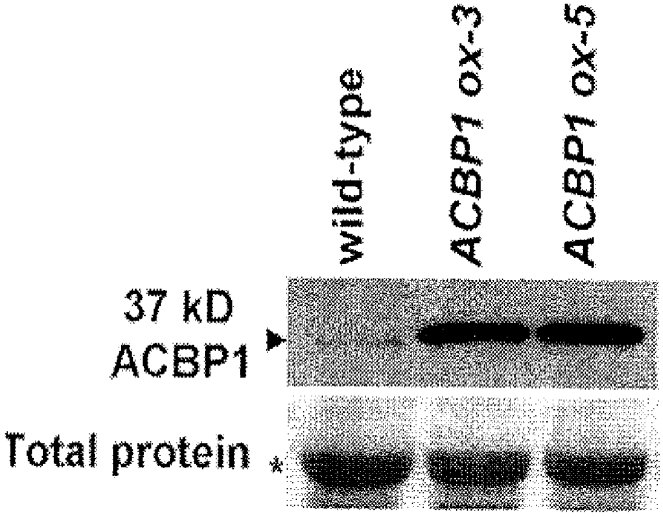

wild-type
ACBP1 ox-3
ACBP1 ox-5
37 kD
ACBP1
Total protein

FIG. 4B

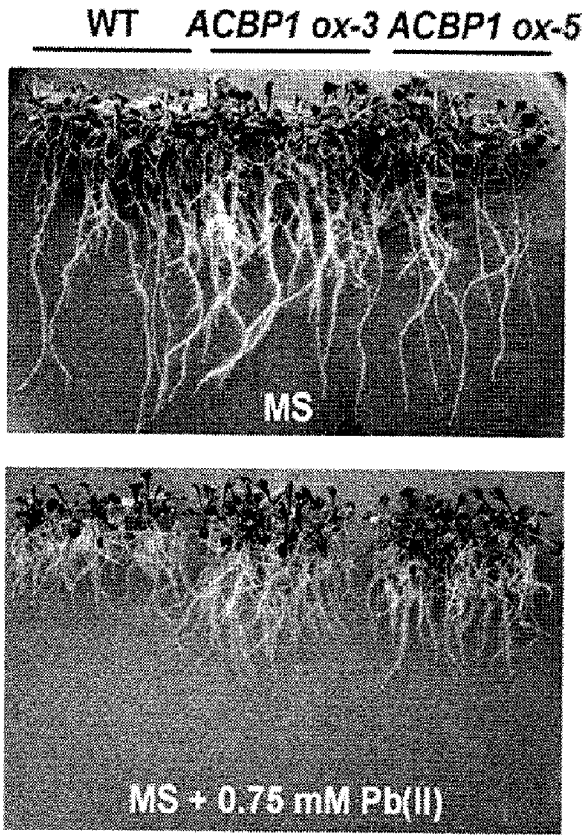

WT
ACBP1 ox-3
ACBP1 ox-5
MS
MS + 0.75 mM Pb(II)

FIG. 4C

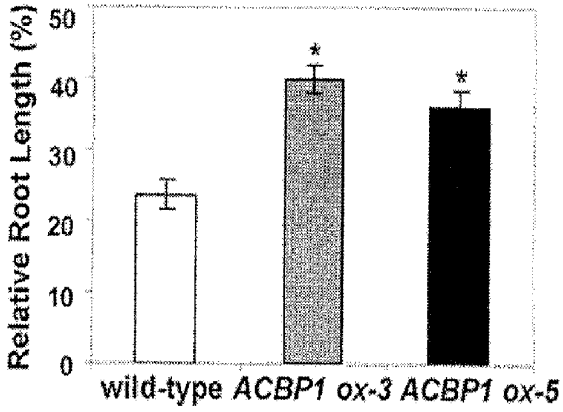

Relative Root Length (%)
50
40
30
20
10
0
*
*
wild-type
ACBP1 ox-3
ACBP1 ox-5

FIG. 4D

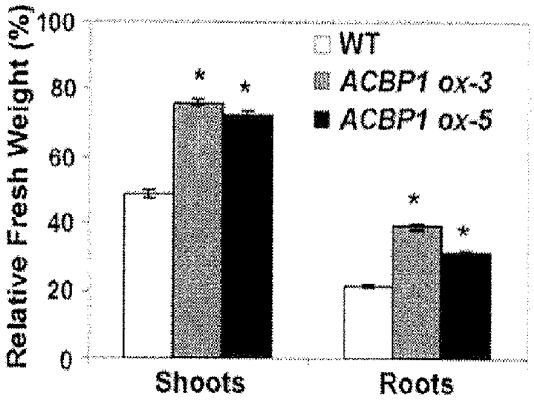


FIG. 4E

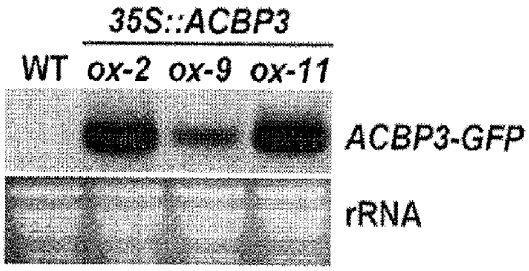


FIG. 5A

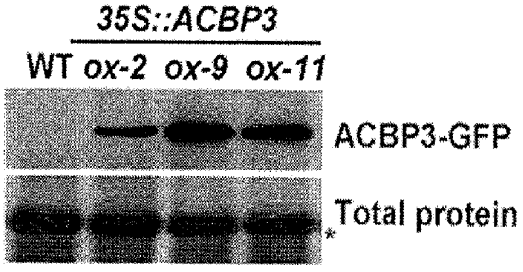


FIG. 5B

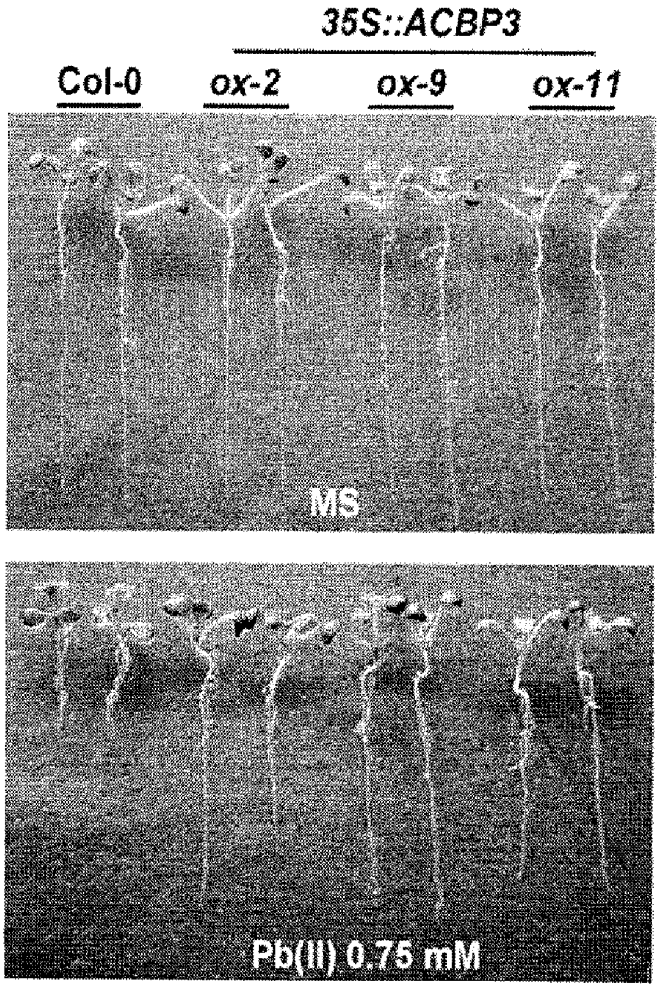

35S::ACBP3
Col-0
ox-2
ox-9
ox-11
MS
Pb(II) 0.75 mM

FIG. 5C

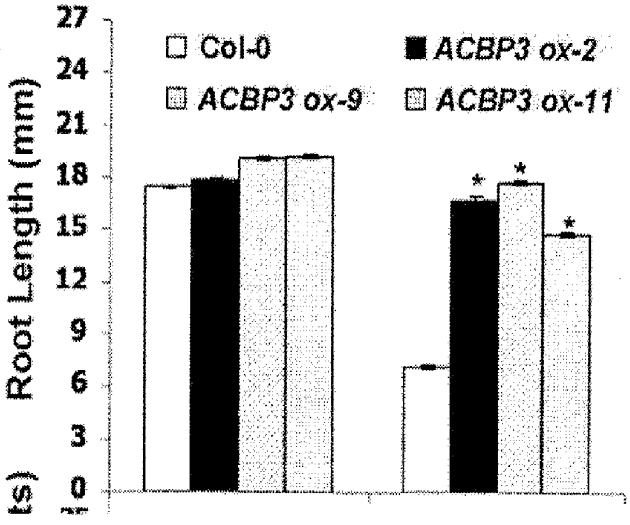

Root Length (mm)
27
24
21
18
15
12
9
6
3
0
Col-0
ACBP3 ox-2
ACBP3 ox-9
ACBP3 ox-11
*
*
*

FIG. 5D

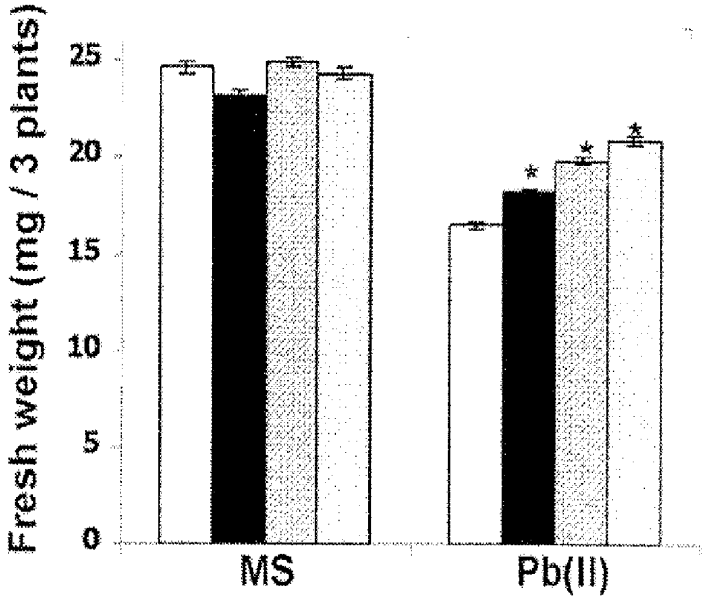

Fresh weight (mg / 3 plants)
25
20
15
10
5
0
*
*
*
MS
Pb(II)

FIG. 5E

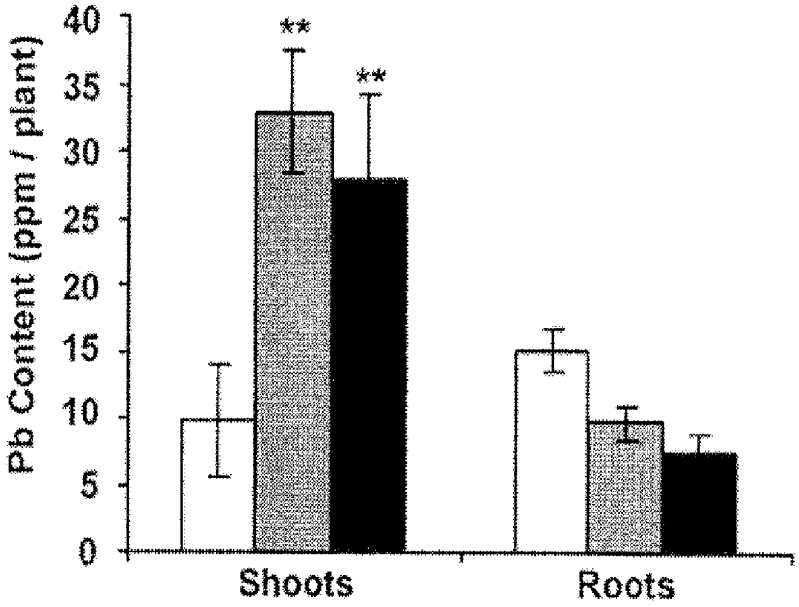

Pb Content (ppm / plant)
40
35
30
25
20
15
10
5
0
**
**
Shoots
Roots

FIG. 6A

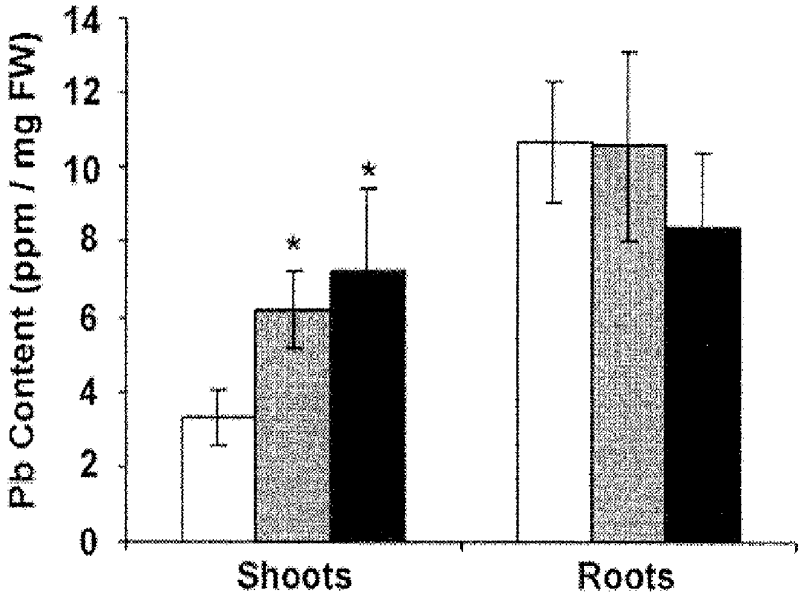

Pb Content (ppm / mg FW)
14
12
10
8
6
4
2
0
*
*
Shoots
Roots

FIG. 6B

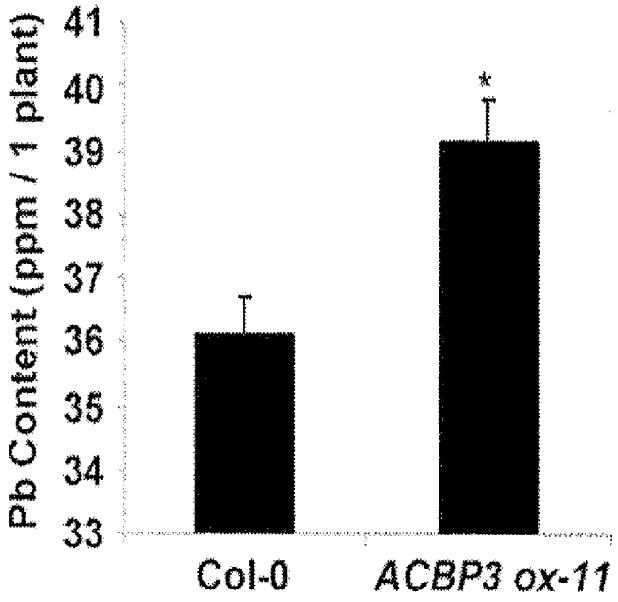

Pb Content (ppm / 1 plant)
41
40
39
38
37
36
35
34
33
*
Col-0
ACBP3 ox-11

FIG. 7A

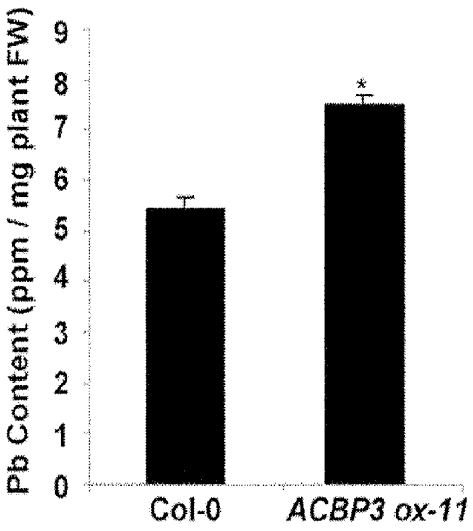

Pb Content (ppm / mg plant FW)
9
8
7
6
5
4
3
2
1
0
*
Col-0
ACBP3 ox-11

FIG. 7B

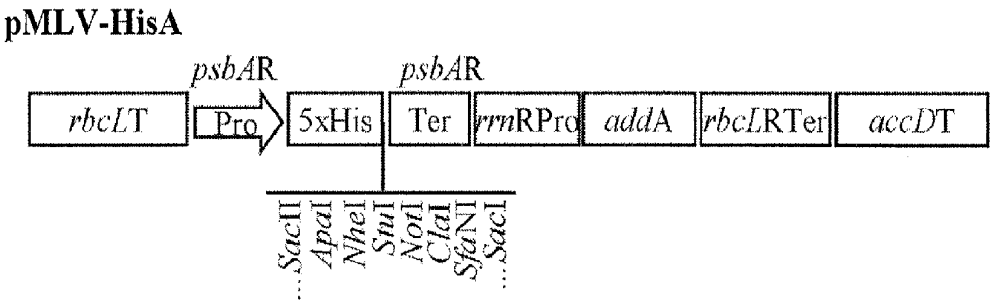

pMLV-HisA
psbAR
psbAR
rbcLT
Pro
5xHis
Ter
rrnRPro
addA
rbcLRTer
accDT
...SacII
ApaI
NheI
SnaI
NotI
ClaI
SfaNI
...SacI

