Research Paper

**CEP genes regulate root and shoot development in response to environmental cues and are specific to seed plants**

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**Abstract**

The manifestation of repetitive developmental programmes during plant growth can be adjusted in response to various environmental cues. During root development, this means being able to precisely control root growth and lateral root development. Small signalling peptides have been found to play roles in many aspects of root development. One member of the CEP (C-TERMINALLY ENCODED PEPTIDE) gene family has been shown to arrest root growth. Here we report that CEP genes are widespread among seed plants but are not present in land plants that lack true branching roots or root vasculature. We have identified 10 additional CEP genes in Arabidopsis. Expression analysis revealed that CEP genes are regulated by environmental cues such as nitrogen limitation, increased salt levels, increased osmotic strength, and increased CO₂ levels in both roots and shoots. Analysis of synthetic CEP variants showed that both peptide sequence and modifications of key amino acids affect CEP biological activity. Analysis of several CEP over-expression lines revealed distinct roles for CEP genes in root and shoot development. A cep3 knockout mutant showed increased root and shoot growth under a range of abiotic stress, nutrient, and light conditions. We demonstrate that CEPs are negative regulators of root development, slowing primary root growth and reducing lateral root formation. We propose that CEPs are negative regulators that mediate environmental influences on plant development.

**Key words:** CEP, environmental regulation, lateral root formation, root development, small signalling peptide.

**Introduction**

Plant roots exhibit remarkable plasticity in their ability to adapt and react to environmental stimuli. From a single embryonic primary root the entire lateral root network is formed to exploit the surrounding soil and maximize plant fitness. This highly regulated process requires integration of intrinsic developmental pathways and environmental information (Malamy, 2005; Peret et al., 2009) but how this occurs is not understood. Phytohormones play a major role in these processes, however, other signalling molecules are also required.

Small, secreted regulatory peptides are a growing class of signalling molecules involved in many aspects of shoot and root development. They arise from genes that typically encode an N-terminal secretion signal, one or more conserved peptide domains, and variable regions that flank one or both sides of the discrete peptide domains. The precursor proteins undergo processing to form the mature peptide product. Many peptides also undergo post-translational modifications, such as hydroxylation, sulphation, and arabinosylation (Matsubayashi, 2012). Most regulatory peptides act as extracellular signalling molecules that are ligands for membrane bound receptors. Several families of regulatory peptides have been described and are defined by homology of the peptide domain including
CLAVATA3/ESR-related (CLE; Cock and McCormick, 2001), ROOT GROWTH FACTOR (RGF; Matsuzaki et al., 2010; Meng et al., 2012; Whitford et al., 2012), INFLORESCENCE DEFICIENT IN ALCISSON (IDA; Butenko et al., 2003), and C-TERMINALLY ENCODED PEPTIDE (CEP; Ohyama et al., 2008). Recent work has implicated small peptide signals in many aspects of root growth and development, including meristem maintenance, gravitropism, lateral root development, and protoxylem differentiation (Delay et al., 2013).

The CEP family was discovered using an in silico approach (Ohyama et al., 2008). The founding five members of this family in Arabidopsis were characterized by a conserved 15-amino acid peptide domain at or near the C-terminus. The mature product was shown to be a 14- or 15-amino acid peptide containing one or two hyroxylated proline residues and the 15-amino acid peptide was reported to be biologically active on roots. Over-expression of AtCEP1, which was mainly expressed in the shoot apical meristem and lateral root primordia during development, resulted in reduced primary and lateral root elongation as well as a smaller shoot system. Confocal imaging showed that CEP1-over-expressing roots had a reduced number of meristem cells (Ohyama et al., 2008).

Aside from the above study on CEP1, little is known about the CEP family. This includes their distribution beyond Arabidopsis, what controls CEP expression, the roles of different CEP family members in Arabidopsis, their molecular mode of action, and mutant studies.

Here we have used several approaches to fill these gaps. We report that CEP genes are widely distributed in seed plants. Using a bioinformatic approach we found a further 10 Arabidopsis CEPs. We show that CEP expression is regulated by environmental cues such as nitrogen limitation, increased salt levels, increased osmotic strength, and increased CO2 levels in both roots and shoots. Analysis of synthetic CEP variants showed that both peptide sequence and modifications of key amino acids affect CEP biological activity. Over-expression of several CEP genes gave differing root and shoot phenotypes. A cep3 knockout mutant showed enhanced root growth under a range of environmental conditions and enhanced shoot growth when grown hydroponically. We show that CEPs decrease lateral root formation and slow primary root growth. Collectively our results indicate CEPs mediate developmental pathways in response to environmental cues.

Materials and methods

Plant materials and growth

For root assays, seeds of Arabidopsis thaliana (accession Col-0) were surface-sterilized with 6.25% bleach, stratified for 3–4 days, and sown onto plates. Standard growth medium was ½ Murashige and Skoog (MS) medium with Gamborg’s vitamins (M0404; Sigma-Aldrich, St Louis, MO, USA) adjusted to pH 5.7 and solidified with 1% phytagel (P8169; Sigma-Aldrich). Modified ½ MS medium consisted of basal micronutrient solution (M0529; Sigma-Aldrich) with macronutrients added to the concentrations described (Murashige and Skoog, 1962) as indicated in the text. Plates were placed vertically in a growth chamber at 22 °C with a 16h photoperiod and photosynthetically active radiation of 100 μmol m-2 s-1. Plates were imaged using an Epson scanner and images were analysed using the SmartRoot plugin (Lobet et al., 2011) in ImageJ. Statistically significant differences were determined using a two-sample t test (Genstat 14th edition) where appropriate.

