Short title: Local CEPR1 activity controls seed size and yield

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The peptide hormone receptor CEPR1 functions in the reproductive tissue to control seed size and yield

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One sentence summary: CEP receptor signalling in maternal tissues controls delivery of nitrogen to reproductive sinks.

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Abstract

The interaction of C-Terminally Encoded Peptides (CEPs) with CEP RECEPTOR 1 (CEPR1) controls root growth and development, as well as nitrate uptake, but has no known role in determining yield. We used physiological, microscopic, molecular and grafting approaches to demonstrate a reproductive tissue-specific role for CEPR1 in controlling yield and seed size. Independent Arabidopsis (Arabidopsis thaliana) cepr1 null mutants showed disproportionately large reductions in yield and seed size relative to their decreased vegetative growth. These yield defects correlated with compromised reproductive development predominantly in female tissues, as well as chlorosis, and the accumulation of anthocyanins in cepr1 reproductive tissues. The thinning of competing reproductive organs to improve source-to-sink ratios in cepr1, along with reciprocal bolt-grafting experiments, demonstrated that CEPR1 acts locally in the reproductive bolt to control yield and seed size. CEPR1 is expressed throughout the vasculature of reproductive organs, including in the chalazal seed coat, but not in other seed tissues. This expression pattern implies that CEPR1 controls yield and seed size from the maternal tissue. The complementation of cepr1 mutants with transgenic CEPR1 rescued the yield and other phenotypes. Transcriptional analyses of cepr1 bolts showed alterations in the expression levels of several genes of the CEP-CEPR1 and nitrogen homeostasis pathways. This transcriptional profile was consistent with cepr1 bolts being nitrogen-deficient, and with a reproductive tissue-specific function for CEP-CEPR1 signalling. The results reveal a local role for CEPR1 in the maternal reproductive tissue in determining seed size and yield, likely via the control of nitrogen delivery to the reproductive sinks.

Introduction

Leucine-rich repeat receptor-like kinases (LRR-RLK) are one of the largest gene families in plants, comprising more than 220 members in Arabidopsis (Arabidopsis thaliana) (Gou et al., 2010). Research over the past decade has implicated LRR-RLKs and their selective interactions with secreted peptide hormones in a myriad of developmental processes including reproduction, chemotropism, biotic and abiotic stress tolerances, symbiosis, root architecture, regulation of organ number, stomatal function and development, abscission, and general interactions with the environment (Czyzewicz et al., 2013; Delay et al., 2013a; Djordjevic et al., 2015; Santiago et al., 2016; Shabala et al., 2016; Shinohara et al., 2016; Imin et al., 2018; Roy et al., 2018; Chapman et al., 2020).
A conserved LRR-RLK with a growing list of important roles is C-TERMINALLY ENCODED PEPTIDE RECEPTOR 1 (CEPR1) in Arabidopsis, and its functional orthologue COMPACT ROOT ARCHITECTURE 2 (CRA2) in Medicago (Medicago truncatula). In Arabidopsis, CEPR1’s ectodomain specifically interacts with peptide hormones of the C-terminally Encoded Peptide (CEP) family (Tabata et al., 2014). Arabidopsis has 12 canonical CEP genes, each encoding one or more conserved 15-amino acid CEP domains from which the secreted, mature CEP peptide hormones are derived (Ogilvie et al., 2014). Since the identification of CEPR1 as a CEP receptor (Tabata et al., 2014), its developmental and physiological role has been defined primarily in the context of roots. For example, CEPR1/CRA2 signalling controls the extent of root growth and development, root nodule number in legumes, and nitrate uptake in roots (Tabata et al., 2014; Mohd-Radzman et al., 2016; Roberts et al., 2016; Taleski et al., 2016; Taleski et al., 2018; Chapman et al., 2019; Delay et al., 2019; Chapman et al., 2020). Grafting and split root studies show that CEPR1 influences nitrate uptake in Arabidopsis via a systemic mechanism, and that the rate of nitrate uptake is reduced in cepr1-1 (Tabata et al., 2014). The identification of mature CEPs in the xylem streams of various plants also supports the existence of systemic mechanisms (Tabata et al., 2014; Okamoto et al., 2015; Patel et al., 2018). The interaction of the root-derived CEPs with CEPR1 in the shoot vasculature triggers the upregulation of phloem-mobile shoot-to-root signals, namely the glutaredoxins CEP DOWNSTREAM 1 (CEPD1) and CEPD2, that upregulate the level of nitrate transporter expression in roots selectively exposed to high nitrate (Ohkubo et al., 2017). Recently, we demonstrated that local and systemic CEP-CEPR1 signalling curtails the expenditure of resources to control lateral root growth in response to elevated shoot-derived carbon (Chapman et al., 2019), and that systemic CEP-CEPR1 signalling controls aspects of root system architecture in soil (Chapman et al., 2020). CEP-CEPR1 signalling also controls main root growth in Arabidopsis (Delay et al., 2013b; Delay et al., 2019). In Medicago, CRA2 controls root nodulation systemically from the shoot, however a local interaction of CEPs with CRA2 controls the growth of lateral roots (Huault et al., 2014; Mohd-Radzman et al., 2015; Laffont et al., 2019).

Whether CEP-CEPR1 signalling plays a role in the growth of shoots has not been thoroughly explored. Aboveground, the cepr1-1 mutant has been described as dwarfed, producing smaller rosettes with pale green leaves and a shorter floral stem that over accumulates anthocyanins (Bryan et al., 2012; Tabata et al., 2014). Since these traits are typical responses to nitrogen deficiency (Vidal and Gutiérrez, 2008; Takatani et al., 2014), it could be
reasonable to dismiss any cepr1 aboveground defects as simply the result of reduced nitrate acquisition by the cepr1 roots. Our anecdotal observations of two cepr1 null mutants, however, indicated that their yield was reduced much more dramatically than expected based on their modest reduction in vegetative growth. In addition, we observed that the cepr1 mutants produced smaller seeds. These phenotypes appear inconsistent with an effect of CEPR1 on nitrate uptake alone, given that wild-type plants grown at low nitrogen, or mutants with impaired nitrate uptake, produce normally sized but fewer seeds such that their yield losses are proportional to the decreases in vegetative growth (Schulze et al., 1994; Masclaux-Daubresse and Chardon, 2011).

In contrast to plants with impaired root nitrate uptake, an impairment in the remobilisation of assimilated nitrogen from vegetative to reproductive tissues leads to a reduction in both seed size and yield (Guan et al., 2015; Li et al., 2015; Di Berardino et al., 2018; Moison et al., 2018). Smaller seeds also result from knocking out particular UMAMIT genes, which encode transporters required for delivering assimilated nitrogen to seeds (Müller et al., 2015). It is not known if CEPR1 signalling affects nitrogen mobilisation and delivery to reproductive sinks, however the phenotypic similarities of cepr1 with mutants impaired in these processes hint at this possibility, and suggest that CEPR1’s control over seed size and yield extends beyond its influence over root nitrate uptake. Supporting this hypothesis, we noted that several Arabidopsis CEPs are expressed in reproductive tissues (Roberts et al., 2013), and that rice (Oryza sativa) CEPs OsCEP5 and OsCEP6 are specifically expressed at defined stages of reproductive development (Ogilvie et al., 2014; Sui et al., 2016). CEPR1 is expressed throughout the shoot and root vasculature (Bryan et al., 2012; Huault et al., 2014; Tabata et al., 2014), suggesting roles in multiple tissues.