FIG. 8A

pAT385

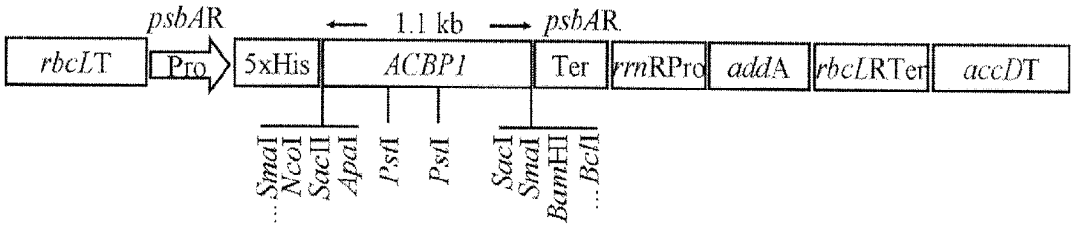


FIG. 8B

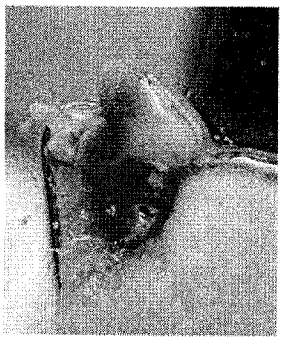


FIG. 9A

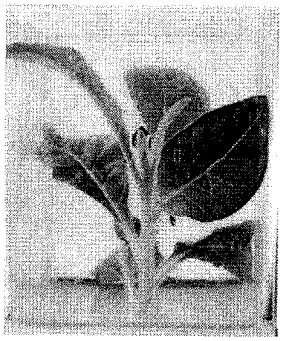


FIG. 9B

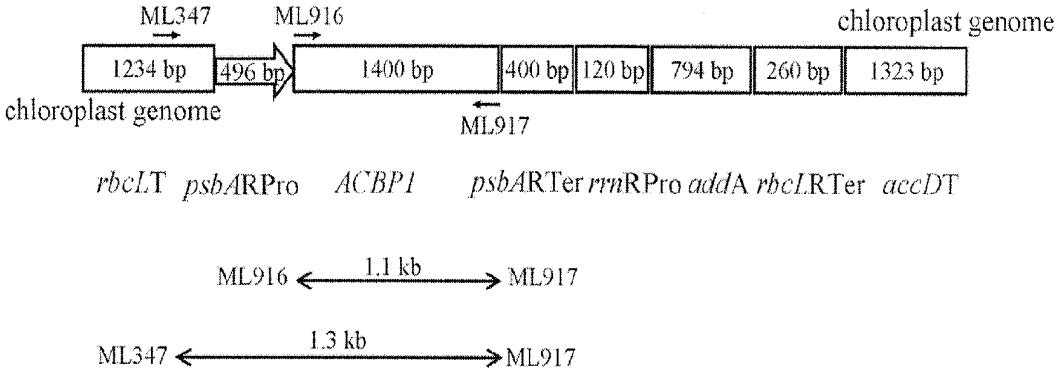

ML347
ML916
chloroplast genome
1234 bp
496 bp
1400 bp
400 bp
120 bp
794 bp
260 bp
1323 bp
chloroplast genome
ML917
rbcLT psbARPro ACBP1 psbARTer rrnRPro addA rbcLRTer accDT
ML916 1.1 kb ML917
ML347 1.3 kb ML917

FIG. 9C

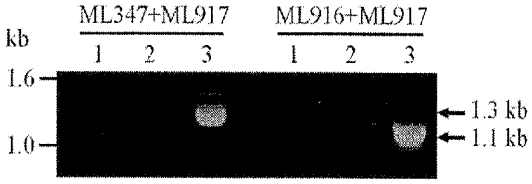

ML347+ML917
ML916+ML917
kb
1 2 3 1 2 3
1.6
1.0
1.3 kb
1.1 kb

FIG. 9D

METHODS OF USING TRANSFORMED PLANTS EXPRESSING PLANT-DERIVED ACYL-COENZYME-A-BINDING PROTEINS IN PHYTOREMEDIATION

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. provisional application Ser. No. 60/922,847, filed Apr. 11, 2007.

TECHNICAL FIELD

[0002] The present invention relates generally to a method of bioremediation, and more specifically to using genetically-transformed plants for phytoremediation of lead and other metal pollutants.

BACKGROUND OF THE INVENTION

[0003] Phytoremediation is the process by which plants are used to remove pollutants like heavy metals from the environment. The roots of the plant "suck up" the pollutants from the environment and these can be stored within the plant. Plants thrive by photosynthesis, hence phytoremediation is solar-driven, environmentally-friendly, low-cost and remediation occurs in sit u.

[0004] Heavy metals, the undesirable products from industries like mining and manufacturing, as well as agriculture, contaminate the environment by polluting streams, sediment, sludge, groundwater, and soil. Transgenic plants have been successfully used to detoxify heavy metals like mercury, cadmium, arsenate, and selenate from soil (Kramer, *Curr. Opin. Biotech.* 16: 133-141, 2005). Such heavy metals, toxic to humans and animals, adversely affect the human nervous system and induce cancers. These pollutants are also known to stress the growth and development of wild-type plants growing on contaminated soils. Transgenic plants that contain heterologous gene(s), with the ability to "detoxify" the pollutant, can tolerate the heavy metal stress and will concurrently clean-up the environment. The toxin will be absorbed and concentrated in the plant tissue such as in leaves and stems. Subsequently, these plants (if the toxin is yet not degraded) can be harvested and then incinerated safely. This is especially applicable to metal pollutants including arsenate, cadmium, and mercury which cannot be easily broken down (Powell, *Nature* doi: 10.1038/news021100-14, 2002).

[0005] Genetic engineering has made it possible to transfer non-plant derived genes for expression in plants. Examples in phytoremediation of genetically-transformed plants expressing non-plant genes include those that express bacterial enzymes that breakdown arsenic compounds in transgenic *Arabidopsis* (Dhankher et al., *Nature Biotech.* 20: 1140-1146, 2002) and others that detoxify mercury (Kramer, *Curr. Opin. Biotech.* 16: 133-141, 2005). The generation of transgenic *Arabidopsis* and *Brassica* plants that detoxify selenate has also been reported (Kramer, *Curr. Opin. Biotech.* 16: 133-141, 2005).

[0006] There is an apparent lack of plant genes that encode proteins capable of binding lead. Transgenic plants that can potentially phytoremediate lead have been generated by the expression of a yeast YCF1 protein (Song et al., *Nature Biotech.* 21: 914-919, 2003). Bacterial P-type ATPases which remove lead are deemed unsuitable for phytoremediation because their use will not culminate in the accumulation of lead in plants cells (Song et al., *Nature Biotech.* 21: 914-919, 2003). Since lead toxicity is of prime concern to human health, particularly that of children (http://www.epa.gov/seahome/leadenv.html), phytoremediation would provide a useful strategy to eliminate lead accumulation and its concentration in food chains. Hence, such procedures in lead bioremediation are invaluable for the protection of human health and the environment worldwide.

[0007] It has been previously reported that two low molecular weight cytosolic proteins isolated from human kidney tissue have been observed to bind physiologic lead in vivo with high affinities. The two human proteins were identified as thymosin beta-4 of molecular mass 5 kDa and a 9-kDa acyl-CoA-binding protein (Smith et al., *Chemico-Biological Interactions* 115: 39-52, 1998). These small proteins, known to be highly-conserved in mammals, have been suggested to be the specific molecular targets for lead in environmentally-exposed humans (Smith et al., *Chemico-Biological Interactions* 115: 39-52, 1998).

[0008] The 9-kDa human ACBP is homologous to the bovine 10-kDa cytosolic ACBP (diazepam-binding inhibitor/enzepine), and such 10-kDa ACBPs have already been well-characterized in many organisms (reviewed in Kragelund et al., *Biochim Biophys Acta* 1441: 150-161, 1999) including man (Swinnen et al., *DNA Cell Biol.* 15: 197-208, 1996). Bovine 10-kDa ACBP and rat 10-kDa ACBP have been demonstrated to bind palmitoyl-CoA and oleoyl-CoA (Rasmussen et al., *Biochem. J.* 265: 849-855, 1990). The 10-kDa ACBP has been implicated to mediate intracellular acyl-CoA transport by binding long-chain acyl-CoA esters (reviewed in Kragelund et al., *Biochim Biophys Acta* 1441: 150-161, 1999). These long-chain acyl-CoAs esters not only function as intermediates in lipid metabolism but have been implicated in protein trafficking, vesicular trafficking, and gene regulation (reviewed in Faergman and Knudsen, *Biochem. J.* 323: 1-12, 1997).

[0009] In the model plant *Arabidopsis*, a 10-ia ACBP (GenBank Accession No. NP_174462) that is homologous to the previously characterised human and bovine ACBPs, has been reported by Engeseth et al. (*Arch. Biochem. Biophys.* 331: 55-62, 1996). However, our recent work has shown that other forms of ACBPs are also known to occur in *Arabidopsis* (Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004). The *Arabidopsis* complete ACBP gene family of six members encode proteins ranging from 92 amino acids to 668 amino acids, each containing a conserved acyl-CoA-binding domain (Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004). Specifically, they are the *Arabidopsis* ACBP6 (10-kDa ACBP, GenBank Accession No. NP-174462, Engeseth et al., *Arch. Biochem. Biophys.* 331: 55-62, 1996), membrane-associated ACBP1 (GenBank Accession No. AAD03482, Chye et al., *Plant J.* 18: 205-214, 1999), membrane-associated ACBP2 (GenBank Accession No. NP_194507, Chye et al., *Plant Mol. Biol.* 44: 711-721, 2000; Li and Chye, *Plant Mol. Biol.* 51: 483-492, 2003), ACBP3 (GenBank Accession No. NP_194154, Leung et al., *Planta* 223: 871-881, 2006) and the two kelch-motif-containing ACBPs, ACBP4 (GenBank Accession No. NP_187193, Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004) and ACBP5 (GenBank Accession No. NP_198115, Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004).

[0010] Many ACBPs identified in other organisms are 10-kDa homologs of the 10-kDa bovine ACBP, consisting of 86-104 amino acids, or variants thereof arising from alternative first exon usage (Nitz et al., *Int. J. Biochem. Cell Biol.* 37: 2395-2405, 2005). The membrane-associated domains of

Arabidopsis ACBP1 (consisting of 338 amino acids) and ACBP2 (consisting of 354 amino acids) are located at the N-terminus and both have C-terminal ankyrin repeats. ACBP1 and ACBP2 share 76.9% amino acid identity. Highly-conserved (81.4% identity) ACBP4 and ACBP5 both contain C-terminal kelch motifs. Ankyrin repeats and kelch motifs are domains that can potentially mediate protein-protein interactions, suggesting these *Arabidopsis* ACBPs can interact with protein partners (Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004).

[0011] Thus, according to presence of structural domains, the *Arabidopsis* ACBP family can be divided into four classes: (1) small 10-kDa ACBP6 of 92 amino acids; (2) ACBP1 (338 amino acids) and ACBP2 (354 amino acids) with N-terminal membrane-associated domains and C-terminal ankyrin repeats; (3) ACBP3 of 362 amino acids; and (4) kelch-motif containing large ACBP4 (668 amino acids) and ACBP5 (648 amino acids). To establish the significance of each acyl-CoA-binding domain in binding acyl-CoA esters, *Arabidopsis* ACBPs have been expressed as recombinant (His)-tagged proteins in *Escherichia coli* for in vitro binding assays, and residues within the acyl-CoA-binding domain essential in binding have been identified by site-directed mutagenesis (Chye et al., *Plant Mol. Biol.* 44: 711-721, 2000; Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004; Leung et al., *Planta* 223: 871-881, 2006). The differential binding affinities of *Arabidopsis* ACBPs to various acyl-CoA esters suggest they may possess different cellular functions.

[0012] Since the expression of plant-derived gene(s) in transformed plants may generally be more acceptable to the public, we provide herein below a method for the phytoremediation of lead and other metals by the expression or overexpression of plant-derived acyl-CoA-binding proteins (ACBPs) in transformed plants.

BRIEF SUMMARY OF THE INVENTION

[0013] An aspect of the present invention is based on the observation that genetically modified plants and progeny thereof expressing acyl-CoA-binding proteins (ACBP) can be applied for phytoremediation of heavy metals like lead (Pb(II)). Presented herein are plant transformation vectors that each comprises a nucleic acid sequence encoding an ACBP that can be used to generate genetically-transformed plants via nuclear transformation or plastid transformation. The resultant plants that overexpress ACBPs, exemplified herein by *Arabidopsis* ACBPs, are conferred the ability to grow in an environment that contains a heavy metal pollutant, for example, lead, copper, and cadmium. These plants are able to uptake such metals and incorporate them in their tissue, and hence are useful for the phytoremediation of lead and other metals.

[0014] Since all six Acyl-Coenzyme-A-Binding Proteins (ACBPs) from *Arabidopsis* can bind the heavy metal lead, and other metal contaminants present as divalent cations (such as, for example, copper and cadmium), growth of genetically-transformed plants overexpressing these ACBPs will absorb such contaminants from the environment. The plants can subsequently be harvested, thereby removing the contaminant. In specific embodiments, the generation of transgenic plants overexpressing *Arabidopsis* ACBPs from plant transformation vectors is described.