For hydroponic growth assays, the lids of microcentrifuge tubes were separated and a hole was punched in the top. The lids were filled with 0.5% agar and a single stratified seed was placed in the hole. Lids were placed in floating holders in tubs containing ¼ MS medium (M0404; Sigma-Aldrich). Tubs were aerated for 15min every 2h.

SALK_105856C, which has a T-DNA insertion in the CEP3 gene (Alonso et al., 2003), was obtained from the Arabidopsis Biological Resource Centre in Columbus, OH, USA. As it was initially expected that redundancy would occur in the CEP family, this mutant was crossed with SALK_075885, which has a T-DNA insertion in the CEP9 gene. This line, cep3-1a, was confirmed to be homozygous for the T-DNA in CEP3 and hemizygous for the T-DNA insertion in CEP9. Quantitative real-time PCR (qRT-PCR) was used to confirm that CEP3 expression was absent and CEP9 expression was not reduced in this line (Fig. S1). Additionally, phenotypes were extremely consistent within treatments, indicating that the hemizygous insertion in CEP9 was not affecting the phenotype. cep3-1a was used in the majority of phenotyping assays. From the progeny of cep3-1a, a line with a single homozygous T-DNA insertion in the CEP3 gene and no insertion in the CEP9 gene, cep3-1b, was obtained. This line showed phenotypes consistent with cep3-1a in selected assays (Fig. S2A). qRT-PCR was used to confirm that CEP3 expression was absent and CEP9 expression was not reduced in this line (Fig. S1).

Over-expression constructs and plant transformation

To make over-expression constructs, CEP2, CEP3, CEP4, CEP5, CEP6, and CEP9 coding sequences were PCR-amplified from genomic DNA and cloned into pENTR D-TOPO. An LR recombination reaction (Life Technologies) was performed with the pK7WG2D destination vector (Karimi et al., 2002). Constructs were transformed into Agrobacterium tumefaciens strain LBA4404 (Invitrogen, Carlsbad, CA, USA), which was used to transformed the vector into Col-0 plants using the floral dip method (Clough and Bent, 1998). Over-expression was confirmed by qRT-PCR in selected independent lines (independent lines are identified by different numbers). All lines were at least generation T3.

RNA extraction, cDNA synthesis, and qRT-PCR analysis

RNA was extracted using the Trizol reagent (Life Technologies, Carlsbad, CA, USA) and purified using spin columns (RNasey plant mini kit; Qiagen, Valencia, CA, USA). cDNA was synthesized using the Superscript III Kit (Invitrogen). Taqman reactions were set up and run according to manufacturer’s specifications (Life Technologies) using gene specific probes and a control probe (PP2AA3) designed by the manufacturer. Three biological replicates and three technical replicates were used. Outliers were omitted from analysis. Data were analysed using the ΔΔCt method (Livak and Schmittgen, 2001) and statistical analysis was performed as described using a t test (Yuan et al., 2006). For CEP3 and CEP9 expression assays in the cep3-1 mutants and for confirmation of CEP over-expression lines, primers were used together with Fast Sybr Green Mastermix (Invitrogen) according to manufacturer’s instructions. Melting curves were analysed to ensure specific primer binding. Data were analysed as described above.

Microscopy and imaging

To define the stages of lateral root development, differential interference contrast microscopy was performed on cleared roots as described (Malamy and Benfey, 1997).
Sequence and conserved domain analysis of CEP proteins

To identify potential members of the CEP gene family in plants, amino acid sequences of Arabidopsis CEP family members were used as a query in BLAST searches against NCBI non-redundant, expressed sequence tag (EST) and genomic databases (blast.ncbi.nlm.nih.gov/), JGI (genome.jgi-psf.org/), and Phytozome v9.0 (www.phytozome.net/). The BLAST analyses were performed by automatically adjusting input to short sequence for a TBLASTN search in all six open reading frames. Full-length open reading frames were also used in the BLAST searches to confirm the matches from short sequence input searches. SignalP was used to search for signal sequences in all resulting open reading frames using both neural network (NN) and hidden Markov model (HMM) modes. In several cases, where SignalP yielded low scores, SecretomeP (www.cbs.dtu.dk/services/SecretomeP-2.0/) was used to detect non-classical secretion signals. Only the sequences with either N-terminal signal peptide (SignalP) or non-classical secretion signals were reported. Using the BLAST search parameters described above we also searched the 1000-plants (oneKP) EST database in which the transcripts of 1000 different species of plants are being sequenced by next generation sequencing (http://www.onekp.com). Due to the incomplete nature of the oneKP database, no attempt was made to identify putative signal peptides. Arabidopsis CEP assignment and naming was aligned with the results from Roberts et al. (2013).

Data-mining analyses

To instigate our analysis of CEP expression profiles in Arabidopsis we used Genevestigator (Hruz et al., 2008). Data were filtered to show only results with a fold change greater than 1.5 and a P value of <0.05.