To explore a potential role for CEP-CEPR1 signalling in reproduction, we addressed several questions. Firstly, what is the physiological basis for yield reduction in cepr1 mutants? Second, can yield losses in cepr1 be restored by complementation with transgenic CEPR1 or by manipulating nutrient allocation from the vegetative tissues? Third, is CEPR1 expressed in reproductive tissues, and is its effect on yield controlled systemically via vegetative tissues or locally in reproductive tissues? Finally, does CEPR1 regulate genes involved in nitrogen homeostasis/nutrient mobilisation in the reproductive tissues?

In this study, we demonstrate that CEPR1 has a specific role in reproductive tissues in the promotion of fecundity, seed yield and size. The two cepr1 knockout mutants showed yield
reductions of between 88% and 98%, which were associated with the production of smaller seeds and a diminished number of reproductive units. These yield defects correlated with poorly developed reproductive tissues as well as chlorosis and the accumulation of anthocyanins in cepr1 reproductive tissues, all of which could be restored by transgenic complementation with CEPR1. Bolt grafting and manipulation of nutrient allocation to reproductive sinks showed that local CEP-CEPR1 signalling underpins the poor fecundity of the cepr1 mutants. We found that CEPR1 expression in the reproductive organs occurred specifically in the vasculature. Notably, CEPR1 expression in the seed was restricted to the chalazal seed coat, the site where nutrients for seed filling are unloaded from the terminating maternal vasculature (Müller et al., 2015). This result supported a local role for CEPR1 in the control of seed size and yield through activity in the mother tissue. Finally, we used transcriptional profiling of key marker genes to demonstrate a perturbation of nitrogen status in cepr1 bolts. Collectively, the results reveal a role for CEP-CEPR1 signalling that involves local activity in the bolt to control nitrogen mobilisation and delivery to reproductive sinks.

Results

CEPR1 controls vegetative growth, reproductive development and seed yield

We explored whether CEPR1 plays a role in vegetative and reproductive development by examining two independent knockout mutant alleles in the No-0 and Col-0 backgrounds (cepr1-1 and cepr1-3, respectively) (Tabata et al., 2014; Chapman et al., 2019). Both cepr1 knockout mutants displayed a ~30% retardation in rosette growth (Fig. 1, A-C). Since vegetative leaves mobilise resources to the bolt and other reproductive tissues, we examined whether loss of CEPR1 function affected yield. The cepr1 mutants displayed a reduction in the growth of reproductive tissues (Fig. 1, D and E), and the number of reproductive units (siliques, flowers and buds) on the main inflorescence was reduced by ~10% in cepr1-1 and ~40% in cepr1-3 (Supplemental Fig. S1). The diminished reproductive capacity correlated with a substantial reduction in the total seed yield per plant of ~88% and ~98% in cepr1-1 and cepr1-3, respectively (Fig. 1, F and G). The reduced seed yield resulted from a lower seed number and a 12-25% reduction in seed weight (Fig. 1, H and I). In general, the limited seeds produced by the cepr1 mutants were smaller and more diverse in size compared to wild-type (WT) seeds (Fig. 1, J and K). These results demonstrate that CEPR1 knockout reduces vegetative growth as well as seed size and yield.

CEPR1 activity controls the proper development of reproductive organs
The severe reductions in cepr1 seed yield (~88-98%) seemed disproportionate to the decrease in vegetative growth (~30%) and reduced number of reproductive units per main inflorescence (~10-40%). Therefore, we examined if CEPR1 knockout mutants had additional effects on reproductive development. An examination of floral development in the cepr1 mutants (Fig. 2) revealed chlorosis in the sepals of the developing buds and those of the open flowers (Fig. 2, A-H), and anthocyanin accumulation in the apical region of the main stem, pedicle and valves of the siliques, especially in cepr1-3 (Fig. 2, E-H). This anthocyanin accumulated in the cepr1 siliques valves shortly after fertilisation (stage 14; Smyth et al., 1990), intensified during siliques elongation (stages 15-17) and subsided as the siliques reached full length (late stage 17) (Fig. 2E-H).

We examined the preanthesis floral structure in dissected stage 12 flowers of WT No-0 and cepr1-1 (Fig. 2, I and J). The cepr1-1 flowers were smaller than WT No-0, but retained the relative dimensional ratios between the different floral organs. There was a clear differentiation of the sub-structures of the cepr1-1 gynoecium (i.e. stigma, style, valves and replum), but, like the sepals, these tissues were chlorotic compared to WT No-0 (Fig. 2J).

Post-anthesis cepr1-1 flowers (stage 13) were smaller and chlorotic compared to WT No-0, but the anthers still elongated and deposited pollen on the stigma similarly to WT No-0 (Fig. 2, M and N).

Floral development in the Col-0 cepr1-3 mutant was impaired more severely than in the No-0 mutant, and it was not possible to determine floral stage by the conventional landmark developmental events (Smyth et al., 1990). Instead we determined floral buds equivalent to WT Col-0 stage 12, based on the relative position of the bud within the inflorescence from the most recent anthesed flowers. Although the cepr1-3 sepals were of similar size to those of Col-0, there was a severe underdevelopment of the organs in the inner whorls (Fig. 2, K and L). The cepr1-3 gynoecium was typically ‘pear-shaped’ and stunted, with a translucent appearance (Fig. 2L). In cepr1-3, the petals expanded and the anthers elongated and dehisced, however, the stunting of the gynoecium resulted in a state of near hercogamy (i.e. reduced self-pollination; Fig. 2, O and P). We observed only occasional deposition of cepr1-3 pollen from the shorter medial stamens onto the poorly developed stigma. Collectively, these results show that loss of CEPR1 activity perturbs floral and reproductive organ development, with the impact being more severe in Col-0 cepr1-3 compared to No-0 cepr1-1. This suggests that...
background genetic differences between the Col-0 and No-0 accessions modify the severity of the reproductive development defects resulting from a CEPI1 knockout.

**CEPI1 positively influences seed yield on a per silique basis**

Since the loss of CEPI1 function affected flower development, we investigated whether there were effects on yield per silique in cepr1 (Fig. 3). First, we assessed siliques of self-pollinated plants and observed that cepr1 mutants had a higher incidence of unfertilised ovules, and of seeds that had aborted at various stages of development (Fig. 3, A and B). The reduction in seed set per silique was particularly severe in cepr1-3, and worsened acropetally. Therefore, we quantified seed set across silique positions (Fig. 3, C and D). In WT No-0, seed set steadily improved with increasing silique positions up the stem, reaching a maximum at approximately silique 14 (Fig. 3C). The cepr1-1 mutant also showed an improving seed set with increasing silique position up the main shoot, albeit at a decreased rate compared to WT No-0. Maximum seed set in cepr1-1 was lower compared to WT No-0 (Fig. 3C). Seed set in WT Col-0 was minimal in the first silique, and increased to a maximum at approximately silique 6 (Fig. 3D). In contrast to WT Col-0, cepr1-3 had maximal seed set in the first silique, which decreased with increasing silique position (Fig. 3D). We observed nil seed set from silique number 12 onwards in cepr1-3. For siliques with non-zero fecundity, the average seed set was significantly lower in cepr1-1 and cepr1-3 compared to their respective WT lines (Fig. 3, E and F). Moreover, the frequency of clearly fertilised and then aborted seeds (i.e. late aborting seed) was higher in both cepr1 mutants compared to their respective WT lines, and this phenotype was particularly severe in cepr1-3 (Fig. 3, G and H).