[0015] In accordance with another aspect of the present invention, there are provided plant transformation vectors comprising polynucleotides which encode fragments, derivatives, analogs, or variants of ACBP polypeptides. In specific embodiments, the invention provides for transformed plants such as transgenic *Arabidopsis* and transplastomic tobacco plants. The present invention provides modified plants that comprise ACBP polypeptides or fragments, derivatives, analogs, or variants thereof having similar activities as the ACBP polypeptides. The present invention also provides a method of producing the modified plants which comprises transforming a plant with a plastid and/or nuclear transformation vector comprising at least one ACBP-encoding polynucleotide, or fragments, derivatives, analogs, or variants thereof.

[0016] In another specific embodiment, plant transformation vectors are engineered to yield plants that can be used to phytoremediate heavy metals, particularly those present as divalent cations such as lead, from contaminated soil/water environments. One aspect of the invention relates to methods of detecting Pb(II) in a sample by growth of *Arabidopsis* test plants and detection of lead Pb(II) by observation in the mRNA induction of mRNAs encoding ACBP 1 to ACBP5. The sample may be a liquid or solid (e.g. soil) from the environment.

[0017] In one specific embodiment, the invention provides for a plastid transformation vector comprising at least one polynucleotide encoding an ACBP, or encoding a fragment, derivative, analog, or variant of an ACBP. In another specific embodiment, the invention provides for a nuclear transformation vector comprising at least one polynucleotide encoding an ACBP, or encoding a fragment, derivative, analog, or variant thereof. In another embodiment, the invention provides for a nuclear transformation vector which expresses at least one ACBP polypeptide, or a fragment, derivative, analog, or variant thereof, having a similar lead-binding activity as the ACBP polypeptide. In a specific embodiment, the present invention provides a method of producing transgenic plants via the exemplary nuclear transformation vectors pAT31 and pAT314.

[0018] Plant cells containing a vector comprising a polynucleotide encoding a polypeptide exhibiting ACBP activity are also an aspect of this invention. Plant parts of the modified plants, such as for example, fruits, leaves, tubers, seeds, flowers, stems or roots, which comprise cells expressing ACBP polypeptides, derivatives, analogs, or variants thereof are provided in the invention. The plant parts include parts that are separated from the whole plant or attached onto the whole plant.

[0019] In a specific embodiment, a nuclear transformation vector is used to cause expression of one or more ACBPs including ACBP polypeptides or fragments, derivatives, analogs, or variants thereof having similar lead-binding activities as the *Arabidopsis* ACBP polypeptides. In a specific embodiment, a plastid transformation vector is used to cause expression of one or more ACBPs including ACBP polypeptides or fragments, derivatives, analogs, or variants thereof having similar lead-binding activities as the *Arabidopsis* ACBP polypeptides. In a specific embodiment, the present invention provides a method of producing transgenic plants via the exemplary plastid transformation vector pAT385. In a specific embodiment, a plastid transformation vector and a nuclear transformation vector are used to express one or more ACBPs, including ACBP polypeptides, or fragments, derivatives, analogs, or variants thereof having similar lead-binding activities as the ACBP polypeptides. Such nuclear and plastid transformation vectors can be used alone or in conjunction

with other recombinant vectors that can enhance the phytoremediation capabilities of plants transformed therewith.

[0020] The present invention provides a method of producing ACBPs in plants, or fragments, derivatives, analogs, or variants thereof having similar lead-binding activities as the ACBP polypeptides. The method comprises transforming a plant with a vector which comprises a polynucleotide coding for one or more ACBP polypeptides. The vector can optionally also comprise a promoter, operably linked to the coding sequence, and a terminator, and/or other regulatory elements. The vector can be designed to introduce the heterologous polypeptide so that it will be expressed under the control of a plant's own endogenous promoter, such as, for example, in the pseudogene technique taught by Hahn and Kuehnle (US 2003-003362641). Alternatively, or in addition, the vector can contain a constitutive and/or inducible and/or tissue specific promoter operatively linked to the ACBP-encoding polypeptide. Plant cells containing a vector which comprises one or more nucleic acid sequences encoding ACBPs, including polypeptides or fragments, derivatives, analogs, or variants thereof having the similar lead-binding activities as the ACBP polypeptides are also an aspect of this invention. Alternatively, the plant cells may contain one or more vectors of the present invention. Each vector may contain an exogenous polypeptide encoding one polypeptide exhibiting the lead-binding activity of an ACBP, or optionally may contain an operon encoding more than one such ACBP polypeptide. The present invention provides plant parts, such as for example, fruits, leaves, tubers, seeds, flowers, stems, roots, and all other anatomical parts of the modified plant.

[0021] The *Arabidopsis* ACBP proteins were first tested for binding lead using recombinant His-tagged proteins expressed in *E. Coli* to mass-produce and facilitate the purification of the His-tagged ACBPs for in vitro lead-binding assays, because isolation of the native forms from *Arabidopsis* is more difficult to achieve and to mass-accumulate for in vitro assays. Subsequently, we demonstrated that in vitro translated ACBP2 also binds copper and cadmium, thus providing the expectation that the ACBPs taught herein are useful for binding not only lead, copper, and cadmium, but other metals present as divalent cations.

[0022] Upon demonstration of the abilities of all six *Arabidopsis* ACBPs in binding lead using lead-binding assays, we next investigated if the overexpression of an ACBP in a transgenic plant would confer tolerance to lead in the plant growth medium. Plasmid constructs containing nucleic acids that encode ACBPs were generated for plant transformation. Subsequently, the growth of ACBP-overexpressing transgenic lines was compared to wild-type *Arabidopsis*. The ACBP-overexpressing lines showed better growth in medium containing lead than wild type, indicating that ACBP confers the ability to bind lead and tolerate the presence of lead in the growth environment. The introduction of an ACBP transgene in the transformed plant provided the plant a higher tolerance to lead stress in the growth environment.

[0023] To confirm that these ACBP overexpressing plants can phytoremediate lead from the environment, the concentration of lead in shoots and roots of these plants was determined and compared with wild type growing on the lead-containing medium. Results demonstrate that the ACBP-overexpressing plants accumulate lead in their shoots, thus indicating they can phytoremediate lead from the environment, and providing the expectation that they can phytoremediate other metals present in the environment as divalent cations.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] FIG. **1** shows Reverse-Transcription Polymerase Chain Reaction (RT-PCR) analysis of lead Pb(II)-induced elevation of *Arabidopsis* ACBP transcripts (ACBP1 to ACBP5 but not ACBP6) in root. Total RNA was extracted from roots of 3-week-old Col-0 *Arabidopsis* seedlings in the presence (+) or absence (−) of 1 mM $Pb(NO_3)_2$ treatment under continuous light for 24 h. The 18S rDNA transcript was used as a loading control (bottom of figure).

[0025] Primers used for RT-PCR analysis of ACBP6 were ML750 (SEQ ID NO:1) and ML751 (SEQ ID NO:2); ACBP1, ML179 (SEQ ID NO:3) and ML759 (SEQ ID NO:4); ACBP2, ML194 (SEQ ID NO:5) and ML205 (SEQ ID NO:6); ACBP3, ML783 (SEQ ID NO:7) and ML784 (SEQ ID NO:8); ACBP4, ML849 (SEQ ID NO:9) and ML850 (SEQ ID NO:10); ACBP5, ML352 (SEQ ID NO:11) and ML353 (SEQ ID NO:12); 18S, 18S-F (SEQ ID NO:13) and 18S-R (SEQ ID NO:14).

[0026] FIGS. **2**A-**2**D compares binding of $(His)_6$-ACBPs and in vitro translated ACBPs to Pb(II), Cd(II), and Cu(II). (A) Binding of $(His)_6$-ACBPs to Pb(II) by fluorescence analysis. The fluorescence intensities were measured with excitation wavelength set at 360/40 nm and emission wavelength set at 530/25 nm n for the dansylated $(His)_6$-ACBP in the absence and presence of various concentrations (1, 2, 3, 4, 5, 7, and 9 μM) of Pb(II). The relative fluorescence of each ACBP was obtained by deduction of its own blank. The maximum value for relative fluorescence was set at 1 against which others were then compared to obtain the percentage relative fluorescence. Each point shows the average and standard error of three independent experiments. Bars represent SE (n=3). (B) Binding of in vitro translated ACBPs to Pb(II) by metal-chelate affinity chromatography. The left panel (Input) shows equal loadings of [^{35}S]methionine-labeled ACBP1, ACBP2, and ACBP6. The right panel shows in vitro binding between radiolabeled proteins and Pb(II). Pb(II)-equilibrated matrix was incubated with [^{35}S]methionine-labeled proteins, washed 3 times and the binding protein was eluted with an imidazole elution buffer followed by extraction with 2% SDS, 50 mM DTT. The eluted protein was analyzed by SDS-PAGE followed by autoradiography. (C) Binding of in vitro translated ACBPs to Pb(II), Cd(II), and Cu(II) by metal-chelate affinity chromatography. The first panel on the left (Input) shows equal loadings of [^{35}S]methionine-labeled ACBP2 and ACBP6. The next panels show in vitro binding between radiolabeled proteins and heavy metals as indicated. Pb(II), Cd(II), or Cu(II)-equilibrated matrix was incubated with [^{35}S]methionine-labeled proteins, washed 3 times and the binding protein was eluted with an imidazole elution buffer followed by extraction with 2% SDS, 50 mM DTT. The eluted protein was analyzed by SDS-PAGE followed by autoradiography. Binding of ACBP2 to Pb(II), Cd(II), and Cu(II) was inhibited by the metal chelator, ethylenediaminetetraacetic acid (EDTA at 50 mM final concentration), indicating that binding is dependent on divalent cations. (D) Comparison of the acyl-CoA-binding domains of *Arabidopsis* ACBPs. Dots indicate identity to ACBP6. "Con" (Conserved), amino acid residues highly conserved in *Arabidopsis* ACBPs. All *Arabidopsis* ACBPs (ACBP1 to ACBP6) show 100% conservation in 13 amino acid residues (marked with asterisks in "Con"

sequence in FIG. **2**D). Ten other amino acid residues (underlined amino acids in "Con" sequence in FIG. **2**D) within the acyl-CoA-binding domain, are conserved in ACBP1 to ACBP5, but not in ACBP6. From these 10 (underlined amino acids in "Con"), 3 amino acid residues that are further conserved in the 9-kDa human ACBP (GenBank Accesssion No. NM_020548) are encircled; these 3 amino acid residues conserved in human ACBP and ACBP1 to ACBP5 may play an important role in binding (Pb(II). Below "Con", the "Com" (Common) amino acids that are common in ACBPs are displayed. "Com" lists the amino acids within the acyl-CoA-binding domains of ACBP1 to ACBP5 that are common in at least 2 of these 5 ACBPs (ACBP1 to ACBP5). At positions in which 2 different residues occur in 2 or more ACBPs, both are shown, with one below the other.

[0027] FIGS. **3**A-**3**B show construction of pAT31 (35S::ACBP1) and pAT314 (35S::ACBP3-GTP) plant transformation vectors. (A) Construct of 35S:ACBP1 in plasmid pAT31 in which the ACBP1 cDNA is expressed from the Cauliflower Mosaic Virus (CaMV) 35S promoter. The ACBP1 full-length cDNA was cloned in the SmaI site of binary vector pBI121 (Clontech). This pBI121 derivative was used in the generation of ACBP1-overexpressing *Arabidopsis* plants. (B) Construct of 35S:ACBP3-GFP in plasmid pAT314. The ACBP3 full-length cDNA was cloned into BamHI site of binary vector pBI-GFP which is a pBI121 derivative obtained by replacement of the GUS gene in pBI121 with eGFP (Shi et al., *Plant Cell* 17: 2340-2354, 2005). ACBP3 is translationally fused with GFP and expressed from Cauliflower Mosaic Virus (CaMV) 35S promoter. The primer 35SB (designated SEQ ID NO:15) used to genotype transformed plants is located within the CaMV 35S promoter region.

[0028] FIGS. **4**A-**4**E show analyses on ACBP1-overexpressing *Arabidopsis* plants and their improved tolerance to Pb(II) stress. (A) RNA gel blot analysis of ACBP1 transcript levels in wild-type *Arabidopsis* (Col-0) and 3 independent ACBP1-overexpressing *Arabidopsis* transgenic lines: ACBP1 ox-2, ox-3 and ox-5 using an ACBP1 cDNA probe. Ethidium bromide-stained rRNA is shown below the blots to indicate the relative amounts of total RNA loaded. (B) Western blot analysis of ACBP1 protein levels in wild-type *Arabidopsis* (Col-0) and ACBP1-overexpressing *Arabidopsis* transgenic lines ACBP1 ox-3 and ACBP1 ox-5 using ACBP1-specific antibodies (Chye, *Plant Mol. Biol.* 38: 827-838, 1998). Bottom, gel identically loaded stained with Coomassie Blue shows the 54-kDa band of RuBisCO large subunit (asterisk). (C) Phenotype of 2-week-old wild-type (Col-0), ACBP1 ox-3 and ACBP1 ox-5 seedlings grown in MS medium and MS medium containing 0.75 mM $Pb(NO_3)_2$. (D) Comparison in relative root length of *Arabidopsis* plants grown in MS medium containing 0.75 mM $Pb(NO_3)_2$ as shown in FIG. **4**C. Bars represent SE (n=10). *P<0.05 by Student's t-test. (E) Comparison in relative fresh weights of shoots and roots of *Arabidopsis* plants grown in MS medium containing 0.75 mM $Pb(NO_3)_2$ as shown in FIG. **4**C. Bars represent SE (n=10). Roots lengths and fresh weights were expressed relative to the values obtained from seedlings grown on MS (100%). *P<0.05 by Student's t-test.

[0029] FIGS. **5**A-**5**E show analyses on ACBP3-overexpressing *Arabidopsis* plants and their enhanced tolerance to Pb(II) treatment. (A) RNA gel blot analysis of ACBP3-GFP transcript levels in wild type (Col-0) and 3 independent ACBP3-overexpressing *Arabidopsis* transgenic lines ACBP3 ox-2, ox-9, and ox-11. Ethidium bromide-stained rRNA is shown below the blots to indicate the relative amounts of total RNA loaded. (B) Western blot analysis of ACBP3-GFP fusion protein levels in wild-type *Arabidopsis* (Col-0) and ACBP3-overexpressing *Arabidopsis* transgenic lines ACBP3 ox-2, ACBP3 ox-9, and ACBP3 ox-11 using the GFP-specific antibodies. Bottom, gel identically loaded stained with Coomassie Blue shows the 54-kDa band of RuBisCO large subunit (asterisk). (C) Three-week-old wild-type (Col-0) and transgenic (ACBP3 ox-2, ACBP3 ox-9, and ACBP3 ox-11) seedlings grown in MS medium and MS medium containing 0.75 mM $Pb(NO_3)_2$. (D) Root length analysis of the plants grown in MS medium in the presence and absence of Pb(II) as depicted in FIG. **5**C. (E) Fresh weight analysis of the plants grown in MS in the presence and absence of Pb(II), as depicted in FIG. **5**C. Bars represent SE (n>10). *P<0.05 by Student's t-test.