Results and discussion

CEP loci are widely distributed among seed plants but not ancient lineages of land plants

Although broad structural and developmental diversity exists between ancient (e.g. Physcomitrella and Selaginella) and more recently evolved lineages (e.g. angiosperms) of land plants, phylogenetic analyses demonstrate that they share key genes involved in developmental regulation and phytohormone biosynthesis (Raven and Edwards, 2001; Bowman et al., 2007; Floyd and Bowman, 2007; Rensing et al., 2008; Pils and Heyl, 2009; Prigge and Bezanilla, 2010). In contrast to this, a search for CEP-domain-containing genes using plant genomic and EST collections found that they occur only in seed plants (Fig. 1). CEPs were absent from the Selaginella, Physcomitrella patens, and other mosses, while groups I and II CEPs were found in a variety of angiosperm and gymnosperm species. The distribution of CEP genes in plant genomes and EST collections is shown in Fig. 1.

Fig. 1. Distribution of CEP genes found in plant genomes and EST collections. Genomes were searched using TBLASTN, with the Arabidopsis CEP domains as query. Parameters were adjusted to search for a short input sequence, at JGI (genome.jgi-psf.org/) and Phytozome (phytozome.net/). EST collections were searched at JGI and dbEST (ncbi.nlm.nih.gov/nucest). No data or insufficient data refers to species for which no genome sequence was available, or for which <0.5 × 10^5 EST sequences were available. The tree was adopted from Phytozome (Goodstein et al., 2012). Weblogo plots (Crooks et al., 2004) were used to show the 15-amino acid CEP domain conservation. Weblogos for group I and II CEP domains were derived from Datasets S1 and S3, respectively.
Physcomitrella, and algal genomes. Selaginella has vascularized roots and shoots and dominant sporophyte generation (Banks et al. 2011). Physcomitrella is non-vascular and uses free-swimming motile sperm during fertilization (Priegge and Bezanilla, 2010). The absence of CEP coincides with a lack of root branching in Selaginella. Root system growth in Selaginella occurs by bifurcation of the apical meristem (Banks, 2009).

Analysis of angiosperm CEP genes showed that they encode an N-terminal secretion signal and consist of one to seven 15-amino acid CEP domains (Dataset S1). Apart from the secretion signals and the CEP domains themselves, CEPs displayed little sequence conservation. CEPs also universally lacked introns. Our analysis identified 126 dicotyledon CEP genes (encoding 187 CEP domains) and 33 monocotyledon CEP genes (encoding 41 CEP domains; Dataset S1). Dicot and monocot CEP domains were distinctive (Fig. 1). Monocot CEP domains lacked the phenylalanine residue at position two, which was highly conserved in dicots, and all dicot CEP domains terminated with histidine whereas monocot CEP domains terminated with histidine or asparagine (Fig. 1). We also searched the 1000-plants (oneKP) EST database and found 78 CEP-coding genes only in angiosperms, including three in Magnoliids, the largest clade of early diverging angiosperms (Solits et al., 2005; Dataset S2).

We also found a distinctive group of CEP genes, group II CEPs, in angiosperms (Dataset S3). The group II CEP domain contains a strongly conserved nine-amino acid C-terminal region and exhibits divergence in the first six N-terminal amino acids (Fig. 1). However, the SPG(I/V)GH sequence at the C-terminus is highly conserved throughout group I and II CEPs. We found a family of CEP-like genes in gymnosperm EST databases (Fig. 1; Dataset S4). The gymnosperm CEP domains possessed a highly conserved GHSPG(I/V)GH sequence at the C-terminal region and a divergent N-terminus (Fig. 1). How these are evolutionarily related requires further investigation.

Recently it was reported that CEP genes are present outside plants only in root knot nematodes (RKNs) but not in other plant-parasitic or free-living nematodes (Bobay et al., 2013). A comparison of plant and RKN CEP domains showed that RKN domains were more similar to group I CEP domains than to other RKN domains (Fig. S3). In some instances, the RKN CEP domains were identical to the CEP domains of angiosperm group I CEPs (Fig. S3). This result may point to RKN and plant CEPs sharing an overlapping functional space and the possibility of RKNs utilizing CEP mimics for parasitism. It also raises the question of whether CEP genes were acquired by RKNs through horizontal gene transfer (Jones et al., 2005; Haegeman et al., 2011).

CEP genes in Arabidopsis

Five CEP genes were found previously in the Arabidopsis genome (Ohyama et al., 2008). Using a bioinformatic approach, we and Roberts et al. (2013) identified an additional ten CEP genes in Arabidopsis (Table 1). Four of the novel CEP genes were un-annotated (CEP7, CEP8, CEP10, and CEP11) and one (CEP6) was annotated as the first exon of an unrelated gene (TAIR10). Each AtCEP encodes a small protein (77–133 amino acids) with a predicted signal peptide and one or more CEP domains except AtCEP9, which possesses five CEP domains and encodes for a larger protein of 230 amino acids. The internal expansion of the CEP domain in CEP2, CEP6, CEP9, and CEP10 is intriguing as the domain sequences are not always identical to each other (Fig. S4A). Furthermore, CEP genes were often located in close proximity to each other. For example, CEP3 and CEP11 are located in tandem on chromosome 2 and CEP5, CEP6, CEP7, and CEP8 are also arranged sequentially on chromosome 5. Analysis of the amino acid sequences of these preproteins shows no significant similarity in the N-terminal signal peptide or variable region and the domain sequences are not identical, indicating these genes did not arise through a recent duplication event (Fig. S2B, C). These data indicate that evolution may be favouring diversity in CEP domain sequence as opposed to an increase in domain dosage.