We conducted reciprocal crosses to determine whether the more severe seed set reduction in cepr1-3 was due to a male and/or female reproductive defect (Fig. 3I). Pollen from cepr1-3 fertilized WT pistils without impairment, which suggested that there was no appreciable decrease in cepr1-3 pollen viability. The reduced fecundity of the cepr1-3 pistil compared to the WT held true regardless of pollen genotype. As mechanical pollination would overcome pollen deposition defects resulting from asynchronous anther-gynoecium development in cepr1-3 (Fig. 2, O and P), we reasoned that a female reproductive defect limits cepr1-3 seed set.

To further explore this female fertility defect, we examined fertilisation frequency depending on ovule position in the pistil. Such analysis helps distinguish between problems with pollen
transmission and ovule-specific defects (Kay et al., 2013; Groszmann et al., 2020). As cepr1 mutants in both backgrounds had fewer ovules per pistil than WT (Supplemental Fig. S2), we assessed seed set at normalised ovule positions within the carpels, from position zero (closest to stigma) to position one (closest to gynophore) (Fig. 3, J and K). Compared to WT No-0, cepr1-1 showed a similar, albeit more accentuated, position-dependent fertilisation pattern in the pistil (Fig. 3J). As expected, ovules of WT Col-0 pistils showed a high and mostly position-neutral fertilisation frequency (Fig. 3K). In stark contrast, the fertilisation rate in cepr1-3 was very low at the stigma end and gradually increased towards the proximal region of the pistil, eventually reaching WT Col-0 levels (Fig. 3J). The high fertilisation rate of ovules furthest from the stigma in cepr1-3 demonstrates that pollen tubes can traverse the entire distance of the transmitting tract tissue. This indicates that the defect responsible for the reduced ovule fertility is not in the female pollen transmitting tissues (e.g. stigma and transmitting tract), but rather is specific to the ovules. This fertilisation pattern of cepr1-3 correlated spatially with the poor development of the distal region of the gynoecium (Fig. 2 L and P).

CEPR1 activity controls seed filling

One component of the yield decrease in cepr1 is a reduction in mature seeds per silique due to a reduced ovule number, lower fertility rate and an increased incidence of seed abortion. In addition, cepr1 seeds that successfully progress to maturity are smaller than WT seeds, further contributing to the lower yield. Normally plants with a reduced seed number tend to compensate by having larger seeds, which is due to a greater proportional allocation of the available nutrients being remobilised from the rosette (Bennett et al., 2012). Therefore, the small mature seeds of cepr1 may reflect a deficiency in nutrient supply to the seeds. This may arise due to its smaller source rosette and hence, a proportionately lower per seed availability of nutrients; or alternatively, due to a reduced capacity of cepr1 plants to deliver nutrients from the source rosette to the seed. To test these possibilities, we manipulated nutrient allocation from the rosette (source) by thinning the number of competing reproductive organs (sinks), to improve the source-to-sink ratio in favour of larger seeds in the siliques that remained (Bennett et al., 2012). As expected, we found that seed size increased in response to the thinning of WT plants, however, an increase in seed size did not occur in cepr1-1 despite improving the source-to-sink ratio (Fig. 4). This result suggests that
CEPR1 is required for the redistribution and/or delivery of resources for seed filling, and in doing so, controls seed size.

**CEPR1 control of seed size depends on CEPR1 activity in the reproductive bolt**

We undertook reciprocal bolt grafting between WT and cepr1-1 to elucidate if CEPR1 activity in the vegetative tissues or reproductive tissues determined seed size (Fig. 5, A and B; Supplemental Fig. S3). Early observations of established grafted bolts revealed that both the chlorosis, and the accumulation of anthocyanins in the siliques and inflorescence stem, persisted in cepr1 bolts even when grafted onto WT stock (Fig. 5C). This result suggested that juvenile cepr1 bolts derived little or no additional nutritional benefit from the WT stock. At maturity, we harvested the seeds and other dry bolt materials (i.e. stem, cauline leaves, floral and silique material) to assess the effect of graft combination on seed size and yield. We found that the smaller size of seeds produced by cepr1 bolts could not be rescued by grafting to a WT stock (Fig. 5D). In contrast, there was no penalty to the seed size of WT bolts when grafted to cepr1-1 stock (Fig. 5D). Furthermore, the distribution of seed size for WT bolts was more uniform than for cepr1-1 bolts regardless of the stock (Fig. 5E). This suggests that local CEPR1 activity in the bolt controls seed size and its uniformity. A weak, compensatory effect of vegetative CEPR1 activity was observed for cepr1-1 bolts grafted onto a WT stock (i.e. cepr1-1/WT) with mild improvements in seed size uniformity compared to cepr1-1 homografts (Fig. 5E).

**CEPR1 activity controls total seed yield primarily in the reproductive bolt**

Since the decrease in total seed yield of the cepr1-1 bolt could not be rescued by grafting to a WT stock (Fig. 6A), a lack of CEPR1 activity in the bolt most likely limits yield in cepr1-1 plants. Moreover, there was no yield penalty to a WT bolt when grafted to a cepr1 stock, but rather an apparent increase in total seed yield relative to the WT homografts (Fig. 6A). An examination of the dry mass of harvested bolt material (excluding seed) revealed that WT on cepr1-1 grafts had significantly greater bolt dry mass than the other graft combinations (Fig. 6B), consistent with the greater bolt growth observed for this graft combination at 26 days (Fig. 5B). To account for differences in bolt growth, we calculated the ratio of seed yield to total bolt biomass (bolt harvest index) (Fig. 6C). The bolt harvest index of WT on cepr1-1 grafts was not different to WT homografts. Compared to the cepr1-1 homografts, the bolt harvest index of cepr1-1 slightly improved when grafted to a WT stock (increasing from
~37% to ~50% of WT homografts). Therefore, whilst a WT stock could partially compensate for a lack of CEPR1 in the bolt, the bolt harvest index was primarily determined by CEPR1 activity in the bolt. These results, together with the seed size data, show that CEPR1 bolt activity controls reproductive development, seed size, and subsequently total yield.

**CEPR1 is expressed in the vasculature of floral and reproductive organs**

To help elucidate how CEPR1 influences reproductive development and fecundity, we examined *CEPR1* expression in reproductive organs using a 2kb *CEPR1* promoter-*GUS* reporter (*pCEPR1:GUS*). *pCEPR1:GUS* was expressed in the vasculature of the inflorescence stem, the base of floral buds, as well as floral and reproductive organs (Fig. 7, A and B). In flowers, expression was detected in the vasculature of well-developed sepals and petals, elongated stamen filaments (Fig. 7; A, C and D), developing gynoecium prior to fertilisation (Fig. 7E), mature gynoecium (Fig. 7F), and the growing silique (Fig. 7G).