[0030] FIGS. **6**A and **6**B show Pb(II) contents in wild-type *Arabidopsis* (Col-0) and in transgenic *Arabidopsis*, ACBP1 ox-3 and ACBP1 ox-5. *Arabidopsis* plants were grown on MS medium for 2 to 3 weeks and then transferred into 1 mM $Pb(NO_3)_2$ solution for 48 h. The shoots and roots were collected for Pb(II) content measurement. Samples were digested overnight at 200° C. with 11 N HNO_3. After dilution with 0.5 N HNO_3 according to Lee et al. (*Plant Physiol.* 138: 827-836, 2005), the samples were analyzed using an atomic absorption spectrometer (PERKIN ELMER-AA Spectrometer 3110). Six replicates were tested for each plant line and each replicate contains 5 plants. The Pb(II) contents were normalized on either a per-plant basis (FIG. **6**A) or a per fresh weight basis (FIG. **6**B) and the significant difference of ACBP1 ox-3 and ACBP1 ox-5 from wild type was determined by Student's t-test (*P<0.05, **P<0.001). Bars represent SE (n=30).

[0031] FIGS. **7**A and **7**B show comparison in Pb(II) content between wild-type *Arabidopsis* (Col-0) and *Arabidopsis* transgenic line ACBP3 ox-11. Six replicates were tested for each plant line and each replicate contains 5 plants. Pb(II) content was normalized on either a per-plant basis (FIG. **7**A) or a per-fresh weight basis (FIG. **7**B). The significant difference between ACBP3 ox-11 and wild type was determined by using the Student's t-test (*P<0.05). Bars represent SE (n=30).

[0032] FIG. **8** shows restriction map of plastid transformation vector pAT385. The 1.1-kb ACBP1 cDNA fragment was generated by RT-PCR using primer pairs ML916 (SEQ ID NO:16) and ML917 (SEQ ID NO:17) and was cloned in pGEM-T EASY vector (Promega). The ACBP1 fragment was then obtained by digestion with restriction enzymes ApaI and SacI and was subsequently inserted into the ApaI-SaeI sites of plastid transformation vector pMLV-HisA (FIG. **8**A; Li et al. *Exp. Biol. Med.* 231: 1346-1352, 2006) to generated pAT385 (FIG. **8**B). rbcLT, tobacco gene encoding chloroplast RuBisCO large subunit; psbARPro, rice promoter of the herbicide binding D1 protein of the photosystem (PSII) reaction center; psbARTer, tobacco terminator of the herbicide binding D1 protein of the photosystem (PSII) reaction center; rrnRPro, rice promoter of rRNA operon; aadA, aminoglycoside 3'-adenylyltransferase; rbcLRTer, rice terminator of chloroplast RuBisCO large subunit in rice; accDT, tobacco gene encoding chloroplast acetyl-CoA carboxylase-subunit.

[0033] FIGS. **9**A-**9**D show plastid transformation and identification of tobacco transformed with plasmid pAT385. (A) Shoot regeneration following tobacco plastid transformation on shoot-inducing medium (RMOP medium) containing 500

mg/l spectinomycin dihydrochloride, one month after bombardment. (B) Transplastomic tobacco with roots after 1 month of culture on MS medium containing 500 mg/l spectinomycin. (C) Schematic map showing integration of pAT385-derived DNA in the tobacco chloroplast genome by homologous recombination. ML347 (SEQ ID NO:18), ML916 (SEQ ID NO:16) and ML917 (SEQ ID NO:17) are primers used in PCR amplification for detection of recombinant DNA inserts following plastid transformation. (D) PCR analyses on pAT385 transplastomic line. Total DNA samples extracted from wild type (FIG. **9**D, lane 1), pMLV-HisA line (FIG. **9**D, lane 2) and the pAT385 transplastomic line (FIG. **9**D, lane 3) were amplified using primers pairs ML347/ML917 and ML916/ML917. The expected 1.3-kb and 1.1-kb bands were obtained from the pAT385 transplastomic line.

BRIEF DESCRIPTION OF THE SEQUENCES

[0034]

SEQ ID NO:1
5'-ATATGGATCCCACGCGTTGTCCTCGTCTTCT-3'

SEQ ID NO:2
5'-AATATATCATCTTGAATTCAACTG-3'

SEQ ID NO:3
5'-CGGGATCCGAAAATGTCAATCTTTGGTTTGATCTTCGC-3'

SEQ ID NO:4
5'-GTCTACAATTGGAATCCTTCTTCTC-3'

SEQ ID NO:5
5'-TCAAGGGGAGAGTTTCC-3'

SEQ ID NO:6
5'-CGTCACCCAGAGGAGTC-3'

SEQ ID NO:7
5'-CTCTCGAGATGGTGGAGAACGATTTGAGT-3'

SEQ ID NO:8
5'-ACGAGCTCACATCATACTCTTAGGGAATACCA-3'

SEQ ID NO:9
5'-AGCTCGAGATGGCTATGCCTAGGGCAAC-3'

SEQ ID NO:10
5'-CGGAGCTCAATGGCATTACCGGACCAAA-3'

SEQ ID NO:11
5'-CGGATCCAATGGCTCACATGGTGAGAGCAG-3'

SEQ ID NO:12
5'-CGAATTCTCATGGGCACTCATGTTTTAGGC-3'

SEQ ID NO:13
5'-GCTCGAAGACGATCAGATACC-3'

SEQ ID NO:14
5'-AGAAAGAGCTCTCAGCTCGTC-3'

SEQ ID NO:15
5'-CAATCCCACTATCCTTCGCAAGACC-3'

SEQ ID NO:16
5'-TGGGGCCCATGGCTGATTGGTATCAGC-3'

SEQ ID NO:17
5'-GCATCGATCTTTGACACACAATTTTAAAG-3'

SEQ ID NO:18
5'-CACACAAATCGGTAGAGCTTAT-3'

-continued

SEQ ID NO:19
Met Arg Gly Ser His His His His His His Gly Met

Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp

Leu Tyr Asp Asp Asp Asp Lys Asp Arg Trp Ile Arg

Pro Arg Asp Leu Gln Leu Val Pro Trp Asn Ser Arg

SEQ ID NO:20
5'-CGGGATCCGAAAATGTCGCTAATCTCTATCCTCCTCG-3'

SEQ ID NO:21
5'-ATGGGTGATTGGGCTCAACT-3'

SEQ ID NO:22
5'-TTAGTCTGCCTGCTTTGCAG-3'

SEQ ID NO:23
5'-TTCTCCGTCTTACACCGATT-3'

SEQ ID NO:24
5'-CTTGATGAGGCATTTAGTGC-3'

SEQ ID NO:25
5'-TGGGTAAAGCTGAGTAACAAG-3

DETAILED DESCRIPTION OF THE INVENTION

[0035] As used herein, the term "modified plant or plant parts" refers to a plant or plant part, whether it is attached or detached from the whole plant. It also includes progeny of the modified plant or plant parts that are produced through sexual or asexual reproduction.

[0036] The terms "polynucleotide" and "nucleic acid," used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides. Thus, this term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases.

[0037] As used herein, the terms "operon" and "single transcription unit" are used interchangeably to refer to two or more contiguous coding regions (nucleotide sequences that encode a gene product such as an RNA or a protein) that are coordinately regulated by one or more controlling elements (e.g., a promoter). As used herein, the term "gene product" refers to RNA encoded by DNA (or vice versa) or protein that is encoded by an RNA or DNA, where a gene will typically comprise one or more nucleotide sequences that encode a protein, and may also include introns and other non-coding nucleotide sequences.

[0038] The terms "peptide," "polypeptide," and "protein" are used interchangeably herein, and refer to a polymeric form of amino acids of any length, which can include coded and non-coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones.

[0039] The term "naturally-occurring" as used herein as applied to a nucleic acid, a cell, or an organism, refers to a nucleic acid, cell, or organism that is found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by a human in the laboratory is naturally occurring.

[0040] The term "heterologous nucleic acid," as used herein, refers to a nucleic acid wherein at least one of the following is true: (a) the nucleic acid is foreign ("exogenous") to (i.e., not naturally found in) a given host microorganism or host cell; (b) the nucleic acid comprises a nucleotide sequence that is naturally found in (e.g., is "endogenous to") a given host microorganism or host cell (e.g., the nucleic acid comprises a nucleotide sequence endogenous to the host microorganism or host cell); however, in the context of a heterologous nucleic acid, the same nucleotide sequence as found endogenously is produced in an unnatural (e.g., greater than expected or greater than naturally found) amount in the cell, or a nucleic acid comprising a nucleotide sequence that differs in sequence from the endogenous nucleotide sequence but encodes the same protein (having the same or substantially the same amino acid sequence) as found endogenously is produced in an unnatural (e.g., greater than expected or greater than naturally found) amount in the cell; (c) the nucleic acid comprises two or more nucleotide sequences that are not found in the same relationship to each other in nature, e.g., the nucleic acid is recombinant. An example of a heterologous nucleic acid is a nucleotide sequence encoding an ACBP operably linked to a transcriptional control element (for example, a promoter) to which an endogenous (naturally-occurring) ACBP coding sequence is not normally operably linked. Another example of a heterologous nucleic acid is a high copy number plasmid comprising a nucleotide sequence encoding an ACBP. Another example of a heterologous nucleic acid is a nucleic acid encoding an ACBP, where a host cell that does not normally produce ACBPs is genetically modified with the nucleic acid encoding ACBP; because ACBP-encoding nucleic acids are not naturally found in the host cell, the nucleic acid is heterologous to the genetically modified host cell.

[0041] "Recomnbinant," as used herein, means that a particular nucleic acid (DNA or RNA) is the product of various combinations of cloning, restriction, and/or ligation steps resulting in a construct having a structural coding or non-coding sequence distinguishable from endogenous nucleic acids found in natural systems. Generally, DNA sequences encoding the structural coding sequence can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of synthetic oligonucleotides, to provide a synthetic nucleic acid which is capable of being expressed from a recombinant transcriptional unit contained in a cell or in a cell-free transcription and translation system. Such sequences can be provided in the form of an open reading frame uninterrupted by internal non-translated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA comprising the relevant sequences can also be used in the formation of a recombinant gene or transcriptional unit. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where such sequences do not interfere with manipulation or expression of the coding regions, and may indeed act to modulate production of a desired product by various mechanisms (see "DNA regulatory sequences", below).

[0042] Thus, for example, the term "recombinant" polynucleotide or nucleic acid refers to one which is not naturally occurring, for example, is made by the artificial combination of two otherwise separated segments of sequence through human intervention. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a desired combination of functions. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

[0043] By "construct" is meant a recombinant nucleic acid, generally recombinant DNA, which has been generated for the purpose of the expression of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences.

[0044] As used herein, the term "exogenous nucleic acid" refers to a nucleic acid that is not normally or naturally found in and/or produced by a given bacterium, organism, or cell in nature. As used herein, the term "endogenous nucleic acid" refers to a nucleic acid that is normally found in and/or produced by a given bacterium, organism, or cell in nature. An "endogenous nucleic acid" is also referred to as a "native nucleic acid" or a nucleic acid that is "native" to a given bacterium, organism, or cell. For example, the nucleic acids encoding ACBPs in Example 3 represent exogenous nucleic acids to *E. coli*. These nucleic acids were cloned from *Arabidopsis*. In *Arabidopsis*, the gene sequences in their native location on the chromosome encoding those ACBPs would be "endogenous" nucleic acids.

[0045] The terms "DNA regulatory sequences," "control elements," and "regulatory elements," used interchangeably herein, refer to transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, protein degradation signals, and the like, that provide for and/or regulate expression of a coding sequence and/or production of an encoded polypeptide in a host cell.

[0046] The terms "transformation" or "transformed" are used interchangeably herein with "genetic modification" or "genetically modified" and refer to a permanent or transient genetic change induced in a cell following introduction of new nucleic acid (i.e., DNA exogenous to the cell). Genetic change ("modification") can be accomplished either by incorporation of the new DNA into the genome of the host cell, or by transient or stable maintenance of the new DNA as an episomal element. Where the cell is a eukaryotic cell, a permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. In prokaryotic cells, permanent changes can be introduced into the chromosome or via extrachromosomal elements such as plasmids and expression vectors, which may contain one or more selectable markers to aid in their maintenance in the recombinant host cell.

[0047] "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For instance, a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression. As used herein, the terms "heterologous promoter" and "heterologous control regions" refer to promoters and other control regions that are not normally associated with a particular nucleic acid in nature. For example, a "transcriptional control region heter-

ologous to a coding region" is a transcriptional control region that is not normally associated with the coding region in nature.

[0048] A "host cell," as used herein, denotes an in vivo or in vitro eukaryotic cell, a prokaryotic cell, or a cell from a multicellular organism (for example, a cell line) cultured as a unicellular entity, which eukaryotic or prokaryotic cells can be, or have been, used as recipients for a nucleic acid (for example, an expression vector that comprises a nucleotide sequence encoding one or more gene products such as ACBPs), and include the progeny of the original cell which has been genetically modified by the nucleic acid. It is understood that the progeny of a single cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. A "recombinant host cell" (also referred to as a "genetically modified host cell") is a host cell into which has been introduced a heterologous nucleic acid, e.g., an expression vector. For example, a subject prokaryotic host cell is a genetically modified prokaryotic host cell (for example, a bacterium), by virtue of introduction into a suitable prokaryotic host cell a heterologous nucleic acid, e.g., an exogenous nucleic acid that is foreign to (not normally found in nature in) the prokaryotic host cell, or a recombinant nucleic acid that is not normally found in the prokaryotic host cell; and a subject eukaryotic host cell is a genetically modified eukaryotic host cell, by virtue of introduction into a suitable eukaryotic host cell a heterologous nucleic acid, for example, an exogenous nucleic acid that is foreign to the eukaryotic host cell, or a recombinant nucleic acid that is not normally found in the eukaryotic host cell.

[0049] As used herein the term "isolated" is meant to describe a polynucleotide, a polypeptide, or a cell that is in an environment different from that in which the polynucleotide, the polypeptide, or the cell naturally occurs. An isolated genetically modified host cell may be present in a mixed population of genetically modified host cells.

[0050] Expression cassettes may be prepared comprising a transcription initiation or transcriptional control region(s) (for example, a promoter), the coding region for the protein of interest, and a transcriptional termination region. Transcriptional control regions include those that provide for overexpression of the protein of interest in the genetically modified host cell; those that provide for inducible expression, such that when an inducing agent is added to the culture medium, transcription of the coding region of the protein of interest is induced or increased to a higher level than prior to induction.

[0051] A nucleic acid is "hybridizable" to another nucleic acid, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid can anneal to the other nucleic acid under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), particularly Chapter 11 and Table 11.1 therein; and Sambrook, J. and Russell, W., *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2001). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Hybridization conditions and post-hybridization washes are useful to obtain the desired determine stringency conditions of the hybridization. One set of illustrative post-hybridization washes is a series of washes starting with 6×SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer), 0.5% SDS at room temperature for 15 minutes, then repeated with 2×SSC, 0.5% SDS at 45° C. for 30 minutes, and then repeated twice with 0.2×SSC, 0.5% SDS at 50° C. for 30 minutes. Other stringent conditions are obtained by using higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 minute washes in 0.2×SSC, 0.5% SDS, which is increased to 60° C. Another set of highly stringent conditions uses two final washes in 0.1×SSC, 0.1% SDS at 65° C. Another example of stringent hybridization conditions is hybridization at 50° C. or higher and 0.1×SSC (15 mM sodium chloride/1.5 mM sodium citrate). Another example of stringent hybridization conditions is overnight incubation at 42° C. in a solution: 50% formamide, 5×SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5×Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1× SSC at about 65° C. Stringent hybridization conditions and post-hybridization wash conditions are hybridization conditions and post-hybridization wash conditions that are at least as stringent as the above representative conditions.