AtCEPs are induced by environmental cues and show tissue-specific expression

To investigate CEP gene regulation in Arabidopsis we searched publicly available data for CEP expression profiles. AtCEP3, CEP5, and CEP9 were significantly induced by environmental conditions, particularly nutrient and biotic stress. For these three genes, nitrate starvation was one of the top three conditions under which a significant perturbation in gene expression occurred (Dataset S5). Additionally, AtCEP9 is one of the 31 signature genes up-regulated by elevated field CO2 (Li et al., 2006). CEP1, CEP13, and CEP14 were significantly induced under a range of different stimuli (data not shown).

To deepen our understanding of CEPs we explored the expression of nine CEP genes under various growth conditions. Plants were grown for six days on standard medium before being transferred to various treatments for 24h (Table 2). We found that the expression of all the CEP genes was perturbed by the environmental stimuli tested in the root, shoot, or both, except for CEP15. As the environmental stimuli tested were by no means comprehensive, it is possible that CEP15 expression is responsive to other environmental factors. Our results suggest that CEP1 and CEP2 are not expressed in the root under the conditions tested. Using a promoter-GUS fusion, Ohyama et al. (2008) showed that CEP1 was expressed in 14-day-old plants in the shoot apical meristem and in developing lateral root primordia. Using qRT-PCR it was also shown that CEP2 was expressed in roots. The discrepancy in our results may be due to the fact that 7-day-old plants do not have many lateral root primordia and as we were taking whole-root samples the expression may have been diluted. Additionally, the expression of CEP1 and CEP2 in the roots may be induced by other factors not tested in this assay.

The most notable perturbation was a 10-fold increase in CEP3 expression in the roots under nitrogen depletion. This strong induction was not seen in the shoots, or under nitrogen-limiting conditions in the roots. However, significant induction in the shoots was seen under nitrate, but not ammonium,
Table 1. CEP genes in Arabidopsis

The CEP genes identified in previous study (Ohyama et al., 2008) are indicated with underscores. CEPs identified in our study were aligned with those obtained by Roberts et al. (2013).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Locus</th>
<th>AGI coordinates</th>
<th>SignalP score</th>
<th>Peptide domain name</th>
<th>Peptide domain sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEP1</td>
<td>At1g47485</td>
<td>17422448–17423066</td>
<td>0.84</td>
<td>CEP1</td>
<td>DFRPTNPNGNSPGVGH</td>
</tr>
<tr>
<td>CEP2</td>
<td>At1g5835</td>
<td>22025041–22025421</td>
<td>0.68</td>
<td>CEP2.1</td>
<td>DFRTPNPGPSGPGVH</td>
</tr>
<tr>
<td>CEP3</td>
<td>At2g23440</td>
<td>9979405–9979819</td>
<td>0.96</td>
<td>CEP3</td>
<td>TFRPTEPGPSGPGVH</td>
</tr>
<tr>
<td>CEP4</td>
<td>At2g35612</td>
<td>14965521–14965501</td>
<td>0.83</td>
<td>CEP4</td>
<td>AFRPTHQPNSGPGVH</td>
</tr>
<tr>
<td>CEP5</td>
<td>At5g68815</td>
<td>26677365–26677865</td>
<td>0.81</td>
<td>CEP5</td>
<td>DFRPPTPGPSGPGVH</td>
</tr>
<tr>
<td>CEP6</td>
<td>At5g68816</td>
<td>26681496–26681800</td>
<td>0.82</td>
<td>CEP6.1</td>
<td>DFPTSPGNSGPGVH</td>
</tr>
<tr>
<td>CEP7</td>
<td>Between At5g68816 and At5g68820</td>
<td>26683388–26683615</td>
<td>0.99</td>
<td>CEP7</td>
<td>DFPTPGPSGPGVH</td>
</tr>
<tr>
<td>CEP8</td>
<td>Between At5g68816 and At5g68820</td>
<td>26686261–26686521</td>
<td>0.97</td>
<td>CEP8</td>
<td>AFRPTPQNSGPGVH</td>
</tr>
<tr>
<td>CEP9</td>
<td>At3g50610</td>
<td>18779723–18780412</td>
<td>0.77</td>
<td>CEP9.1</td>
<td>DFPVTSQGNSGPGVH</td>
</tr>
<tr>
<td>CEP10</td>
<td>Between At1g36040 and At1g36050</td>
<td>13448921–13449316</td>
<td>0.90</td>
<td>CEP10.1</td>
<td>DFAPTNPQHNSGPGVH</td>
</tr>
<tr>
<td>CEP11</td>
<td>Between At2g23440 and At2g23450</td>
<td>9986193–9986504</td>
<td>0.84</td>
<td>CEP11</td>
<td>AFRSTPEPSGPGVH</td>
</tr>
<tr>
<td>CEP12</td>
<td>Exon 1 of At1g31670</td>
<td>11337558–11337836</td>
<td>0.94</td>
<td>CEP12</td>
<td>AFRPTPGQPSGPGVH</td>
</tr>
<tr>
<td>CEP13</td>
<td>At1g16950</td>
<td>5796009–5796559</td>
<td>0.90</td>
<td>CEP13</td>
<td>IYRRLVPSVPSGPGVH</td>
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<tr>
<td>CEP14</td>
<td>At1g29290</td>
<td>10244966–10245572</td>
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<td>CEP14</td>
<td>VDRYRLSVPSVPSGPGVH</td>
</tr>
<tr>
<td>CEP15</td>
<td>At2g40530</td>
<td>16927502–16928208</td>
<td>0.45</td>
<td>CEP15</td>
<td>IYRQQGDVPSVPSGPGVH</td>
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</tbody>
</table>