*CEPR1* expression in the maternal reproductive tissues appeared throughout the entire vasculature network including the medial and lateral vascular strands, the terminating vascular bundles in the style, and the vasculature of the funiculus right to the point of termination within the chalazal seed coat (Fig. 7, F-H). We observed *CEPR1* expression within the funiculus and chalazal seed coat only after fertilisation (Fig. 7E vs Fig. 7F) and it persisted throughout seed development (Fig. 7, H and I). We detected no expression of *CEPR1* in the endosperm or embryo.

The restriction of seed *pCEPR1:GUS* expression to the chalazal seed coat is consistent with publicly available expression data (Fig. 7J) (Belmonte et al., 2013; Arabidopsis eFP browser, https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). *CEPR1* expression in the chalazal seed coat is important since it represents the final delivery point of nutrients via the vasculature from the mother tissue prior to their uptake via a variety of transporters to the symplasmically isolated filial tissues (Stadler et al., 2005; Chen et al., 2015; Müller et al., 2015; Phan et al., 2020). The lack of *pCEPR1:GUS* or *CEPR1* mRNA expression in the endosperm or embryo tissue indicates that *CEPR1* control of seed size occurs strictly through activity in the mother tissue. Consistent with this, restoring WT *CEPR1* in only the filial tissue of the cepr1-3 mutant via crossing (i.e. cepr1-3 ♀ x WT Col-0 ♂) did not improve seed size (Supplemental Fig. S4).

**Transgenic CEPR1 rescues cepr1 reproductive and yield defects**
We complemented both cepr1 mutants using a transgene containing CEPR1 driven by the same 2kb upstream sequence used in our GUS reporter expression analysis (Fig. 8). The examination of multiple complemented lines revealed that the transgene rescued several obvious cepr1 developmental defects (e.g. anthocyanin accumulation in stem and silique, smaller chlorotic flowers, and aberrant floral organ morphology; Fig. 8, A and B). Further analysis of two independent complemented lines of each cepr1 allele demonstrated full or substantial rescue of ovule number (Fig. 8, C and G), seed set (Fig. 8, D and H), seed abortion (Fig. 8 E and I), and seed size (Fig. 8 F and J). These results indicate that the 2kb of sequence upstream of CEPR1 is sufficient for native CEPR1 function and imply that a loss of CEPR1 expression in the vasculature is the causal factor leading to the developmental, reproductive, and yield defects associated with the cepr1 mutants.

**CEPR1 regulates the expression of genes involved in nitrogen homeostasis in the reproductive bolt**

Anthocyanin accumulation and chlorosis are signs of imbalances in nitrogen and carbon, especially where nitrogen is low and carbon is in proportional excess (Takatani et al., 2014). Therefore, nutritional limitation may account for the cepr1 defects in floral morphology, lower fecundity, the high incidence of seed abortion and its smaller, more variable seed sizes. To investigate this, we harvested bolt and inflorescence tissues and surveyed the expression of several genes involved in the CEP-CEPR1 signalling pathway along with several genes involved in nitrogen and carbon homeostasis (Fig. 9). *CEP DOWNSTREAM 1* (*CEPD1*), which is positively regulated by CEP-CEPR1 signalling in shoots (Ohkubo et al., 2017), was downregulated ~8-fold in the cepr1 bolt tissue (Fig. 9A). Three out of four CEP ligand-encoding genes known to be expressed in the bolt (Roberts et al., 2013; Col-0 accession) were also differentially expressed in the cepr1 mutants. *CEP5* and *CEP9* were strongly upregulated (~32-fold), *CEP2* was downregulated in cepr1-3 (~3-fold) and undetectable in the No-0 background, and *CEP1* expression was not significantly altered (Fig. 9A). The cepr1 mutants had a ~60% reduction in the expression of *GLUTAMINE SYNTHETASE 1;2* (*GLN1;2*) (Fig. 9B), which encodes the main isozyme contributing to glutamine synthetase activity in the shoot (Guan et al., 2016), and is known to be involved in nitrogen mobilisation and yield formation (Diaz et al., 2008; Guan et al., 2016; Moison et al., 2018). The expression of the nitrate reductase gene *NITRATE REDUCTASE1* (*NIA1*), also involved in nitrogen metabolism (Wilkinson and Crawford, 1993), was not significantly different. The
expression of *USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 14 (UMAMIT14)*, an amino acid transporter gene linked to seed filling (Müller et al., 2015), was strongly downregulated in the *cepr1* mutants (>80% reduction; Fig. 9B). In contrast, *UMAMIT11* and *UMAMIT18/SILIQUES ARE RED 1 (SIAR1)*, which also supply assimilated nitrogen as amino acids to reproductive tissues (Ladwig et al., 2012; Müller et al., 2015), were not differentially expressed in the *cepr1* mutants (Fig. 9B). Consistent with the observed anthocyanin accumulation in *cepr1* stems, there was a substantial upregulation in the *cepr1* mutants of one or both of the low nitrogen-induced MYB transcription factors *PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1)* and *PAP2*, which are involved in anthocyanin production when nitrogen is limited (Scheible et al., 2004; Lea et al., 2007; Rubin et al., 2009) (Fig. 9B).

Unlike some of the nitrogen-associated genes, marker genes responding to elevated carbon (Nunes et al., 2013; Cookson et al., 2016) were not substantially altered in the *cepr1* mutants. Specifically, *AKINBETA1* and the putative trehalose-6-phosphate synthase (TPS) genes, *TREHALOSE PHOSPHATE SYNTHATSE 8 (TPS8)* and *TPS10*, which are normally downregulated in response to elevated carbon levels, were either mildly downregulated or unaltered in the *cepr1* mutants (Fig. 9C). The *ARABIDOPSIS THALIANA BASIC LEUCINE-ZIPPER 11 (bZIP11)* and *TPS5* genes, which positively respond to elevated carbon levels, remained unchanged (Fig. 9C). Together with the nitrogen-associated transcripts, the expression profiles of *cepr1* bolts implies a state of nitrogen limitation with minimal or no perturbations to carbon status.

**Discussion**

In this study we demonstrated a function for CEPR1 in controlling seed yield via a local circuit in the reproductive tissues of Arabidopsis. The severely decreased yield of *cepr1* mutants is due to a loss of vasculature-expressed *CEPR1*, which compromises bolt growth, female reproductive development, seed set, and mature seed size, and in addition, causes chlorosis and anthocyanin accumulation in inflorescence tissues. The complementation of *cepr1* mutants using a *CEPR1* genomic fragment rescues these diverse phenotypes. Underpinning these phenotypes of the *cepr1* mutants is the apparent common theme of an inability to mobilise nutrients, specifically nitrogen, either from the vegetative tissues to the young bolts or from within bolt tissues to the developing floral organs and seeds. Several lines of evidence support this view.
First, the cepr1 reproductive tissues showed chlorosis in the bolt tissues and floral organs, and anthocyanin accumulation in the inflorescence stems and siliques. The grafting of young cepr1 bolts onto WT vegetative stocks did not remedy these well-documented phenotypes of nutrient limitation stress, suggesting a compromise of nutrient delivery from the vegetative tissue to the young cepr1 bolt. Concordantly, young WT bolts did not develop chlorosis or accumulate anthocyanins when grafted onto cepr1 vegetative stocks.