[0052] Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of the melting temperature (Tm) for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher Tm) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating Tm have been derived (see Sambrook et al., supra, 9.50 9.51). For hybridizations with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., supra, 11.7 11.8). Typically, the length for a hybridizable nucleic acid is at least about 10 nucleotides. Illustrative minimum lengths for a hybridizable nucleic acid are: at least about 15 nucleotides; at least about 20 nucleotides; and at least about 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

[0053] The term "conservative amino acid substitution" refers to the interchangeability in proteins of amino acid residues having similar side chains. For example, a group of amino acids having aliphatic side chains consists of glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains consists of serine and threonine; a group of amino acids having amide-containing side chains consists of asparagine and glutamine; a group of amino acids having aromatic side chains consists of phenylalanine, tyrosine, and tryptophan; a group of amino acids

having basic side chains consists of lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains consists of cysteine and methionine. Exemplary conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

[0054] "Synthetic nucleic acids" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments which are then enzymatically assembled to construct the entire gene. "Chemically synthesized," as related to a sequence of DNA, means that the component nucleotides were assembled in vitro. Manual chemical synthesis of DNA may be accomplished using well-established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. The nucleotide sequence of the nucleic acids can be modified for optimal expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available. Fragments of full-length proteins can be produced by techniques well known in the art, such as by creating synthetic nucleic acids encoding the desired portions; or by use of Bal 31 exonuclease to generate fragments of a longer nucleic acid.

[0055] A polynucleotide or polypeptide has a certain percent "sequence identity" to another polynucleotide or polypeptide, meaning that, when aligned, that percentage of bases or amino acids are the same, and in the same relative position, when comparing the two sequences. Sequence similarity can be determined in a number of different manners. To determine sequence identity, sequences can be aligned using the methods and computer programs, including BLAST, available over the world wide web at nebi.nlm.nih.gov/BLAST. See, e.g., Altschul et al. (1990), *J. Mol. Biol.* 215: 403-410. Another alignment algorithm is FASTA, available in the Genetics Computing Group (GCG) package, from Madison, Wis., USA, a wholly owned subsidiary of Oxford Molecular Group, Inc. Other techniques for alignment are described in *Methods in Enzymology*, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, Calif., USA. Of particular interest are alignment programs that permit gaps in the sequence. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See *Meth. Mol. Biol.* 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. See *J. Mol. Biol.* 48: 443-453 (1970).

[0056] As used herein, the term "variant" refers either to a naturally occurring genetic mutant of ACBP or a recombinantly prepared variation of ACBP, each of which contain one or more mutations in its DNA. The term "variant" may also refer to either a naturally occurring variation of a given peptide or a recombinantly prepared variation of a given peptide or protein in which one or more amino acid residues have been modified by amino acid substitution, addition, or deletion. Preferably, the variants include less than 25, less than 20, less than 15, less than 10, less than 5, less than 4, less than 3, or less than 2 amino acid substitutions, rearrangements, insertions, and/or deletions relative to *Arabidopsis* ACBPs. In this regard, the term "variant" can encompass fragments, derivatives, and analogs of *Arabidopsis* ACBPs.

[0057] To generate a subject genetically modified host cell, one or more nucleic acids comprising nucleotide sequences encoding one or more ACBP polypeptides that relieve lead accumulation-induced growth inhibition is introduced stably or transiently into a parent host cell, using established techniques, including, but not limited to, electroporation, calcium phosphate precipitation, DEAE-dextran mediated transfection, liposome-mediated transfection, particle bombardment, and the like. For stable transformation, a nucleic acid will generally further include a selectable marker, for example, any of several well-known selectable markers such as neomycin resistance, ampicillin resistance, tetracycline resistance, chloramphenicol resistance, kanamycin resistance, and the like.

[0058] Where a parent host cell has been genetically modified to produce two or more ACBPs, nucleotide sequences encoding the two or more ACBPs will in some embodiments each be contained on separate expression vectors. Where the host cell is genetically modified to express one or more ACBPs, nucleotide sequences encoding the one or more ACBPs will in some embodiments be contained in a single expression vector. Where nucleotide sequences encoding the one or more ACBPs are contained in a single expression vector, in some embodiments, the nucleotide sequences will be operably linked to a common control element (for example, a promoter), such that the common control element controls expression of all of the ACBP-encoding nucleotide sequences on the single expression vector.

[0059] Where nucleotide sequences encoding the ACBP(s) are contained in a single expression vector, in some embodiments, the nucleotide sequences will be operably linked to different control elements (for example, a promoter), such that, the different control elements control expression of each of the ACBP-encoding nucleotide sequences separately on a single expression vector.

[0060] A subject screening method can involve introducing an exogenous nucleic acid into a host cell, producing a test cell, where the host cell is one that exhibits growth inhibition when lead (or another metal present as a divalent cation, such as copper or cadmium, etc.) is present in the growth medium in a growth-inhibiting amount. When an exogenous nucleic acid comprising a nucleotide sequence that encodes an ACBP is introduced into the host cell, growth inhibition of the test cell is relieved. Thus, a reduction in growth inhibition indicates that the exogenous nucleic acid encodes ACBP, where the encoded ACBP is produced at a level and/or has an activity that relieves the lead accumulation-induced growth inhibition. A reduction in growth inhibition includes an at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or more, reduction in growth inhibition. In some embodiments, the ACBP encoded by the exogenous nucleic acid reduces the growth inhibition such that the rate of cell growth is restored to the rate of cell growth of the host cell when grown under conditions where lead is not present in growth inhibiting amounts.

[0061] In some embodiments, for example, where the exogenous nucleic acid is a plurality of exogenous nucleic acids (such as, for example, a cDNA library, a genomic library, or a population of nucleic acids, each encoding an

ACBP with a different amino acid sequence, etc.), the exogenous nucleic acids are introduced into a plurality of host cells, forming a plurality of test cells. The test cells are in some embodiments grown in culture under conditions such that lead is present in a growth inhibiting and/or death-inducing amount; those test cells comprising an exogenous nucleic acid that comprises nucleotide sequences encoding an ACBP will grow faster than test cells that do not comprise an exogenous nucleic acid that comprises nucleotide sequences encoding an ACBP, or those test cells comprising an exogenous nucleic acid that comprises nucleotide sequences encoding an ACBP will live, while test cells that do not comprise an exogenous nucleic acid that comprises nucleotide sequences encoding ACBP will die or otherwise be adversely affected.

[0062] In some embodiments, the method further involves isolating an exogenous nucleic acid from a test cell, where the exogenous nucleic acid is one that that relieves growth inhibition in a subject screening method. Methods of isolating the exogenous nucleic acid from a test cell are well known in the art. Suitable methods include, but are not limited to, any of a number of alkaline lysis methods that are standard in the art.

[0063] In some embodiments, a subject screening method will further comprise further characterizing a candidate gene product. In these embodiments, the exogenous nucleic acid comprising nucleotide sequence(s) encoding an ACBP(s) are isolated from a test cell; the gene product(s) are expressed in a cell and/or in an in vitro cell-free transcription/translation system. In some embodiments, the exogenous nucleic acid is subjected to nucleotide sequence analysis, and the amino acid sequence of the gene product deduced from the nucleotide sequence. In some embodiments, the amino acid sequence of the gene product is compared with other amino acid sequences in a public database of amino acid sequences, to determine whether any significant amino acid sequence identity to an amino acid sequence of a known protein exists. In addition, the gene product(s) are expressed in a cell and/or in an in vitro cell-free transcription/translation system; and the effect of the gene product(s) on a metabolic pathway intermediate or other metabolite is analyzed.

[0064] Exogenous nucleic acids that are suitable for introducing into a host cell, to produce a test cell, include, but are not limited to, naturally-occurring nucleic acids isolated from a cell; naturally-occurring nucleic acids that have been modified (for example, by mutation) before or subsequent to isolation from a cell; synthetic nucleic acids, e.g., nucleic acids synthesized in a laboratory using standard methods of chemical synthesis of nucleic acids, or generated by recombinant methods; synthetic or naturally-occurring nucleic acids that have been amplified in vitro, either within a cell or in a cell-free system; and the like.

[0065] Exogenous nucleic acids that are suitable for introducing into a host cell include, but are not limited to, genomic DNA; RNA; a complementary DNA (cDNA) copy of mRNA isolated from a cell; recombinant DNA; and DNA synthesized in vitro, e.g., using standard cell-free in vitro methods for DNA synthesis. In some embodiments, exogenous nucleic acids are a cDNA library made from cells, either prokaryotic cells or eukaryotic cells. In some embodiments, exogenous nucleic acids are a genomic DNA library made from cells, either prokaryotic cells or eukaryotic cells.

[0066] Nucleic acids will in some embodiments be mutated before being introduced into a host cell. Methods of mutating a nucleic acid are well known in the art and include well-established chemical mutation methods, radiation-induced mutagenesis, and methods of mutating a nucleic acid during synthesis. Chemical methods of mutating DNA include exposure of DNA to a chemical mutagen, e.g., ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), N-nitrosourea (ENU), N-methyl-N-nitro-N'-nitrosoguanidine, 4-nitroquinoline N-oxide, diethylsulfate, benzopyrene, cyclophosphamide, bleomycin, triethylmelamine, acrylamide monomer, nitrogen mustard, vincristine, diepoxyalkanes (for example, diepoxybutane), ICR-170, formaldehyde, procarbazine hydrochloride, ethylene oxide, dimethylnitrosamine, 7,12 dimethylbenz(a)anthracene, chlorambucil, hexamethylphosphoramide, bisulfan, and the like. Radiation mutation-inducing agents include ultraviolet radiation, .gammna.-iiradiation, X-rays, and fast neutron bombardment. Mutations can also be introduced into a nucleic acid using, e.g., trimethylpsoralen with ultraviolet light. Random or targeted insertion of a mobile DNA element, e.g., a transposable element, is another suitable method for generating mutations. Mutations can be introduced into a nucleic acid during amplification in a cell-free in vitro system, e.g., using a polymerase chain reaction (PCR) technique such as error-prone PCR. Mutations can be introduced into a nucleic acid in vitro using DNA shuffling techniques (e.g., exon shuffling, domain swapping, and the like). Mutations can also be introduced into a nucleic acid as a result of a deficiency in a DNA repair enzyme in a cell, e.g., the presence in a cell of a mutant gene encoding a mutant DNA repair enzyme is expected to generate a high frequency of mutations (i.e., about 1 mutation/100 genes-1 mutation/10,000 genes) in the genome of the cell. Examples of genes encoding DNA repair enzymes include but are not limited to Mut H, Mut S, Mut L, and Mut U, and the homologs thereof in other species (e.g., MSH 16, PMS 12, MLH 1, GTBP, ERCC-1, and the like). Methods of mutating nucleic acids are well known in the art, and any known method is suitable for use. See, e.g., Stemple (2004) *Nature* 5:1-7; Chiang et al. (1993) *PCR Methods Appl* 2(3): 210-217; Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; and U.S. Pat. Nos. 6,033,861, and 6,773,900.

[0067] In many embodiments, the exogenous nucleic acid is inserted into an expression vector. Expression vectors that are suitable for use in prokaryotic and eukaryotic host cells are known in the art, and any suitable expression vector can be used. Suitable expression vectors are as described above.

[0068] As noted above, an exogenous nucleic acid will in some embodiments be isolated from a cell or an organism in its natural environment. In some embodiments, the nucleic acid of the cell or organism will be mutated before nucleic acid is isolated from the cell or organism. In other embodiments, the exogenous nucleic acid is synthesized in a cell-free system in vitro.

[0069] In some embodiments, the exogenous nucleic acid is a synthetic nucleic acid. In some embodiments, a synthetic nucleic acid comprises a nucleotide sequence encoding a variant ACBP, for example, an ACBP that differs in amino acid sequence by one or more amino acids from a naturally-occurring ACBP or other parent ACBP. In some embodiments, a variant ACBP differs in amino acid sequence by one amino acid, two amino acids, three amino acids, four amino acids, five amino acids, six amino acids, seven amino acids, eight amino acids, nine amino acids, or amino acids, or more, compared to the amino acid sequence of a naturally-occurring parent ACBP. In some embodiments, a variant ACBP differs

in amino acid sequence by from about 10 amino acids to about 15 amino acids, from about 15 amino acids to about 20 amino acids, from about 20 amino acids to about 25 amino acids, from about 25 amino acids to about 30 amino acids, from about 30 amino acids to about 35 amino acids, from about 35 amino acids to about 40 amino acids, from about 40 amino acids to about 50 amino acids, or from about 50 amino acids to about 60 amino acids, compared to the amino acid sequence of a naturally-occurring parent ACBP.

[0070] In some embodiments, a variant ACBP is encoded by a nucleic acid that hybridizes under stringent hybridization conditions to a nucleic acid encoding a known ACBP. In other embodiments, a variant ACBP is encoded by a nucleic acid that hybridizes under moderate hybridization conditions to a nucleic acid encoding a known ACBP.

[0071] In some embodiments, a nucleic acid comprising a nucleotide sequence encoding a naturally-occurring ACBP is mutated, using any of a variety of well-established methods, giving rise to a nucleic acid comprising a nucleotide sequence encoding a variant ACBP. Suitable mutagenesis methods include, but are not limited to, chemical mutation methods, radiation-induced mutagenesis, and methods of mutating a nucleic acid during synthesis, as described supra. Thus, for example, a nucleic acid comprising a nucleotide sequence encoding a naturally-occurring ACBP is exposed to a chemical mutagen, as described above, or subjected to radiation mutation, or subjected to an error-prone PCR, and the mutagenized nucleic acid introduced into a genetically modified host cell(s) as described above. Methods for random mutagenesis using a "mutator" strain of bacteria are also well known in the art and can be used to generate a variant ACBP. See, e.g., Greener et al., "An Efficient Random Mutagenesis Technique Using an *E. coli* Mutator Strain", *Methods in Molecular Biology,* 57:375-385 (1995). Saturation mutagenesis techniques employing a polymerase chain reaction (PCR) are also well known and can be used. See, e.g., U.S. Pat. No. 6,171,820. Nucleic acids comprising a nucleotide sequence encoding a variant ACBP are identified by the ability to relieve growth inhibition caused by lead.

[0072] Nucleotide sequences encoding ACBPs are known in the art, and any known ACBP-encoding nucleotide sequence can be altered to generate a synthetic nucleic acid for use in a subject method.

[0073] An embodiment of the invention provides a host cell comprising a vector according to the invention. Other embodiments include plant plastid transformation vectors or nuclear transformation vectors containing nucleotide sequences encoding *Arabidopsis* ACBPs, such as containing the full-length ACBPs, or variants or fragments thereof, for the expression of ACBPs or ACBP polypeptides or variants exhibiting similar lead-binding activities to full-length ACBPs. These plant vectors may contain other sequences for the generation of chimeric ACBP polypeptides which may contain mutations, deletions, or insertions of the ACBP polypeptides.

[0074] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0075] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0076] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0077] It must be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a genetically modified host cell" includes a plurality of such host cells and reference to "the ACBP" includes reference to one or more *Arabidopsis* ACBPs and equivalents thereof that will become known to those skilled in the art in view of this disclosure, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

EXAMPLES

[0078] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1

Plant Materials, Growth Conditions and Treatment

[0079] *Arabidopsis thaliana* wild-type Columbia (ecotype Col-0), ACBP1- and ACBP3-overexpressing transgenic plants (ecotype Col-0) were grown under 8 h dark at 21° C. and 16 h light at 23° C. cycles. For Pb(II)-inducible gene expression analysis, *Arabidopsis* seedlings were grown for 3 weeks in continuous light, treated with 1 mM $Pb(NO_3)_2$ or

water (as control), and shoot and root samples were collected at 24 h post-treatment. For Pb(II) sensitivity tests, *Arabidopsis* seedlings were surface-sterilized and grown on Murashige and Skoog (*Physiol. Plant.* 15: 473-497, 1962) medium containing 2% sucrose with or without 0.75 mM $Pb(NO_3)_2$ (Sigma-Aldrich, St. Louis) for 2 to 3 weeks.