Table 2. CEPs are induced by environmental cues

Plants were grown on standard medium for 6 days before being transferred to specified treatments. Root and shoot tissue was harvested 24 h after transfer. qRT-PCR was performed using Taqman probes and data were analysed using the ΔΔCT method. Expression shown is relative to a control treatment (transfer to standard medium for 24 h). n.e. indicates no reproducible data could be obtained, suggesting genes are not expressed. n.t indicates not tested. Fold change ± standard error are shown. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>CEP1</th>
<th>CEP2</th>
<th>CEP3</th>
<th>CEP4</th>
<th>CEP5</th>
<th>CEP6</th>
<th>CEP9</th>
<th>CEP10</th>
<th>CEP11</th>
<th>CEP12</th>
<th>CEP13</th>
<th>CEP14</th>
<th>CEP15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>0 mM nitrogen</td>
<td>n.e.</td>
<td>n.e.</td>
<td>1.01±0.58***</td>
<td>1.60±0.23*</td>
<td>2.13±0.21**</td>
<td>1.30±0.14</td>
<td>1.61±0.80</td>
<td>1.04±0.14</td>
<td>1.05±0.08</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.25 mM nitrate</td>
<td>n.e.</td>
<td>n.e.</td>
<td>1.31±0.08</td>
<td>1.17±0.12</td>
<td>1.79±0.08***</td>
<td>1.29±0.35</td>
<td>2.29±0.08</td>
<td>1.09±0.02</td>
<td>0.82±0.06</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.25 mM NH4Cl</td>
<td>n.e.</td>
<td>n.e.</td>
<td>1.36±0.03</td>
<td>1.26±0.05*</td>
<td>0.71±0.02***</td>
<td>0.48±0.02***</td>
<td>2.31±0.26</td>
<td>1.21±0.03</td>
<td>0.84±0.03</td>
<td></td>
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<tr>
<td></td>
<td>100 mM mannitol</td>
<td>n.e.</td>
<td>n.e.</td>
<td>0.57±0.13</td>
<td>2.35±0.49*</td>
<td>1.08±0.21</td>
<td>0.34±0.03</td>
<td>2.67±0.66</td>
<td>0.87±0.06</td>
<td>1.25±0.16</td>
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<tr>
<td></td>
<td>100 mM NaCl</td>
<td>n.e.</td>
<td>n.e.</td>
<td>2.00±0.13*</td>
<td>1.68±0.17**</td>
<td>1.08±0.08</td>
<td>1.70±0.41</td>
<td>1.00±0.15</td>
<td>1.48±0.29</td>
<td>1.04±0.04</td>
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</tr>
<tr>
<td></td>
<td>1000 ppm CO2</td>
<td>n.e.</td>
<td>n.e.</td>
<td>0.18±0.04***</td>
<td>1.02±0.1</td>
<td>1.11±0.13</td>
<td>0.90±0.41</td>
<td>0.58±0.44</td>
<td>1.15±0.04*</td>
<td>1.23±0.06</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Shoot</td>
<td>0 mM nitrogen</td>
<td>4.40±0.97***</td>
<td>0.46±0.06**</td>
<td>1.16±0.16</td>
<td>0.70±0.09</td>
<td>0.65±0.10</td>
<td>1.39±0.18</td>
<td>0.62±0.01*</td>
<td>0.96±0.07</td>
<td>0.89±0.04</td>
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<tr>
<td></td>
<td>0.25 mM nitrate</td>
<td>3.72±0.11**</td>
<td>4.94±3.18*</td>
<td>5.89±0.37***</td>
<td>4.34±0.49</td>
<td>3.62±2.08</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>0.25 mM NH4Cl</td>
<td>0.80±0.03</td>
<td>0.63±0.08*</td>
<td>1.14±0.11</td>
<td>1.01±0.91</td>
<td>1.26±0.51</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td></td>
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<td>100 mM mannitol</td>
<td>4.79±0.56***</td>
<td>1.26±0.08*</td>
<td>2.49±0.34**</td>
<td>1.86±0.16*</td>
<td>1.83±0.16*</td>
<td>1.50±0.04</td>
<td>1.63±0.23</td>
<td>3.92±0.46**</td>
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<td>100 mM NaCl</td>
<td>2.85±0.26**</td>
<td>0.77±0.26</td>
<td>1.75±0.98</td>
<td>1.53±0.93</td>
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<td>1000 ppm CO2</td>
<td>1.29±0.09</td>
<td>0.94±0.14</td>
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limitation. These data indicate that the response of CEP3 to low nitrogen is both tissue- and nitrogen-source-specific. CEP3 was up-regulated 2-fold in response to increased salt in the roots and increased osmotic strength in the shoots. CEP3 was severely down-regulated in response to increased CO2 levels in the roots only.