The grafting of cepr1 bolts onto WT vegetative stocks did not restore yield or a normal seed size distribution, whereas WT bolts had no reduction in yield or seed size when grafted to cepr1 vegetative stocks. In agreement with a role for CEPR1 in seed filling through the control of nitrogen mobilisation and delivery, we found that both GLN1;2 and UMAMIT14 were down-regulated in the cepr1 reproductive bolt. The cytosolic glutamine synthetase (GS) encoded by the vasculature-expressed GLN1;2 contributes to nitrogen remobilisation from source tissues for seed filling (Moison et al., 2018), with gln1;2 loss-of-function mutants displaying reduced seed size and yield similar to cepr1 (Guan et al., 2015). The importance of cytosolic GS in seed filling appears conserved across diverse species including maize (Zea mays) (Martin et al., 2006). The UMAMIT14 amino acid transporter is also expressed throughout the plant vasculature, including the chalazal seed coat, where it is required for the unloading of amino acids transported from source tissues to support seed filling. Consistent with the decreased UMAMIT14 expression in the cepr1 mutants, the umamit14 null mutants produce smaller seeds (Müller et al., 2015).

Improving nutrient allocation by thinning the number of reproductive units resulted in the expected increased seed size in WT plants, but not in cepr1 mutants. The differential effect of thinning between WT and cepr1 is not due to the smaller cepr1 rosettes, because grafting showed that the cepr1 rosettes support the flourishing of WT bolts. Moreover, this grafting result implies that the cepr1 source rosettes are still able to sufficiently load nutrients such as amino acids into the phloem for export to the developing bolts (Santiago and Tegeder, 2016). Therefore, the seeds of cepr1 plants that were thinned appear unable to receive the expected allocation of surplus nutrient that clearly benefits the seeds of thinned WT plants.

CEPR1 expression was detected throughout the vasculature of reproductive tissues, consistent with a function in nutrient mobilisation/delivery. The specific expression of CEPR1 in the chalazal seed coat, but not in any other seed tissues, is pertinent to the cepr1 seed size defect since this tissue is the final delivery point of nutrients from the mother tissue.
via the vasculature prior to their uptake via transporters to the symplasmically isolated filial tissues (Müller et al., 2015). This localisation points to CEPR1 playing an important role in nutrient unloading at the seed and indicates that it controls seed development and size strictly through activity in the maternal tissue. An impairment of nutrient delivery to the seed would be consistent with the more variable seed size and higher rates of seed abortion observed in the cepr1 mutants, with seed abortion occurring in instances where early nutrient demands of the embryo are not met (Di Berardino et al., 2018), and with the more variable mature seed sizes reflecting inconsistency in nutrient delivery.

Our RT-qPCR data indicates that the nutrient stress caused by a loss of CEPR1 function is related to impaired nitrogen status of the reproductive bolt. Consistent with a low nitrogen state and anthocyanin accumulation, cepr1 bolts displayed a strong upregulation of one or both of the MYB transcription factors PAP1 and PAP2. These PAP genes are highly upregulated in response to nitrogen starvation and positively regulate anthocyanin biosynthesis (Lea et al., 2007; Rubin et al., 2009). The downregulation of the glutamine synthetase gene GLN1;2 and UMAMIT14 would be consistent with decreased levels of assimilated nitrogen compounds (Patterson et al., 2010; Besnard et al., 2016). By contrast, the mild downregulation of carbon-repressed TPS genes and the unchanged expression of carbon-induced transcripts in cepr1 bolts implies a sufficient carbon supply. A decreased nitrogen-state but steady carbon-state in cepr1 mutants is further supported by their accumulation of anthocyanins, which in general is not just a symptom of nitrogen limitation, but rather a symptom of excess levels of carbon relative to nitrogen (Takatani et al., 2014).

Moreover, several CEP-CEPR1 pathway genes both upstream (i.e. several CEP genes) and downstream (i.e. CEPD1) of CEPR1 displayed altered expression in cepr1 bolts, consistent with a locally perturbed CEPR1-signalling status involving both feedforward and feedback loops. Similar transcriptional perturbations occur to CEP-CEPR1 pathway genes locally in cepr1 root tissues (Chapman et al., 2019). These transcriptional responses, together with CEPR1 expression in the vasculature of reproductive tissues and the dependence of seed size and yield on local CEPR1 activity, demonstrates that CEPR1 specifically acts in bolt tissues to control aspects of nitrogen mobilisation and delivery to reproductive sinks.

Finally, an impairment in the nitrogen economy of the bolt can also explain the cepr1 fecundity and silique defects. Nutrient deficiency is consistent with the diminished growth of the bolt and the reduced number of reproductive units produced (i.e. flowers and siliques).
The aberrant and asynchronous development of the cepr1 gynoecium and stamens contributing to the reduced seed number on a per silique basis, is comparable with the miscoordination of reproductive development occurring in rice florets when nitrogen is limited by loss-of-function mutations in arginase (OsARG) or ornithine δ-aminotransferase (OsOAT) (Ma et al., 2013; Liu et al., 2018). Similar to cepr1, these rice mutants display reduced seed set and smaller seed/grain. The accumulation of anthocyanins observed in cepr1 developing siliques is identical to the phenotype of mutants in the amino acid transporter gene UMAMIT18/SIAR1 and is likely a stress symptom of inadequate nitrogen provision during the nutrient demanding stages of seed development (Ladwig et al., 2012). The miscoordination of reproductive development along with the altered expression of some nitrogen-related genes in the cepr1 bolt, is consistent with CEPR1 acting as one node of a complex network regulating nitrogen homeostasis (Castaings et al., 2010; Tegeder and Masclaux-Daubresse, 2018).

Conclusions

In this paper, we show that CEPR1 activity in the reproductive tissue is critical for both reproductive development and yield. Our data highlights the importance of CEPR1 activity in the reproductive bolt for the delivery of nutrients for yield formation. The apparent perturbation of nitrogen status in cepr1 bolts suggests that CEPR1 plays a broader role in controlling nitrogen homeostasis at the whole-plant level, beyond roles previously identified in root nitrogen acquisition, root system architecture and nodulation (Huault et al., 2014; Tabata et al., 2014; Mohd-Radzaman et al., 2016; Ohkubo et al., 2017; Chapman et al., 2020). The manipulation of CEPR1-dependent outputs could provide a new avenue to improve nutrient delivery from source tissues to reproductive sinks, with the aim of improving traits such as seed yield and nitrogen use efficiency in plants.

Material and Methods

Plant materials and growth conditions

The previously described Arabidopsis (Arabidopsis thaliana) No-0 cepr1-1 (RATM11-2459; RIKEN) (Bryan et al., 2012; Tabata et al., 2014) and Col-0 cepr1-3 (467C01; GABI-Kat) (Kleinboelting et al., 2012; Chapman et al., 2019) mutant lines were used. Sterilised seedlings were grown on solidified media (1% w/v Type M agar) containing ½ strength Murashige-Skoog (MS) basal salts (Sigma) at pH 5.7 for 7-10 days until seedlings were transferred to...
soil (seed raising mix; Debco, Bella Vista NSW) supplemented with Osmocote Exact fertiliser and grown in chambers at 22 °C. For the grafting experiment, plants were grown with 200 μmol m\(^{-2}\) s\(^{-1}\) light and an 8 h photoperiod. For all other experiments, plants were grown with 100 μmol m\(^{-2}\) s\(^{-1}\) light and a 16 h photoperiod.