Example 2

ACBP Transcripts are Induced by PB(II) Treatment

[0080] To determine if the regulation of *Arabidopsis* ACBP mRNAs responds to Pb(II) stress, we performed reverse transcription-PCR(RT-PCR) using root tissue RNA following treatment with 1 mM $Pb(NO_3)_2$. For RT-PCR, total RNA was extracted with TRIzol (Invitrogen) reagent, according to the manufacturer's protocol, from roots of 3-week-old wild-type *Arabidopsis* (Col-0) seedlings in the presence or absence (water as control) of 1 mM $Pb(NO_3)_2$. RT-PCR analysis was performed as according to the manufacturer (Invitrogen Cat No. 12371-019). Specific primers were designed based on mRNA sequences. Primers used for RT-PCR analysis of ACBP6 were ML750 (SEQ TD NO:1) and ML751 (SEQ ID NO:2); ACBP1, ML179 (SEQ ID NO:3) and ML759 (SEQ ID NO:4); ACBP2, ML194 (SEQ ID NO:5) and ML205 (SEQ ID NO:6); ACBP3, ML783 (SEQ ID NO:7) and ML784 (SEQ ID NO:8); ACBP4, ML849 (SEQ ID NO:9) and ML850 (SEQ ID NO:10); ACBP5, ML352 (SEQ ID NO:11) and ML353 (SEQ ID NO:12); 18S, 18S-F (SEQ ID NO:13) and 18S-R (SEQ ID NO:14).

[0081] The mRNA expressions of ACBP1 to ACBP5 was observed to increase in roots 24 h after Pb(II) treatment on RT-PCR analysis (FIG. **1**). Surprisingly, the transcript of ACBP6 which encodes the homolog of human 9-kDa ACBP implicated as a molecular target for Pb(II) in vivo (Smith et al., *Chemico-Biological Interactions* 115: 39-52, 1998), showed little change upon Pb(II) treatment (FIG. **1**). These results indicate that *Arabidopsis* ACBP1 to ACBP5, to a greater extent than ACBP6, play a role in Pb(II) stress.

Example 3

Expression of Recombinant ACBPs in *E. coli* BL21DE3 Cells

[0082] Each of the six cDNAs encoding *Arabidopsis* ACBPs were cloned in plasmid pRSET (Invitrogen) for expression in *Escherichia coli* according to protocols specified by Invitrogen, as $(His)_6$-tagged ACBP recombinant proteins that were subsequently used to ascertain if these $(His)_6$-ACBPs bind Pb(II) in vitro. The ACBP-pRSET derivatives were expressed in bacterial (*Escherichia coli* BL21DE3) cells and subsequently harvested for purification of the $(His)_6$-tagged proteins following methods described in Leung et al. (*Planta* 223: 871-881, 2006). These purified ACBP recombinant proteins were then tested for the ability to bind lead using Pb(II)-binding assays (Funaba and Mathews, *Mol. Endocrinol.* 14: 1583-1591, 2000).

[0083] *E. coli* BL21(DE3)Star pLysS (Invitrogen, Carlsbad, Calif., USA) were transformed with each of the six plasmids expressing recombinant ACBP6 (10-kDa ACBP) and ACBP1 to ACBP5. Transformed cells were grown to OD_{600nm}=0.4, as measured using a UV-spectrophotometer (Shimadzu Model UV-1206, Japan), and induced with 1 mM IPTG. Each $(His)_6$-ACBP was harvested 3 h after IPTG-induction for extraction of soluble and insoluble proteins following procedures described by Invitrogen (Carlsbad, Calif., USA). The detailed procedure for the purification of each recombinant ACBP has been previously described for ACBP1 (Chye, *Plant Mol. Biol.* 38: 827-838, 1998), ACBP2 (Chye et al., *Plant Mol. Biol.* 44: 711-721, 2000), ACBP3 (Leung et al., *Planta* 223: 871-881, 2006) and ACBP4 and ACBP5 (Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004) and is briefly described below. Harvested protein samples were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (*Nature* 227: 680-685, 1970).

[0084] Recombinant $(His)_6$-ACBP1 fusion protein was expressed from plasmid pAT 61 in *E. coli* as described (Chye, *Plant Mol. Biol.* 38: 827-838, 1998). Plasmid pAT 61 was produced by cloning an ExoIII deletion derivative of the ACBP1 cDNA on a 1.1 kb EcoRI fragment in-frame to the EcoRI site of pRSET B vector (Chye, *Plant Mol. Biol.* 38: 827-838, 1998). The re-combinant $(His)_6$-ACBP1 fusion protein lacks the first 40 amino acids at the N-terminal of ACBP1 (Chye, *Plant Mol. Biol.* 38: 827-838, 1998).

[0085] Recombinant $(His)_6$-ACBP2 fusion protein expression in *E. coli* was achieved by cloning a 1.3-kb ACBP2 cDNA on an EcoRI fragment, in-frame to the EcoRI site of vector pRSET C (Invitrogen Xpress System) to yield plasmid pACBP2. This 1.3 kb cDNA is incomplete at the 5'-end, lacking the 0.19 kb 5'-untranslated region and the methionine start codon. To confirm that the 1.3 kb cDNA was cloned in-frame to the $(His)_6$ tag, the DNA sequence of pACBP2 across the EcoRI site was subsequently verified. The EcoRI site in the pRSET C vector lies downstream from the T7 promoter, which drives expression of the recombinant $(His)_6$-ACBP2 fusion protein. At the N-terminus of this recombinant protein, the peptide sequence encoded by pRSET C (MRGSHHHHHHGMASMTGGQQMGRDLYDDDDIDRWIRPRDLQLVPWNSR), designated SEQ ID NO:19, is fused to amino acid residue Gly-2 on the ACBP2 peptide. The expected molecular mass of this recombinant protein was predicted using the GCG analysis package (Genetics Computer Group). *E. Coli* cells transformed with pACBP2 were cultured and $(His)_6$-ACBP2 fusion protein expression was induced with 1 mM IPTG; soluble protein and insoluble protein were extracted according to the procedures described by Invitrogen. Protein concentrations of these protein extracts were determined by the method of Bradford (*Anal. Biochem.* 72: 248-254, 1976). The optimal time for protein induction was determined at 4 h after IPTG induction, according to the procedure described by Invitrogen. Protein samples (10 μg) of soluble protein extract and of insoluble protein extract, taken at various time intervals after addition of 1 mM IPTG, were analyzed by SDS-PAGE according to Laemmli (*Nature* 227:680-685, 1970).

[0086] Recombinant $(His)_6$-ACBP3, cloned in pRSET B (Invitrogen)-derived vector pAT223, was prepared from *E. coli* BL21(DE3)Star pLys (Invitrogen) transformants as described in Leung et al. (*Planta* 223: 871-881, 2006). Transformed cells were grown to $OD_{600\ nm}$=0.4, as measured using a UV-spectrophotometer (Shimadzu Model UV-1206, Japan), and induced with 1 mM IPTG. $(His)_6$-ACBP3 was harvested 3 h after IPTG-induction for extraction of soluble and insoluble proteins following procedures described by Invitrogen (Carlsbad, Calif., USA).

[0087] Recombinant $(His)_6$-ACBP4 and $(His)_6$-ACBP5, expressed from pRSET B (Invitrogen)-derived vectors pAT184 and pAT185, respectively, were prepared from *E.*

coli BL21(DE3)Star pLys (Invitrogen) transformants as described in Leung et al. (*Plant Mol. Biol.* 55: 297-309, 2004).

[0088] Recombinant $(His)_6$-ACBP6 (size of recombinant protein inclusive of His-tag is 18.9 kDa) cloned in pRSET B (Invitrogen)-derived vector pAT335, was prepared from *E. coli* BL21(DE3)Star pLys (Invitrogen) transformants. A 0.65-kb PCR fragment consisting of the full-length cDNA encoding *Arabidopsis* ACBP6 was generated by RT-PCR using primers ML750 (5'-ATATGGATCCCACGCGT-TGTCCTCGTCTTCT-3'), designated SEQ ID NO:1, and ML751 (5'-AATATATCATCTTGAATTCAACTG-3'), designated SEQ ID NO:2. The 0.65-1 kb fragment was cloned into pGEM-T EASY vector (Promega). After confirmation of the insert by DNA sequencing analysis, the SacI-EcoRI fragment corresponding to the coding region was cloned in-frame to vector pRSET B (Invitrogen) to yield plasmid pAT335. The molecular mass of this $(His)_6$-tagged ACBP expressed from this plasmid was predicted using the GCG (Genetics Computer Group, Wisconsin Software Version 10.2) programme to be 18.9-kDa. The plasmid pAT335 was introduced into *E. coli* BL21 (DE3)Star pLysS (Invitrogen, Carlsbad, Calif., USA) and then cultured to OD_{600nm}=0.4, as measured using a UV-spectrophotometer (Shimadzu Model UV-1206, Japan), and subsequently induced with 1 mM IPTG. After IPTG-induction for 3 h, the $(His)_6$-tagged ACBP expressing cells were harvested for extraction of insoluble protein following procedures described by Invitrogen (Carlsbad, Calif., USA). Protein concentration of bacterial crude extracts was determined following the method of Bradford (Anal Biochem 72: 248-254, 1976). Subsequently, 10 μg of the each protein sample were separated by 15% polyacrylamide gel and then electrophoretically transferred to ECL membrane (Amersham, Buckinghamshire, UK) using the Trans-Blot cell (Bio-Rad) or stained by Coomassie Brilliant Blue.

[0089] Protein concentrations of bacterial crude extracts were determined by the method of Bradford (*Anal. Biochem.* 72: 248-254, 1976) and samples (10 μg) harvested at various time intervals were analyzed by SDS-PAGE according to Laemmli (*Nature* 227: 680-685, 1970). Subsequently the 10% polyacrylamide gel was stained with Coomassie Blue or used for western blotting (Sambrook et al. In: *Molecular Cloning: A Laboratory Manual*, Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 18.60-18. 73, 1989) by electrophoretically transfer of proteins to ECL membranes (Amersham, Buckinghamshire, UK) from the polyacrylamide gel using the Trans-Blot® cell (Bio-Rad) following the manufacturer's instructions. The QIAexpress Ni-NTA Conjugate (Qiagen, Valencia, Calif., USA) was used according to the manufacturer to detect the expression of the $(His)_6$-tagged ACBP proteins.

Example 4

Purification of Recombinant $(His)_6$-Tagged ACBPs from *E. coli* Transformants

[0090] Recombinant $(His)_6$-tagged ACBPs were purified through affinity columns of Ni-NTA Agarose (Qiagen, Valebcia, Calif., USA) following instructions of the manufacturer as previously described (Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004; Leung et al., *Planta* 223: 871-881, 2006).

[0091] $(His)_6$-ACBP1 expressed in the soluble fraction of *E. Coli* extracts and was purified through an affinity column of Ni-NTA Agarose (Qiagen, Valencia, Calif., USA) according to the instructions of the supplier.

[0092] Batch extractions of $(His)_6$-ACBP6, $(His)_6$-ACBP2, $(His)_6$-ACBP3, $(His)_6$-ACBP4 and $(His)_6$-ACBP5 were carried out under denaturing conditions. The $(His)_6$-ACBP2 fusion protein eluted in Buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris pH 4.5, 5% glycerol). The $(His)_6$-ACBP3, $(His)_6$-ACBP4 and $(His)_6$-ACBP5 fusion proteins eluted in both buffer D (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 5.9, 5% glycerol) and buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 4.5, 5% glycerol). After dialysis and refolding in 50 mM HEPES sodium salt, 200 mM NaCl, 2 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, 0.005% (v/v) Tween-20, pH 7.9 at 4 C, each recombinant protein was purified through an affinity column of Ni-NTA Agarose (Qiagen, Valencia, Calif., USA) according to the instructions of the supplier.

[0093] Each purified $(His)_6$-tagged recombinant fusion protein was concentrated using Centricon-10 (Amicon) spin columns and was subsequently used for in vitro binding assays. The concentrations of purified $(His)_6$-ACBPs were determined by weighing freeze-dried recombinant protein and measurements of absorbance at 280 nm (Layne, *Meth. Enzymol.* 3: 447-454, 1957).

Example 5

Lead-Binding Assays

[0094] Lead-binding assays and fluorescence measurements were carried out according to Funaba and Mathews (*Mol. Endocrinol.* 14:1583-1591, 2000) with minor modifications. Briefly, 3.2 μM of each purified $(His)_6$-ACBP protein which was equilibrated in 20 mM phosphate buffer (Ph 7.2) with 200 in M NaCl, were labeled with 8 μl of 200 mM dansyl aziridine (Molecular Probes, Cat. No. D151) and reacted for 2 h in a 1.5 ml Eppendorf tube at room temperature. The dansylated $(His)_6$-ACBP proteins were then distributed to 8 wells of a 96-well microtiter plate (Nunc Cat. No. 236105). Various concentrations (0, 1.0, 2.0, 3.0, 4.0, 5.0, 7.0 and 9.0 μM) of $Pb(NO_3)_2$ (Aldrich, Cat. No. 203580) solutions were added and the reactions were incubated at room temperature for 1 h.

[0095] Fluorescence measurements were performed using a BIO-TEK FL600 Fluorescence Plate Reader (BIO-TEK Instrument, INC, USA) with excitation wavelength set at 360/40 nm and emission wavelength set at 530/25 nm. Prior to binding with lead, the dansyl aziridine coupled $(His)_6$-ACBP recombinant proteins are sensitive to the conformational changes. After binding to Pb(II) molecules, the dansylated $(His)_6$-ACBP recombinant proteins would change their conformations and the dye will show an increase in fluorescence excitation. Hence, a difference in fluorescence in the presence and absence of Pb(II) molecules would indicate binding of a $(His)_6$-ACBP recombinant protein with Pb(II). Each experiment was repeated three times to ascertain results.

[0096] Our results indicate that recombinant ACBPs bind Pb(II) in vitro. The relative fluorescence intensities of $(His)_6$-ACBPs increased in the presence of Pb(II) in a dose-dependent manner from 1 to 9 μM of Pb(II), indicative of binding to Pb(II) (FIG. **2**A). The relative fluorescence intensity of $(His)_6$-ACBP4 and $(His)_6$-ACBP1 were observed to be much higher than the other recombinant ACBPs.

Example 6

Heavy-Metal-Binding Assays Using Metal-Chelate Affinity Chromatography

[0097] The coding regions of ACBP1, ACBP2 and ACBP6 were generated by RT-PCR for construction of pGEM-T

EASY (Promega) derivatives for in vitro transcription/translation. Primers used in RT-PCR were as follows: ACBP1 (ML190 (SEQ ID NO:20) and ML917 (SEQ ID NO:17), ACBP2 (ML902 (SEQ ID NO:21) and ML903 (SEQ ID NO:22)), and ACBP6 (ML812 (SEQ ID NO:23) and ML751 (SEQ ID NO:2)). The PCR products were subsequently cloned into pGEM-T EASY vector (Promega) for in vitro transcription/translation using the TNT® T7/SP6 Coupled Wheat Germ Extract System (Promega) following the manufacturer's instruction. Pb(II)-, Cd(II)-, and Cu(II)-equilibrated matrices for metal-binding assays were prepared by stripping Ni-NTA agarose (Qiagen) of nickel and re-equilibrating with 0.1 M $Pb(NO_3)_2$ (Aldrich), $CdCl_2$ (Aldrich), or $CuCl_2$ (Aldrich). Twenty μl of Pb(II)-, Cd(II)-, or Cu(II)-equilibrated matrix, 545 μl of 50 mM KH_2PO_4, 300 mM NaCl, pH 7.4, and [^{35}S]methionine-labeled protein were added to a microfuge tube (Dykema et al., *Plant Mol. Biol.* 41: 139-150, 1999) and rotated on a wheel at 4° C. for 1 h. Subsequently, the matrix was washed 3 times with 1.0 ml of 50 mM KH_2PO_4, 300 mM NaCl, pH 7.5. The binding protein was eluted with 200 μl of 50 mM KH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 4.5, followed by extraction with 200 μl of 2% SDS, 50 mM DTT, heating at 85° C. for 3 min and analysis by SDS-PAGE followed by autoradiography.