The expression of other CEP genes was also perturbed under the conditions tested. CEP1 was up-regulated in the shoots...
under nitrogen depletion and nitrate limitation, but not ammonium limitation. Increased osmotic strength and increased salt levels also induced CEP1 in the shoots. CEP2 expression was down-regulated in the shoots under nitrogen depletion and ammonium limitation, but was induced by nitrate limitation. CEP4 expression was induced slightly in the roots by nitrogen depletion and ammonium limitation, but not by nitrate limitation. The strongest induction in CEP4 expression was seen under increased osmotic strength in both the roots and shoots. CEP5 was induced in the roots under nitrogen depletion and nitrate limitation, and repressed under ammonium limitation. The only change in CEP13 expression was repression seen in the shoots under nitrogen depletion. CEP14 expression was increased slightly in the roots under increased CO₂ levels and more strongly in the shoots under increased osmotic pressure.

CEP9 expression was repressed under ammonium limitation as well as increased osmotic strength in the roots. The expression of CEP9 was not significantly induced in either roots or shoots. Surprisingly, we did not see a change in CEP9 expression under increased CO₂ levels as it has been reported to be a signature gene induced by elevated field CO₂ levels (Li et al., 2006). There are several differences in experimental conditions that may account for this discrepancy. Firstly, Li et al. (2006) grew plants in the field, in soil. Secondly, above-ground parts of older plants exposed to CO₂ for 12 days were analysed. Thirdly, plants were exposed to CO₂ at 550 ppm. These differences in conditions are likely to contribute to the differences in CEP9 expression observed in our study.

Our data indicated that CEP expression is perturbed by different environmental stimuli. This implicates CEPs as regulators of plant development in response to environmental stress. Expression changes were specific to roots and shoots and each CEP gene tested had a different expression profile. While root architecture response to environmental signals has been well documented, mechanisms behind this regulation remain to be elucidated (Malamy, 2005; Peret et al., 2009; Gruber et al., 2013). It has been recently proposed that under low-nitrogen conditions lateral root development is regulated by NRT1.1-dependent auxin depletion (Krouk et al., 2010; Mounier et al., 2013). It was further shown that NRT1.1 transcription itself is regulated by other environmental factors such as pH (Mounier et al., 2013). How CEP integrates with these systems is yet to be elucidated.

### Activity of synthetic CEP variants in Arabidopsis

A previous report described the mature product of CEP1 as a 14- or 15-amino acid peptide with either one or two hydroxylated proline residues (Ohyama et al., 2008). Treating plants with a synthetic 15-amino acid CEP1 peptide at 10⁻⁸ to 10⁻¹⁷ M inhibited root growth and generated a phenotype similar to CEP1 over-expression (Ohyama et al., 2008). We explored the phenotypic activity of variants of CEP3, CEP5, and CEP9 as well as a scrambled peptide based on the amino acid sequence of CEP5 H (Fig. 2A, B and Fig. S5).

All of the CEP variants, applied to plants at 10⁻⁶ M, not only decreased primary root length, but also significantly decreased emerged lateral root density, except for CEP5 H (Fig. 2C). As expected, the scrambled peptide showed no effect on root growth. The severity of the effect of CEP variants was dependent on the peptide sequence as well as the modification. CEP3 H and CEP5 H both severely affected primary root length, reducing it to about half that of untreated plants. However, only

### Fig. 2. CEP synthetic peptides decrease primary root length and emerged lateral root density. (A) CEP peptides used in growth assays. (hyP) indicates hydroxyproline residues. (B) Alignment of CEP3, CEP5, and CEP9.1 peptide domains. (C) Primary root (PR) length and lateral root (LR) density of 12-day-old Col-0 plants grown on standard medium supplemented with 1 μM of the specified peptide (n≥7 plants). (D) Differential biological activity of CEP3, CEP5, and CEP9 peptide variants. The histogram indicates the lowest concentration at which a peptide elicited a significant reduction in primary root length compared to untreated plants (P<0.05). Col-0 plants were grown vertically for 12 days on standard medium supplemented with peptide concentrations ranging from 10⁻⁶ to 10⁻¹² M (n=7–16 plants). Error bars show standard error. *P<0.05; **P<0.01; ***P<0.001 (two-sample t test, Genstat).
CEP3 H significantly decreased lateral root density. These two peptide variants are very similar in amino acid sequence, with only three residues differing and the last nine residues being identical (Fig. 2B). CEP9.1 and CEP9 H had a much weaker effect on primary root length, but also reduced lateral root density. Two of the terminal nine residues of CEP9.1 are different (but synonymous) to CEP3 H and CEP5 H. Thus, the terminal residues play a crucial role in peptide activity, potentially as they are required for interaction with receptors. It has been proposed that the CLE peptide domain sequence determines functionality in a tissue-specific manner due to optimal interactions with specific receptors (Meng et al., 2010), which may also be the case with CEPs. In particular, the terminal residues of CEP peptide domains are highly conserved.

To determine the differential biological activity of each peptide, a titration was performed using primary root length as an indicator of biological activity (Fig. 2D). CEP3 H, which had the most severe effect on overall root architecture (Fig. 2B), was active at $10^{-8}$ M. Biological activity was seen with CEP5 H at $10^{-9}$ M. CEP9.1 H was active at a lower concentration than the non-hydroxylated CEP9.1, even though the effect of CEP9.1 H on primary root length at $10^{-6}$ M was less severe. These data suggest that both domain sequence and modifications determine functional activity and may present an avenue for regulation of peptide activity through post-translational modification. It has been reported that sulphation of the RGF1 peptide is essential for its function in root meristem maintenance (Matsuzaki et al., 2010). Furthermore, arabinosylation of a hydroxyproline residue in CLV3 increased its affinity for the receptor CLV1 (Ohyama et al., 2009). It is possible that the difference in potency of our CEP peptide variants is due to changes in the affinity of the peptide for its receptor. Our data further highlight the dynamics of peptide-mediated regulation and the importance of the peptide sequence and structure.