**Determination of seed size, seed set and yield parameters**

Inflorescences were covered with microperforated plastic bags to collect seeds. To determine single seed mass, aliquots were weighed and seed number per aliquot counted. Seed area was determined using ImageJ (https://imagej.nih.gov/ij/) using the ‘particle analysis’ function with a consistently applied ‘threshold’ on all microscope images of seeds taken with identical magnification and exposure time within experiments (Herridge et al., 2011). For seed size distributions, the percentage of seeds within bins at 0.002 mm\(^2\) intervals was determined and a running average over six bins was plotted. For controlled pollination, preanthesis flowers (Smyth et al., 1990; stage 12) were emasculated using forceps. One day after emasculation, the pollen from the donor genotype was applied to the stigma of the recipient genotype. Seed development and seed set was determined by dissecting developing siliques and dehisced siliques, respectively (Groszmann et al., 2008; Kay et al., 2013). For the grafting experiment, bolt harvest index was determined as the seed mass as a proportion of total bolt biomass (seed mass/total bolt dry mass including seed).

**Vector Construction**

To generate the pCEPR1:GUS reporter, the 2 kb of sequence upstream of the start codon of *CEPR1* was amplified from Col-0 gDNA, cloned into pENTR/D-TOPO, and then transferred into the pHGWFS7 destination vector (Karimi et al., 2002) by LR recombination. To generate the *CEPR1* complementation construct, the kanamycin marker for plant selection in the pBI121 vector was first removed by digestion with PmeI and ApaI and was replaced using Gibson Assembly (NEB) with a Basta selection marker amplified from the MIGS2.1 vector (de Felippes et al., 2012). Using Gibson Assembly, the Col-0 *CEPR1* genomic fragment and 2 kb of upstream sequence, was cloned upstream of a *NOS* terminator in the modified pBI121 vector cut with SacI and Clal. Primer sequences used for cloning are listed in Supplemental Table S1.

**Plant transformation and introgression of constructs**
Plants were transformed by floral dipping (Clough and Bent, 1998) with the *A. tumefaciens* strain LBA4404 harbouring the described vectors to create the *pCEPR1:GUS* reporter line (No-0 background) and the *cepr1-1* complementation lines (comp #1 and comp #2). Due to the severely impaired female fertility in *cepr1-3*, which prevented the direct transformation of this mutant, the complementation lines in the *cepr1-3* background (comp #3 and comp #4) were generated by crossing with WT Col-0 transformed with the complementation construct. Flowers from independent Col-0 lines harbouring the *CEPR1* complementation construct were emasculated and then pollinated using *cepr1-3* pollen. F2 plants homozygous for the *cepr1-3* allele (i.e. no endogenous WT *CEPR1*) and carrying the *CEPR1* transgene were identified using PCR genotyping (see Supplemental Table S1 for primers).

### Thinning experiment

Six-week-old No-0 (WT) and *cepr1-1* plants were thinned by removal of siliques and flowers in addition to secondary and lateral apexes so that only three or four open flowers or very early stage siliques remained on the primary stem. Seeds from thinned plants were compared to those from unpruned controls.

### Inflorescence stem (bolt) grafting

Bolt grafting was carried out as described in Nisar et al. (2012). Briefly, young No-0 (WT) and *cepr1-1* bolts (~80 mm long) were excised ~20 mm from the base. The end of the donor bolt was cut into a wedge, which was inserted into a vertical incision made in the remaining basal stem section of the recipient stock. The graft junction was stabilised with silicon tubing 2-2.5 mm (inner diameter) and covered with parafilm to retain moisture. Plants were kept in a humid environment using a plastic covering until grafted bolts had re-established turgor and growth. Bolts that did not take, or that grew poorly as determined by the appearance of necrotic cauline leaves were discarded. Secondary branches that formed from the stock post grafting were continuously removed so that the final reproductive organs were derived from the donor bolt only.

### Promoter-GUS Reporter analysis

GUS staining and analysis was performed essentially as previously described (Groszmann et al., 2010; Groszmann et al., 2011). Briefly, samples were harvested into cold phosphate buffer (pH 7) with 4% v/v formaldehyde, washed in cold phosphate buffer, transferred into a 2mM X-gluc staining solution, incubated at 37°C for specified durations and then cleared for
24 hrs in 70% ethanol, fixed for 24 hrs in 70% ethanol plus FAA (3.7% (v/v) formaldehyde and 5% (v/v) acetic acid), washed again and stored in 70% ethanol ready for visualisation.

**Microscopy**

Microscopic imaging was carried out with a M205 FA stereomicroscope with a DFC550 camera (Leica).

**Complementation analyses**

T2 complementation lines in the cepr1-l background (comp #1 and comp #2) were grown alongside WT No-0 and cepr1-l. PCR genotyping identified cepr1-l plants harbouring the complementation transgene and null (azygous) segregants. Azygous segregants were pooled with the cepr1-l plants grown in parallel for the analysis.

For complementation lines in the cepr1-3 background (comp #3 and comp #4), segregating F2 plants were grown alongside WT Col-0 and cepr1-3. PCR genotyping identified homozygous cepr1-3 plants harbouring the complementation CEPR1 transgene. For the analysis, WT Col-0 and cepr1-3 segregants identified from the F2 populations were pooled with the WT Col-0 and cepr1-3 plants grown in parallel, respectively. See Supplemental Table S1 for genotyping primers used.

**RT–qPCR analyses**

WT and cepr1 primary inflorescence tissue (including stem, inflorescence meristem, buds and flowers) was harvested for analyses upon opening of the first flower. The lateral organs (cauline leaves, developing branches and buds) were removed from the harvested tissue before they were snap-frozen in liquid nitrogen. Three biological samples per genotype, each containing two bolts, were used, and total RNA was isolated by a modified Trizol extraction method using columns from the RNeasy Plant Mini Kit (QIAGEN) (Delay et al., 2013b).

cDNA synthesis was carried out using oligo(dT)_{12-18} primers and Superscript III reverse transcriptase (Invitrogen), and was followed by treatment with RNase H (Invitrogen). For RT–qPCR, Fast SYBR Green fluorescent dye (Applied Biosystems) was used and samples were run on a ViiA 7 Real-Time PCR System (Applied Biosystems) following manufacturer’s specifications. Data were analysed using the ΔΔC\text{_T} method (Livak and Schmittgen, 2001), with EF1α (At1g07920) expression used for normalisation (Czechowski et al., 2005). RT-qPCR primers are listed in Supplemental Table. S1.
Accession numbers

The AGI locus codes for genes discussed in this study are as follows: CEPR1 (AT5G49660), CEPD1 (AT1G06830), CEPD2 (AT2G47880), CEPI (AT1G47485), CEP2 (AT1G59835), CEP5 (AT5G66815), CEP9 (AT3G50610), UMAMIT11 (AT2G40900), UMAMIT14 (AT2G39510), UMAMIT18/SIAR1 (AT1G44800), PAP1 (AT1G56650), PAP2 (AT1G66390), NLA1 (AT1G77760), GLN1;2 (AT1G66200), AKINBETA1 (AT5G21170), TPS5 (AT4G17770), TPS8 (AT1G70290), TPS10 (AT1G60140), bZIP11 (AT4G34590).