[0098] Our results indicate that in vitro translated ACBP1 and ACBP2 bind Pb(II) better than ACBP6 (FIG. **2**B). ACBP1 appeared to show slightly better binding than ACBP2 (FIG. **2**B). Our results (FIG. **2**C) also indicate that in vitro translated ACBP2 also binds Cd(II) and Cu(II) better than ACBP6. Binding of ACBP2 to Pb(II), Cd(II), and Cu(II) was inhibited by the metal chelator, ethylenediaminetetraacetic acid (EDTA), indicating that binding is dependent on divalent cations (FIG. **2**C).

[0099] Disparity in Pb(II)-binding between in vitro translated ACBP2 (FIG. **2**B) and $(His)_6$-ACBP2 (FIG. **2**A) may have arisen from differences in protein preparation procedures. $(His)_6$-ACBP2 was prepared from *E. coli* insoluble extracts under denaturing conditions followed by refolding (Chye et al., *Plant Mol. Biol.* 44: 711-721, 2000) in contrast to in vitro translated ACBP2. In comparison, $(His)_6$-ACBP1 was prepared directly from soluble *E. coli* extracts (Chye, *Plant Mol. Biol.* 38: 827-838, 1998). $(His)_6$-ACBP6 (FIG. **2**A) and in vitro translated ACBP6 (FIG. **2**B) seem to bind Pb(II) less well than ACBP1 despite being a closer homolog to the human ACBP, a molecular target for Pb(II) in vivo (Smith et al., *Chemico-Biological Interactions* 115: 39-52, 1998). Since larger homologs of *Arabidopsis* ACBPs have not been identified in man, the smaller ACBP may be the only available ACBP in humans that binds Pb(II). A comparison of *Arabidopsis* ACBPs at the acyl-CoA-binding domain is shown in FIG. **2**D. Ten amino acid residues within the acyl-CoA-binding domain, that are conserved in ACBP1 to ACBP5 but not in ACBP6, are identified (underlined amino acids in "Con" sequence in FIG. **2**D). Those 3 conserved amino acid residues that are further conserved in the 9-kDa human ACBP (GenBank Accesssion No. NM_020548) are encircled; these 3 amino acid residues conserved in human ACBP and ACBP1 to ACBP5 may play an important role in binding lead.

[0100] Other plant-derived ACBPs can be identified by searching known databases for sequences demonstrating homology to any of the *Arabidopsis* ACBPs 1-6 using techniques well known to those of ordinary skill in the art. Alternatively, to obtain other plant-derived ACBPs, those of ordinary skill in the art can obtain and probe DNA libraries of plant species of interest using routine techniques and probes based on any of *Arabidopsis* ACBPs 1-6 to identify homologous sequences which can then be isolated and expressed. The protein expression products can then be tested to confirm whether they have the ability to bind acyl-CoA esters.

[0101] All *Arabidopsis* ACBPs (ACBP1 to ACBP6) show 100% conservation in 13 other amino acid residues (marked with asterisks in "Con" sequence in FIG. **2**D). Plant-derived ACBPs and ACBP variants conserved in these residues, at a putative acyl-CoA-binding domain, should retain at least 7 of these 13 conserved residues. While the first 2 conserved residues (marked with asterisks) "F" and "V" may be variably separated (by a spacing of 3 to 6 residues), the last 11 conserved residues are specifically LxxLxxAxxGxxxxxx-PxxxxxxxxKWxxWxxxxxxxxEAM, where x denotes any amino acid residue. In ACBP variants or other plant-derived ACBPs, retaining at least 7 of these 13 conserved residues, presence of this domain can be further tested to confirm whether it confers the ability to bind acyl-CoA esters.

[0102] FIG. **2**D also shows the "Com" (Common) amino acids, displayed below the "Con" (Conserved) amino acids, occurring within the acyl-CoA-binding domain. By definition, "Com" lists the amino acids within the acyl-CoA-binding domains of ACBP1 to ACBP5 that occur at least twice in any of these 5 ACBPs (ACBP1 to ACBP5). FIG. **2**D indicates that there is high conservation in amino acids at the acyl-CoA-binding domain of ACBP1 to ACBP5; hence the nucleotide sequence encoding this domain can be PCR-amplified and used as a hybridization probe to identify homologs of *Arabidopsis* ACBP1 to ACBP5 in other organisms.

[0103] For example, the nucleotide sequence encoding the acyl-CoA-binding-domain in ACBP1 (amino acids 94 to 180) can be generated using forward primer 5'-CTTGATGAG-GCATTTAGTGC-3' (designated as SEQ ID NO:24) and reverse primer 5'-TGGGTAAAGCTGAGTAACAAG-3' (designated as SEQ ID NO:25) to produce a 0.26-kb DNA fragment that should be able detect other plant-derived nucleotide sequences encoding acyl-CoA-binding domains when it is used as a hybridization probe when screening DNA libraries. For PCR amplification of the acyl-CoA-binding domain in ACBP1, each 25-μl PCR reaction consisted of 25 ng plasmid pAT31 DNA, 10 pmol of each primer of SEQ ID NO:24 and of SEQ ID NO:25, 1 U Taq polymerase (Perkin Elmer), 2.5 μl 10×PCR buffer, 1.5 μl of 25 mM $MgCl_2$ and 0.5 μl each of 10 mM dATP, dGTP, dTTP, and ^{32}P-labeled dCTP. PCR-amplification was initiated with denaturation at 95° C. for 3 min, followed by 40 cycles of 94° C. for 1 min, 55° C. for 1 min and 68° C. for 4 min and extension at 72° C. for 10 min.

[0104] The 0.26-kb PCR-generated radiolabeled probe can be used to screen DNA libraries to identify hybridizing clones that encode ACBP homologs. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), particularly Chapter 11 and Table 11.1 therein; and Sambrook, J. and Russell, W., *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2001). A BLAST search of the NCBI database (www.ncbi.nlm.nih.gov/blast) using, as query sequence, this 0.26-kb nucleotide sequence of ACBP1 that encodes its acyl-CoA-binding domain, yielded 56 "Blast hits" from other species confirming its feasibility as a hybridization probe in screen-

ing DNA libraries. These "Blast hits" (with % DNA identity shown in brackets) include GenBank entries, AM476905 (*Vitis vinifera;* 75% identity), AC189237 (*Brassica rapa;* 75%); CT831642 (*Oryza sativa;* 73%); NM_001060827 (*Oryza saliva;* 73%), DQ908250 (*Gossypium hirsutum;* 81%), and EF086012 (*Picea sitchensis;* 69%). We have previously detected in Western blot analysis, using anti-ACBP1 antibodies on total plant protein from *Brassica juncea, Solanum melongena*, lettuce, carrot and potato, the presence of cross-reacting bands similar in molecular mass as *Arabidopsis* ACBP1, suggesting that ACBP1 homologs occur in many diverse plants species (Chye et al., *Plant J.* 18: 205-214, 1999).

[0105] Upon the isolation of other plant-derived ACBPs, the presence of a functional acyl-CoA-binding domain in these ACBPs can tested in vitro by Lipidex assays using (His)-tagged ACBPs and radiolabeled acyl-CoA esters including, but not limited to, ^{14}[C]oleoyl-CoA, ^{14}[C]palmitoyl-CoA, and ^{14}[C]arachidonyl-CoA as previously described (Chye et al., *Plant Mol. Biol.* 44: 711-721, 2000; Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004; Leung et al., *Planta* 223: 871-881, 2006). Non-radiolabeled acyl-CoA esters can be used in conjunction with radiolabeled acyl-CoA esters to test binding as described in Leung et al., *Planta* 223: 871-881, 2006). (His)-tagged proteins can be generated as described in Examples 3 and 4. The methods for using these (His)-tagged ACBPs in Lipidex assays have already been described in detailed for *Arabidopsis* ACBP 1 to 5 (Chye et al., *Plant Mol. Biol.* 44: 711-721, 2000; Leung et al., *Plant Mol. Biol.* 55: 297-309, 2004; Leung et al., *Planta* 223: 871-881, 2006).

Example 7

Generation of ACBP1- and ACBP3-Overexpressing Transgenic *Arabidopsis*

[0106] Plant transformation vectors pAT31 (35S:ACBP1) and pAT314 (35S:ACBP3) shown in FIGS. **3**A-**3**B were mobilised from *E. coli* into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (Roger et al., *Plant Molecular Biology Manual pp. A*2: 1-12, 1988), and were subsequently introduced into *Arabidopsis* wild-type plants (ecotype Columbia) using the floral dip method (Clough and Bent, *Plant J.* 16: 735-743, 1998). The transformed T_0 seeds were screened on the plant growth medium containing 50 mg/mL kanamycin and the positive transformants were transferred into soil to obtain the T_1 population. The positive transformants were further confirmed by PCR using a 35S promoter-specific forward primer 35SB (SEQ ID NO:15) together with reverse gene-specific primers: ML759 (SEQ ID NO:4; for 35S:ACBP1) and ML784 (SEQ ID NO:8; for 35S:ACBP3). RNA gel blot analysis was carried out using the Digoxigenin Northern blot kit (Roche) according to the protocol supplied by the manufacturer that has been also described in Xiao et al. (*Plant Cell* 16:1132-1142, 2004). Briefly, total RNA was extracted from the T_2 transformants and 30 fig of total RNA were separated on a 1.5% agarose gel containing 6% formaldehyde and transferred to Hybond N membranes (Amershaam). Northern (RNA) blot analysis was performed to detect the 35S:ACBP1 and 35S:ACBP3 transcripts using ACBP1- and ACBP3-specific cDNA probes. To generate probes for Northern blot analyses, specific primers of ACBP1 (ML179, SEQ ID NO:3 and ML759, SEQ ID NO:4) and those of ACBP3 (ML783, SEQ ID NO:7 and ML784, SEQ ID NO:8) were utilized for PCR amplification. The fragments were labeled with the PCR Digoxigenin Probe Synthesis Kit according to the manufacturer's instructions (Roche, Germany). Hybridization and detection were performed according to the standard procedures as advised by the manufacturer (Roche). Western blot analyses were carried out as described by Chye (*Plant Mol. Biol.* 38: 827-838, 1998). Total plant protein was extracted from mature silique-bearing plants. Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bradford, 1976). Ten fg of total protein was loaded per well in an SDS-PAGE gel. The proteins were electrophoretically transferred to Hybond-C membrane (Amersham) from the SDS-PAGE gel using a Trans-Blot cell (Bio-Rad). Affinity-column purified ACBP1-specific antibodies (Chye, *Plant Mol. Biol.* 38: 827-838, 1998) or GFP-specific antibodies (Invitrogen) were used in Western blot analysis. The ECL Western Blotting Detection Kit (Amersham) was used following the manufacturer's instructions to detect cross-reacting bands.

[0107] The T_3 stable transformants were tested for Pb(II)-sensitivity and measurement of Pb(II) content. To measure Pb(II) content in ACBP1-overexpressing transgenic *Arabidopsis*, plants were grown on MS medium for 2 to 3 weeks and then transferred into 1 mM $Pb(NO_3)_2$ solution for 48 h. The shoots and roots were weighted and collected for Pb(II) content measurement. To measure Pb(II) content in ACBP3-overexpressing transgenic *Arabidopsis*, the shoots of 2-week-old *Arabidopsis* were collected from seedlings germinated and grown on medium containing 0.75 mM $Pb(NO_3)_2$. The samples were digested overnight with 11 N HNO_3 at 200° C. as described previously (Lee et al., *Plant Physiol.* 138: 827-836, 2005). After dilution with 0.5 N HNO_3, the samples were analyzed using an atomic absorption spectrometer (PERKIN ELMER-AA Spectrometer 3110). Each plant line was tested by harvesting shoot samples from 30 independent plants divided into 6 groups (comprising 5 plants per group), i.e. six replicates were tested for each plant line and each replicate contains 5 plants. The average value for each plant line is derived from three independent experiments.

Example 8

ACBP1-Overexpressing Transgenic Plants are More Tolerant to Pb(II) Stress than Wild Type

[0108] To substantiate whether ACBP1 confers Pb(II) resistance, ACBP1-overexpressing transgenic plants were generated by *Agrobacterium* transformation (Clough and Bent, *Plant J.* 16: 735-743, 1998). To this end, the ACBP1 full-length cDNA was cloned into binary vector pBI121 in which ACBP1 is expressed from the CaMV 35S promoter. The resultant plant transformation vector pAT31 (FIG. **3**A) was subsequently introduced into wild-type (Col-0) *Arabidopsis* plants using *Agrobacterium*-mediated transformation by the floral dip approach (Clough and Bent, *Plant J.* 16: 735-743, 1998). Three independent T_2 transgenic lines were identified to over-produce ACBP1 mRNA in RNA gel blot analysis (FIG. **4**A). Among them, two lines (designated ACBP1 ox-3 and ACBP1 ox-5) showed a 3:1 (resistant/sensitive) segregation ratio in the T_2 population grown on growth medium containing the selective antibiotic kanamycin, indicating that they contain only one copy of the 35S::ACBP1 transgene. The ACBP1 protein levels in wild type, ACBP1 ox-3 and ACBP1 ox-5 were further confirmed by Western blot

analyses using ACBP1-specific antibodies (FIG. **4**B) and their resultant T_3 stable transgenic plants hence selected for Pb(II) treatment.

[0109] Wild-type (Col-0) and the T_3 35S::-ACBP1 transgenic plants (ACBP1 ox-3 and ACBP1 ox-5) were grown on MS medium for 3 days after germination and then transferred to MS medium and MS medium containing 0.75 mM $Pb(NO_3)_2$ for vertically growth. As presented in FIG. **4**C, although the growth rates of wild type and T_3 transgenic plants were similar seventeen days after germination on MS (Murashige and Skoog, *Physiol. Plant.* 15: 473-497, 1962) medium (FIG. **4**C), growth of wild type lagged behind ACBP1-transformed lines on MS supplemented with 0.75 mM $Pb(NO_3)_2$. Data (FIG. **4**D) revealed that the relative root lengths of the two transgenic lines ACBP1 ox-3 and ACBP1 ox-5 grown on Pb(1)-containing medium were 40.0±2.0% and 36.2±2.3%, respectively, of similar plants grown in MS medium. These values were significantly ($P<0.05$) higher than those of wild type (23.7±2.1%). Also, the relative fresh weights (FIG. **4**E) of the ACBP1 ox-3 and ACBP1 ox-5 transgenic lines grown in the presence of Pb(II) were respectively, 1.6- and 1.5-fold higher in shoots, and 1.8- and 1.4-fold higher in roots, than wild type. These changes in fresh weights, expressed as a percentage to that obtained from plants grown in the absence of Pb(II), are significant ($P<0.05$).