**Over-expression reveals a role for CEPs in root and shoot development**

To investigate the roles of CEPs in plant development, we over-expressed six CEP genes under the control of a constitutive 35S promoter. When grown on standard medium for 12 days, we observed a significant decrease in primary root length in all CEP over-expression lines (Fig. 3A and Fig. S6).
The most severe decrease was seen in the p35S::CEP3 and p35S::CEP4 lines. While the primary root length of all lines tested was reduced, some unique phenotypes were observed on plates. The shoots of the p35S::CEP3 and p35S::CEP4 lines were significantly larger than in the wild-type lines, even though the roots were severely impaired. Fresh weight measurements showed the shoots of these lines were double the weight of the wild type (Fig. 3B). This increase in shoot size was not observed in the other over-expression lines (Fig. S6).

To further investigate the shoot phenotypes the over-expression lines were grown in pots (Fig. 3C–J and Fig. S7). The phenotypes seen were diverse and unique to each over-expression line. Five-week-old p35S::CEP2 plants had fewer rosette leaves, delayed flowering, and altered leaf morphology, showing flat, round leaves (Fig. 3D). p35S::CEP3 lines displayed leaf morphology defects including epinasty, leaf yellowing, and reduced rosette size (Fig. 3E, F). p35S::CEP4 plants showed a similar phenotype to p35S::CEP3, although plants appeared to be larger overall (Fig. 3G). p35S::CEP6
and p35S::CEP9 plants were not as severely affected, but showed epinasty and yellowing (Fig. 3H–J). p35S::CEP9 lines also show reduced rosette size.

Combining the results from plate and pot assays it appears that CEPs that elicit a more severe root phenotype also display a much more severe shoot phenotype. The differences in phenotypes seen, taken together with the expression data, indicate that each CEP plays a distinct role in root and/or shoot development. This raises the possibility that they may interact with different receptors. Conversely, with peptide families such as CLE and RGF, redundancy among family members is prevalent and over-expression or knock-out/knockdown of several different peptide genes tends to give the same phenotype (Jun et al., 2010; Fernandez et al., 2013).

A knockout mutant confirms the role of CEPs in plant development in response to environmental cues.

The role of CEPs as negative regulators of root development was confirmed by CEP3 T-DNA insertion knockout lines (Fig. 4A). When grown on standard medium, cep3-1a and cep3-1b showed no significant difference in root architecture compared to Col-0 (Fig. 4B). However, when grown under nitrogen-limiting conditions these lines had significantly larger root systems (Fig. S2A). We also observed increased root and shoot growth rates when cep3-1a was grown hydroponically (Fig. S2B–D).

To further investigate the role of environmental conditions on cep3-1a growth, several abiotic stress, nutrient, light, and temperature regimes were assayed (Fig. 4B). The largest increase in root system size was found when cep3-1a was grown under increased salt and nitrogen-limiting conditions. This coincides well with our finding that CEP3 is significantly induced under these two conditions in the roots. cep3-1a root systems were also significantly larger than Col-0 when grown in acidic or high salt conditions, under increased osmotic strength (mannitol), in the presence of sucrose and under decreased or increased irradiance, but not when grown with different day lengths. Elevated temperature did not affect the size of the root system significantly whereas decreased temperature reduced the lateral root density but not primary root

Fig. 5. CEP3 peptide slows primary root growth and affects lateral root formation. (A) Effect of CEP3 H on primary root (PR) growth over time. Col-0 plants were grown vertically on standard medium supplemented with 1 µM of CEP3 H or no peptide and imaged every day for 12 days (n>36 plants). (B) Representative phenotypes of 12-day-old Col-0 plants treated with no peptide or 1 µM of CEP3 H. Scale bar=1 cm. (C, D) Total number of emerged lateral roots (ELRs) plus lateral root primordia (LRP) (C) and proportion of LRP at each stage of development divided by total number of lateral roots (D). Six-day-old Col-0 plants were grown on standard MS medium with or without 1 µM of CEP3 H peptide (n=15). Error bars show standard error. *P≤0.05; **P≤0.01; ***P≤0.001 (two-sample t test, Genstat).
length. These data, together with gene expression analysis, suggest CEPs may act as intermediates between environmental conditions and root development.

To explore the effect of cep3 knockout on lateral root formation we examined lateral root primordia of plants grown under nitrogen-limiting conditions. The total number of emerged lateral roots plus lateral root primordia was significantly increased in the cep3-1a mutant compared to Col-0 (Fig. 4C). When lateral root stages were audited as described (Malamy and Benfey, 1997) we observed no significant difference in the number of lateral root primordia at any developmental stage (as a percentage of total lateral roots; Fig. 4D). This lateral root phenotype may be the product of increased root growth.