Supplemental data

Supplemental Figure S1. CEPR1 mutants have reduced reproductive units per main stem.
Supplemental Figure S2. CEPR1 knockout results in a reduced number of ovules per siliques.
Supplemental Figure S3. Rosette diameter of plants grown under a short day (8 hr) photoperiod for bolt grafting.
Supplemental Figure S4. CEPR1 controls seed size via activity in the maternal tissue.

Supplemental Table S1. List of oligos used.

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Figure legends

Figure 1. CEPR1 affects above-ground plant growth, yield and seed size. (A-C) CEPR1 mutants show decreased vegetative leaf growth. (A) Rosette diameter after 30 days post germination (dpg) for No-0 and cepr1-1. n= 10. (B) Rosette diameter at 31 dpg for Col-0 and cepr1-3. n=4-8. (C) Representative images of plants in B. Note, cepr1-3 rosette leaves displayed no obvious chlorosis. Scale bar = 5 cm. (D-J) Loss of CEPR1 function results in
impaired yield. (D) Inflorescence stems of No-0 and cepr1-1 at 50 dpg. (E) Inflorescence stems of Col-0 and cepr1-3 at 59 dpg. Scale bars in D and E = 100 mm. (F, G) CEPR1 mutants show reduced yield. Total seed yield per plant for (F) No-0 and cepr1-1 (n=6-10), and for (G) Col-0 and cepr1-3 (n=4-8). (H-I) CEPR1 mutants have reduced seed size. Mass of one seed for (H) No-0 and cepr1-1 (n=3 plants), and for (I) Col-0 and cepr1-3 lines (n=4 plants) determined from ~100 seeds per plant. Percentages above bars indicate mean as a percentage of WT. Significant differences were determined by a two-sample t-test; **p<0.01, ***p<0.001. Error bars = SE. Distribution of seed size for WT and cepr1 in the (J) No-0 and (K) Col-0 backgrounds. n = 4-7 plants, 60-120 seeds per plant.

Figure 2. CEPR1 affects the development of reproductive organs. Representative images showing floral and reproductive organ phenotypes of WT and cepr1 mutant plants in the No-0 and Col-0 accession. (A-D) Inflorescence of WT and cepr1 mutants. The developing buds of CEPR1 knockout mutants are chlorotic. (E-H) Side view of the inflorescence showing chlorotic sepals (sep.) and anthocyanin accumulation in the siliques, pedicle and main stem of cepr1 (arrows). Arrow head in F indicates the fading of anthocyanin in older siliques. (I-P) Medial view of dissected WT and cepr1 flowers showing the aberrant floral development associated with CEPR1 knockout. (I-L) Preanthesis stage 12 WT and cepr1 flowers. Arrow head in L indicates poorly developed distal end of cepr1-3 gynoecium. (M, N) Post-anthesis, stage-13 flowers of WT No-0 and cepr1-1. (O, P) Post-anthesis, stage-14 flowers of WT Col-0 and cepr1-3. Scale bars = 1 mm.

Figure 3. Loss of CEPR1 function results in decreased seed set and a higher frequency of seed abortion. (A,B) Representative images of dissected siliques from self-pollinated WT and cepr1 lines in the (A) No-0 and (B) Col-0 backgrounds. Arrowheads indicate unfertilised ovules. Asterisks indicate aborted seeds. Scale bars = 1 mm. (C,D) Seed set at silique positions on the main stem (position 1 = first silique produced) for self-pollinated WT and cepr1 lines in the (C) No-0 (n=4-6), and (D) Col-0 (n=3-6) backgrounds. Error bars show SE. For each plant line, two separate linear trendlines were applied to the distinct subset of silique positions before and after a plateau in seed set. (E,F) Average seed set determined from siliques with non-zero seed set irrespective of silique position for self-pollinated WT and cepr1 in the (E) No-0 background (n=6 plants, 7-14 siliques per plant) and (F) Col-0 background (n=5-6 plants, 2-15 siliques per plant). (G,H) Frequency of aborted seeds observed at fertilised ovule positions for self-pollinated WT and cepr1 in the (G) No-0 background (n=6 plants, 7-14 siliques per plant) and (H) Col-0 background (n=5-6 plants, 2-15 siliques per plant). Significant differences in E-H determined by a two-sample t-test; *p<0.05, **p<0.01, ***p<0.001. Error bars show SE. (I) Percentage seed set for controlled pollination between Col-0 and cepr1-3 plants. Preanthesis flowers were emasculated prior to deposition of donor pollen onto recipient pistils. Significant differences determined by ANOVA followed by Tukey HSD test (alpha=0.05). n=4-6 siliques from 3 plants. Error bars show SE. (J,K) Fertilisation frequency at ovule positions for self-pollinated WT and cepr1 in the (J) No-0 background (n=6 plants, 7-14 siliques per plant) and (K) Col-0 background (n=5-6 plants, 2-15 siliques per plant). Each position is represented as a fraction of the highest
Figure 4. CEPR1 determines the extent of seed filling. The resource availability per silique was increased by thinning the number of reproductive sinks. (A) Images of No-0 (WT) and cepr1-1 before (left) and after (right) thinning. Three to four open flowers or early stage siliques were left at the apex of the main stem. (B) Relative seed area for thinned and untreated plants. ANOVA followed by Tukey HSD test (alpha = 0.05), n=3 plants, 100-200 seeds per plant. Error bars show SE.

Figure 5. Seed size depends upon CEPR1 activity in the bolt. Young No-0 (WT) and cepr1-1 bolts were excised approximately 2 cm from the base and grafted to a recipient stock, which provided vegetative rosette and root tissues to the transplanted reproductive tissue. Secondary branches that formed from the stock post grafting were continuously removed so that the final reproductive organs were derived from the donor bolt only. The bolt and stock genotypes are labelled above and below the horizontal line, respectively. (A,B) Reciprocal grafting of WT and cepr1-1 plants. Representative images of plants (A) 0 and (B) 26 days after grafting (bolt/stock genotype). Arrow heads in A indicate the graft junction. Scale bars = 5 cm. (C) Representative images of inflorescences from hetero-grafted plants. Note the accumulation of anthocyanin in the siliques of cepr1-1 bolt-grafts. (D) Quantification of single seed mass for grafted plants. Statistically significant differences determined by ANOVA followed by Tukey HSD test (alpha = 0.05), n = 6-9. (E) Distribution of seed size. n = 6-9 plants, 100-200 seeds per plant. Error bars show SE.

Figure 6. Yield is primarily determined by CEPR1 activity in the reproductive bolt. (A-C) Analysis of yield on a per plant basis for reciprocal WT and cepr1-1 bolt grafted plants. Quantification of (A) total seed yield, (B) bolt dry mass (minus seed), and (C) bolt harvest index (the ratio of seed yield to total bolt biomass). Significant differences determined by ANOVA followed by Tukey HSD test (alpha=0.05). Error bars show SE. n = 6-9 plants.