Example 9

ACBP3-Overexpressing Transgenic Plants are More Tolerant to Pb(II) Stress than Wild Stress

[0110] To substantiate whether ACBP3 confers Pb(II) resistance, ACBP3-overexpressing transgenic plants were generated by *Agrobacterium* transformation (Clough and Bent, *Plant J.* 16: 735-743, 1998). To this end, the ACBP3 full-length cDNA was cloned into binary vector pBI-GFP, a pBI121 derivative obtained by replacement of the GUS gene with eGFP (Shi et al., *Plant Cell* 17: 2340-2354, 2005), to generate plasmid pAT314, in which ACBP3 is expressed from the CaMV 35S promoter. Plant transformation vector pAT314 (FIG. **3**B) was subsequently introduced into wild-type (Col-0) *Arabidopsis* plants using *Agrobacterium*-mediated transformation by the floral dip approach (Clough and Bent, *Plant J.* 16: 735-743, 1998). Three independent T_2 transgenic lines were identified to over-produce ACBP3-GFP mRNA in RNA gel blot analysis (FIG. **5**A). Among them, all three lines (designated ACBP3 ox-2, ACBP3 ox-9 and ACBP3 ox-11) showed a 3:1 (resistant/sensitive) segregation ratio in the T_2 population grown on growth medium containing the selective antibiotic kanamycin, indicating that they contain only one copy of the 35S::ACBP3 transgene. The ACBP3-GFP fusion protein levels in wild type, ACBP3 ox-2, ACBP3 ox-9 and ACBP1 ox-11 were further confirmed by Western blot analyses using the GFP-specific antibodies (FIG. **5**B) and their resultant T_3 stable transgenic plants were hence selected for Pb(II) treatment.

[0111] Although wild type (Col-0) and the T_3 35S::ACBP3 transgenic plants (ACBP3 ox-2. ACBP3 ox-9 and ACBP3 ox-11) showed similar growth on MS medium, only growth of wild type was inhibited when these lines were cultured on medium containing 0.75 mM $Pb(NO_3)_2$ for 3 weeks (FIG. **5**C). The ACBP3 ox-2, ACBP3 ox-9 and ACBP3 ox-11 transformed plants clearly exhibited better growth than wild type upon $Pb(NO_3)_2$ treatment (FIG. **5**C). Quantitative analyses also showed that the root lengths (FIG. **5**D) and fresh weights (FIG. **5**E) of wild type, ACBP3 ox-2, ACBP3 ox-9 and ACBP3 ox-11 transformed plants did not show significant differences in MS medium but the root lengths of the three independent transgenic lines were longer (16.8±0.12, 17.8±0.12 and 14.8±0.12 mm, respectively) compared to wild type (7.2±0.08 mm). Also, the fresh weights of the ACBP3 ox-2, ACBP3 ox-9 and ACBP3 ox-11 transformed lines were 1.1-, 1.2- and 1.27-fold higher than wild type in Pb(II)-containing medium (FIG. **5**E).

Example 10

Greater Accumulation of Pb (II) in ACBP-Overexpressing Transgenic Plants

[0112] We further investigated whether ACBP-mediated resistance to Pb(II) was due to Pb(II) extrusion like AtPDR12-overexpressing *Arabidopsis* plants (Lee et al., *Plant Physiol.* 138: 827-836, 2005) or translocation that would result in Pb(II) accumulation in plant cells, thus resembling the overexpression of AtATM3 and YCF1 in transgenic plants (Kim et al., *Plant Physiol.* 140: 922-932, 2006; Song et al., *Nat. Biotech.* 21: 914-919, 2003).

[0113] To measure Pb(II) content in the ACBP1-overexpressing transgenic plants, wild type (Col-0), ACBP1 ox-3 and ACBP1 ox-5 lines were grown on MS for 3 weeks before seedlings were transferred to immerse roots in 1 mM $Pb(NO_3)_2$ for 48 h. The plants were washed three times in distilled water and blotted dry. Root and shoot samples were weighed and subsequently digested overnight with 11 N HNO_3 at 200° C. After dilution with 0.5 N HNO_3, the samples were analyzed using an atomic absorption spectrometer (PERKIN ELMER-AA Spectrometer 3110). As shown in FIG. **6**A, When the Pb(II) contents of wild type and transgenic lines were normalized on a per-plant basis, ACBP1 ox-3 and ACBP1 ox-5 lines showed 3.4- and 2.9-fold increases over wild type, respectively, in shoot Pb(II) content ($P<0.001$) with little variation in roots. When the Pb(II) contents in wild-type and transgenic *Arabidopsis* were compared on the per-fresh weight basis, ACBP1 ox-3 and ACBP1 ox-5 lines showed 1.9- and 2.2-fold increases, respectively, in shoot Pb(II) content ($P<0.05$; FIG. **6**B), confirming that the overexpression of ACBP1 resulted in Pb(II) accumulation in shoots.

[0114] The Pb(II) contents in the ACBP3-overexpressing transgenic plants were also measured. Wild type (Col-0) and ACBP3 ox-11 line were germinated and grown on MS containing 0.75 mM $Pb(NO_3)_2$ for 2 weeks before shoots were harvested for measurement of Pb(II) content. FIG. **7**A shows that the ACBP3-overexpressing ACBP3 ox-11 line also accumulates greater Pb(II) content (39.1±0.68 ppm/1 plant) than wild type (36.1±0.6 ppm/1 plant). When the Pb(II) content of wild-type and transgenic *Arabidopsis* were compared on the per-fresh weight basis, ACBP3 ox-11 showed 1.4-fold increase over wild type in shoot Pb(II) content ($P<0.05$; FIG. **7**B), confirming that the overexpression of ACBP3 also resulted in Pb(II) accumulation in shoots.

Example 11

Applications in the Use of ACBP Constructs in Plastid Transformation

[0115] The promoters used for plastid transformation include strong and constitutive promoters in plastid expres-

sion including the psbA promoter (the psbA gene encodes the photosystem II 32 kD protein) and the 16S rRNA operon (rrn) promoter, or modifications thereof of these promoters which have enhanced expression (Suzuki et al., 2003, *Plant Cell* 15: 195-205). In a specific embodiment, each plastid transformation construct containing ACBP-encoding sequences was cloned into plastid transformation vector pMLV-HisA (Li et al. *Exp. Biol. Med.* 231: 1346-1352, 2006) shown in FIG. **8**A, to yield plastid transformation vectors such as pAT385 (FIG. **8**B) for introduction by particle gun bombardment into plant cells (Staub and Maliga, *Plant J.* 6: 547-553, 1994). An example of tobacco plastid transformation using vector pAT385 is illustrated in FIGS. **9**A-**9**B and the molecular analyses of plants after transformation is shown in FIGS. **9**C-**9**D.

Example 12

Generation of Plants Expressing ACBPs

[0116] According to an embodiment of the present invention, a wide variety of plants and plant cell systems can be engineered for the desired physiological and agronomic characteristics described herein using the nucleic acid constructs of the present invention by various transformation methods known in the art, including *Agrobacterium*-mediated transformation (Horsch et al., *Science* 227: 1227-1231, 1985) or plastid transformation (Staub and Maliga, *Plant J.* 6: 547-553, 1994). In preferred embodiments, target plants and plant cells for engineering include, but are not limited to, those monocotyledonous and dicotyledonous plants, such as crops including grain crops (for example, wheat, maize, rice, millet, barley), tobacco, fruit crops (for example, tomato, apple, pear, strawberry, orange), forage crops (for example, alfalfa), root vegetable crops (for example, carrot, potato, sugar beets, yam), leafy vegetable crops (for example, lettuce, spinach); flowering plants (for example, petunia, rose, chrysanthemum), conifers and pine trees (for example, pine fir, spruce); plants used in phytoremediation (for example, heavy metal accumulating plants); oil crops (for example, sunflower, rape seed); and plants used for experimental purposes (for example, *Arabidopsis*).

[0117] According to another embodiment of the present invention, desired plants may be obtained by engineering one or more of the vectors expressing ACBPs as described herein into a variety of plant cell types, including but not limited to, protoplasts, tissue culture cells, tissue and organ explants, pollens, embryos, as well as whole plants. In an embodiment of the present invention, the engineered plant material is selected or screened for transformants (those that have incorporated or integrated the introduced gene construct(s)) following the approaches and methods described below. An isolated transformant may then be regenerated into a plant and progeny thereof (including the immediate and subsequent generations) via sexual or asexual reproduction or growth. Alternatively, the engineered plant material may be regenerated into a plant before subjecting the derived plant to selection or screening for the marker gene traits. Procedures for regenerating plants from plant cells, tissues or organs, either before or after selecting or screening for marker gene (s), are well known to those skilled in the alt.

[0118] According to another embodiment of the present invention, tissue-specific promoters may be used to target the expression of ACBPs in roots or leaves so that an edible plant part is free from heavy metal accumulation. Examples of tissue-specific promoters include those encoding rbsC (Coruzzi et al., *EMBO J.* 3:1671-1697, 1984) for leaf-specific expression and SAHH or SHMT (Sivanandan et al., *Biochimica et Biophysica Acta* 1731:202-208, 2005) for root-specific expression. Another exemplary root-specific promoter is taught by Ekramoddoullah et al., U.S. Pat. No. 7,285, 656 B2. Also, the Cauliflower Mosaic Virus (CaMV) 35S promoter has been reported to have root-specific and leaf-specific modules in its promoter region (Benfey et al., *EMBO J.* 8:2195-2202, 1989). Other tissue-specific promoters are well known and widely available to those of ordinary skill in the art. Further, a wide variety of constitutive or inducible promoters are also well known and widely available to those of ordinary skill in the art.

[0119] A transformed plant cell, callus, tissue, or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection may be performed by growing the engineered plant material on media containing inhibitory amount of the antibiotic or herbicide to which the transforming gene construct confers resistance. Further, transformed plants and plant cells may also be identified by screening for the activities of any visible marker genes (e.g., the β-glucuronidase, luciferase, B or C1 genes) that may be present on the vector of the present invention. Such selection and screening methodologies are well known to those skilled in the art.

[0120] Physical and biochemical methods may also be used to identify plant or plant cell transformants containing the gene constructs of the present invention. These methods include but are not limited to: 1) Southern analysis or PCR amplification for detecting and determining the structure of the recombinant DNA insert; 2) Northern blot, S1 RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme activity, where such gene products are encoded by the gene construct; 4) protein gel electrophoresis (PAGE), Western blot techniques, immunoprecipitation, or enzyme-linked immunoassays, where the gene construct products are proteins. Additional techniques, such as in situ hybridization, enzyme staining, and immunostaining, also may be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for doing all these assays are well known to those skilled in the art. In a specific embodiment, the selectable marker gene nptII, which specifies kanamycin-resistance, is used in nuclear transformation.

[0121] Examples of plants are monocots, dicots, crop plants (i.e., any plant species grown for purposes of agriculture, food production for animals including humans, plants that are typically grown in groups of more than about 10 plants in order to harvest the entire plant or a part of the plant, for example, a fruit, a flower or a crop, for example, tobacco, grain, that the plants bear, etc.), trees (i.e., fruit trees, trees grown for wood production, trees grown for decoration, etc.), flowers of any kind (i.e., plants grown for purposes of decoration, for example, following their harvest), cactuses. Further examples of plants in which the ACBPs may be expressed include Viridiplantae, Streptophyta, Embryophyta, Tracheophyta, Euphyllophytes, Spennatophyta, Magnoliophyta, Liliopsida, Commelimidae, Poales, Poaceae, *Oryza, Oryza sativa, Zea, Zea mays, Hordeum, Hordeum vulgare, Triticum, Triticum aestivum*, Eudicotyledons, Core eudicots, Asteridae,

Euasterids, Rosidae, Eurosids II, Brassicales, Brassicaceae, *Arabidopsis*, Magnoliopsida, Solananae, Solanales, Solanaceae, *Solanum*, and *Nicotiana*. Thus, the embodiments of the invention have used over a broad range of plants including, but not limited to, species from the genera *Anacardium, Arachis, Asparagus, Atropa, Avena, Brassica, Citrus, Citrullus, Capsicum, Carthamus, Cocos, Coffea, Cucumis, Cucurbita, Daucus, Elaeis, Fragaria, Glycine, Gossypium, Helianthus, Heterocallis, Hordeum, Hyoscyamus, Lactuca, Linum, Lolium, Lupinus, Lycopersicon, Malus, Manihot, Majorana, Medicago, Nicotiana, Olea, Oryza, Panieum, Panneserum, Persea, Phaseolus, Pistachia, Pisum, Pyrus, Prunus, Raphanus, Ricinus, Secale, Senecio, Sinapis, Solanum, Sorghum, Theobromus, Trigonella, Titicum, Vicia, Vitis, Vigna*, and *Zea*.

[0122] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

[0123] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

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What is claimed is:

1. A method of phytoremediation of an environment contaminated with a heavy metal comprising

selecting an environment contaminated with a heavy metal, and

growing, in that environment, a transformed organism that expresses a nucleic acid encoding a plant-derived acyl-CoA-binding protein (ACBP) or a variant of a plant-derived ACBP, whereby an amount of heavy metal is removed from the environment.

2. The method according to claim **1**, wherein the transformed organism is a transformed plant.

3. The method according to claim **1**, wherein said nucleic acid is in the nucleus of the transformed plant.

4. The method according to claim **1**, wherein said nucleic acid is in a plastid of the transformed plant.

5. The method according to claim **1**, wherein the heavy metal is one or more of lead, copper, cadmium, nickel, mercury, arsenic, selenium, strontium and zinc.

6. The method according to claim **1**, wherein said ACBP is an *Arabidopsis* ACBP or a variant thereof.

7. The method according to claim **6**, wherein the ACBP is selected from the group consisting of ACBP1, ACBP2, ACBP3, ACBP4, ACBP5, and ACBP6.

8. The method according to claim **2**, wherein the transformed plant is *Arabidopsis*.

9. The method according to claim **2**, wherein the recombinant vector contains a nucleic acid that targets the ACBP to specific tissues within the plant which can be pruned off to remove the heavy metal contaminant without destroying the whole plant.

10. The method according to claim **1**, wherein the transformed organism is a transformed microbe.

11. A method for monitoring/detecting the presence of contaminating metal in an environment comprising

selecting an environment to be tested or monitored for the presence of a contaminating metal;

growing, in that environment, a plant transformed to express a plant-derived ACBP or a variant thereof that binds at least one heavy metal; and

testing at least a portion of said plant for the presence of a contaminating metal,

whereby presence or absence of the contaminating metal in the environment is indicated.

12. The method according to claim **11**, wherein the ACBP is an *Arabidopsis* ACBP or a variant thereof.

13. A cell transformed to express a nucleic acid encoding a plant-derived acyl-CoA-binding protein.

14. The cell according to claim **13**, which is a transformed microbe.

15. The cell according to claim **14**, which is an *E. coli*.

16. The cell according to claim **13**, which is a transformed plant cell.

17. The cell according to claim **16**, wherein the plant cell is an *Arabidopsis*.

18. A plant comprising the cell of claim **16**.

19. The plant according to claim **18**, wherein the plant is an *Arabidopis*.

20. The cell according to claim **13**, wherein the plant-derived acyl-CoA-binding protein is an *Arabidopsis* ACBP or a variant thereof.

21. The cell according to claim **20**, wherein the ACBP is selected from the group consisting of ACBP1, ACBP2, ACBP3, ACBP4, ACBP5, and ACBP6.

* * * * *