**CEPs are negative regulators that slow root growth and reduce lateral root formation**

It has been previously reported that synthetic peptide application or CEP1 over-expression arrested primary root growth (Ohyama et al., 2008). Our synthetic peptide assays and over-expression results indicated that CEPs decrease both primary root length and emerged lateral root density. To determine whether the peptide arrested or slowed root growth, we performed a time course over 12 days using CEP3 H (Fig. 5A, B). We found that the primary roots of plants treated with CEP continued growing, albeit at a significantly slower rate than untreated plants. Ohyama et al. (2008) reported that roots treated with CEP1 peptide had a reduced number of meristematic cells due to a loss of cell division potential. Our results indicate that CEPs do not arrest cell division potential; rather, they just slow the process.

To investigate the lateral root phenotype further, we examined the number (Fig. 5C) and developmental stages (Fig. 5D) of lateral root primordia in CEP3 H treated and untreated plants. The total number of emerged lateral roots plus lateral root primordia was significantly reduced in CEP treated plants. When lateral root stages were audited (Malamy and Benfey, 1997) we observed no significant difference in the number of lateral root primordia at any developmental stage (as a percentage of total lateral roots). This indicated that once lateral roots were successfully initiated, CEP was not specifically inhibiting lateral root development at any particular stage. Therefore, the significant reduction in total lateral roots induced by CEP suggests that it may be acting to stop lateral root formation prior to the first asymmetric cell division.

We have demonstrated that CEPs are negative regulators of plant development. Together, our data indicate that CEPs are able to elicit developmental phenotypes in both roots and shoots and are induced in these two tissues under different conditions, reflecting plasticity in the plants ability to respond to environmental stress.

**Conclusions**

Regulatory peptides are being increasingly recognized for playing key roles in plant development. We have extended the analysis of the CEP family of regulatory peptides. Our *in silico* analysis indicates that CEP genes have a distribution restricted to seed plants. We report that the expression of eight of the nine CEP tested is perturbed by environmental cues such as decreased nitrogen levels, increased salt levels, increased osmotic strength, and increased CO₂ levels. We demonstrate that synthetic CEPs can act at concentrations ranging from micro- to nanomolar. Peptide sequences, particularly the last nine residues and modifications to key amino acids, are both important for biological activity and the extent of activity. Our analyses indicate that CEPs act as negative growth regulators for both root and shoot systems. More specifically, CEPs reduce primary root length by slowing growth and reducing lateral root density prior to lateral root initiation. As more recently evolved regulators, CEPs may serve to fine tune developmental processes in seed plants to enable a rapid adjustment to constantly changing environmental conditions.

**Supplementary material**

Supplementary material is available at JXB online. Dataset S1. List of group I CEP genes found in angiosperms. Both the genomic and EST databases were included in the searches. Signal peptide (SignalP) prediction was done by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). All complete sequences (excluding fragments) have signal peptides predicted by both SignalP-NN (neural networks) and SignalP-HMM (hidden Markov model).

Dataset S2. List of CEP genes found in the oneKP dataset. These are transcripts sequenced by next-generation sequencing available at http://www.onekp.com/ as part of the 1000-plants (oneKP) initiative.

Dataset S3. List of group II CEP genes found in angiosperms. Both the genomic and EST databases were included in the searches. Note that CEP naming continues from Dataset S1.

Dataset S4. List of CEP-like genes found in the gymnosperm EST database.

Dataset S5. Relative change in expression levels for CEP3, CEP5, and CEP9 under various conditions, mutants, and ecotypes. Data obtained from Genevestigator (Hruz et al., 2008) filtered for minimum expression change of 1.5-fold and P<0.05.

Fig. S1. Melt curves from qRT-PCR of cep3 knockout mutants and Col-0. Melt curves for cep3-1a and cep3-1b both show non-specific binding for CEP3 primers compared to Col-0, indicating a lack of CEP3 transcript in these samples. CEP9 and control (At1g13320) melt curves show consistent binding.

Fig. S2. Phenotypes of cep knockout mutants. (A) Primary root length and lateral root density of 12-day-old Col-0, cep3-1a and cep3-1b mutants. Plants were grown modified ½ MS medium containing 0.25mM KNO₃ as the only source of nitrogen (n≥12 plants). Error bars show standard error. *P<0.05; **P<0.01; ***P<0.001 (two-sample t test, Genstat).

(B–D) Representative 26-day-old plants (B), 36-day-old...
plants (C), and 42-day-old root systems (D) of Col-0 and cep3-1a plants grown hydroponically in 1/4 MS medium. Scale bar=0.9 mm. Arrowhead indicates root tip.

Fig. S3. Alignment of selected group I CEP domains from plants with RKN CEPs. Amino acid sequences of (A) Meloidogyne incognita CEP3, Ricinus communis CEP3, RcCEP11, and Jatropha curcas CEP1 and (B) Meloidogyne hapla CEP2, MhCEP11, and Euphorbia esula CEP2 domains, aligned using Geneious.

Fig. S4. Alignments of CEP domains and full-length CEP preproproteins. Amino acid sequences of selected (A) CEP domains and (B, C) CEP preproproteins were aligned using Geneious.

Fig. S5. Phenotypes of 12-day-old Col-0 plants grown on standard medium supplemented with 1 μM of the specified peptide. See Figure 2 for peptide sequences. Scale bar=1 cm.

Fig. S6. Phenotypes of CEP over-expression lines. Plants were grown on standard 1/2 MS medium for 12 days. Scale bar=1 cm.

Fig. S7. Phenotypes of 5-week-old Col-0 and CEP3 over-expression lines grown in soil.

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References