Figure 7. CEPR1 expresses in the vasculature of reproductive organs. pCEPR1:GUS expression in the reproductive organs. (A) whole inflorescence, (B) buds, (C) stage 14 flower, (D) stage 15 gynoecium and stamens, (E) stage 12 gynoecium, (F) stage 14 gynoecium (s.v = style vasculature, m.v = medial vasculature, l.v = lateral vasculature), (G) mature silique, (H) funiculus and chalazal seed coat (CZSC = chalazal seed coat, fun. = funiculus), and (I) mature silique funiculus. Scale bars = 1 mm (A,C-G) or 0.1 mm (B,H,I). Staining carried out for 18 h (A,D,G,I), 24 h (H) or 72 h (B,C,E,F). (J) Microarray expression of CEPR1 in the developing seed from the Arabidopsis eFP browser; https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Belmonte et al., (2013).

Figure 8. Complementation of cepr1 mutants using a CEPR1 genomic fragment. Reproductive development phenotypes were assessed for independent complementation (comp) lines in the No-0 cepr1-1 (#1 and #2) and Col-0 cepr1-3 (#3 and #4) backgrounds compared to their respective cepr1 mutant and WT lines. (A, B) Representative images showing a side view of the inflorescence (upper panels) and dissected post-anthesis flowers (lower panels) for lines in the (A) No-0, and (B) Col-0 ecotypes. (C, G) Quantification of ovule number for siliques with non-zero fecundity for (C) No-0 (n=4-10, 2 siliques per plant), and (G) Col-0 ecotype lines (n=3-11, 1-4 siliques per plant). (D,H) Measurement of seed set for (D) No-0 (n=4-10) and (H) Col-0 ecotype lines (n=3-11). Two siliques were examined per
plant from positions on the stem corresponding to the phase of WT maximal seed set (see Fig. 3C,D). (E,I) Frequency of aborted seeds observed at fertilised ovule positions for lines in the (E) No-0 (n=4-10, 2 silique per plant), and (I) Col-0 ecotypes (n=3-11, 1-4 silique per plant). (F,J) Seed area for (F) No-0 (n=6-11 plants) and (J) Col-0 ecotype lines (n=3-9 plants) determined from 60-120 seeds per plant. Significant differences determined by ANOVA followed by Fisher’s Least Significant Difference test (alpha = 0.05). Error bars show SE.

Figure 9. Loss of CEPR1 function affects the expression of genes involved in nitrogen homeostasis in bolt tissue. For RT-qPCR analyses, the primary inflorescence tissue was harvested from WT and cepr1 plants upon first flower opening. The fold change in expression (log2) was determined for cepr1-1 and cepr1-3 relative to WT (No-0 and Col-0, respectively) for a selection of (A) CEP-CEPR1 pathway genes, (B) genes related to nitrogen (N) homeostasis, and (C) genes related to carbon (C) homeostasis. Significant differences were determined by a two-sample t-test; *p<0.05 , **p˂0.01, *** p˂0.001. Error bars show SE, n=3 biological replicates each consisting of two pooled plants. n.d.; not detected.

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Figure 1. CEPR1 affects above-ground plant growth, yield and seed size. (A-C) CEPR1 mutants show decreased vegetative leaf growth. (A) Rosette diameter after 30 days post germination (dpg) for No-0 and cepr1-1. n=10. (B) Rosette diameter at 31 dpg for Col-0 and cepr1-3. n=4-8. (C) Representative images of plants in B. Note, cepr1-3 rosette leaves displayed no obvious chlorosis. Scale bar = 5 cm. (D-J) Loss of CEPR1 function results in impaired yield. (D) Inflorescence stems of No-0 and cepr1-1 at 50 dpg. (E) Inflorescence stems of Col-0 and cepr1-3 at 59 dpg. Scale bars in D and E = 100 mm. (F, G) CEPR1 mutants show reduced yield. Total seed yield per plant for (F) No-0 and cepr1-1 (n=6-10), and for (G) Col-0 and cepr1-3 (n=4-8). (H-I) CEPR1 mutants have reduced seed size. Mass of one seed for (H) No-0 and cepr1-1 (n=3 plants), and for (I) Col-0 and cepr1-3 lines (n=4 plants) determined from ~100 seeds per plant. Percentages above bars indicate mean as a Student’s t-test; **p<0.01, ***p<0.001. Error bars = SE. Distribution of seed size for WT and cepr1 in the (J) No-0 and (K) Col-0 backgrounds. n = 4-7 plants, 60-120 seeds per plant.
Figure 2. CEPRI affects the development of reproductive organs. Representative images showing floral and reproductive organ phenotypes of WT and cepr1 mutant plants in the No-0 and Col-0 accession. (A-D) Inflorescence of WT and cepr1 mutants. The developing buds of CEPRI knockout mutants are chlorotic. (E-H) Side view of the inflorescence showing chlorotic sepals (sep.) and anthocyanin accumulation in the siliques, pedicle and main stem of cepr1 (arrows). Arrow head in F indicates the fading of anthocyanin in older siliques. (I-P) Medial view of dissected WT and cepr1 flowers showing the aberrant floral development associated with CEPRI knockout. (I-L) Preanthesis stage 12 WT and cepr1 flowers. Arrow head in L indicates poorly-developed disk and end of cepr1-3 gynoecium. (M, N) Post-anthesis, stage-13 flowers of WT No-0 and cepr1-1. (O, P) Post-anthesis, stage-14 flowers of WT Col-0 and cepr1-3. Scale bars = 1 mm.
Figure 3. Loss of CEPR1 function results in decreased seed set and a higher frequency of seed abortion. (A,B) Representative images of dissected siliques from self-pollinated WT and cepr1 lines in the (A) No-0 and (B) Col-0 backgrounds. Arrowheads indicate unfertilised ovules. Asterisks indicate aborted seeds. Scale bars = 1 mm. (C,D) Seed set at silique positions on the main stem (position 1 = first siliques produced) for self-pollinated WT and cepr1 lines in the (C) No-0 (n=4-6), and (D) Col-0 (n=3-6) backgrounds. Error bars show SE. For each plant line, two separate linear trendlines were applied to the distinct subset of silique positions before and after a plateau in seed set. (E,F) Average seed set determined from siliques with non-zero seed set irrespective of silique position for self-pollinated WT and cepr1 in the (E) No-0 background (n= 6 plants, 7-14 siliques per plant) and (F) Col-0 background (n= 5-6 plants, 2-15 siliques per plant). (G,H) Frequency of aborted seeds observed at fertilised ovule positions for self-pollinated WT and cepr1 in the (G) No-0 background (n= 6 plants, 7-14 siliques per plant) and (H) Col-0 background (n= 5-6 plants, 2-15 siliques per plant). Significant differences in E-H determined by a two-sample t-test; *p<0.05, **p<0.01, ***p<0.001. Error bars show SE. (I) Percentage seed set for controlled pollination between Col-0 and cepr1-3 plants. Preanthesis flowers were emasculated prior to deposition of donor pollen onto recipient pistils. Significant differences determined by ANOVA followed by Tukey HSD test (alpha=0.05), n=4-6 siliques from 3 plants. Error bars show SE. (J,K) Fertilisation frequency at ovule positions for self-pollinated WT and cepr1 in the (J) No-0 background (n= 6 plants, 7-14 siliques per plant) and (K) Col-0 background (n= 5-6 plants, 2-15 siliques per plant). Each position is represented as a fraction of the highest position observed in at least 3 plants per genotype (0 = stigma end; 1 = gynophore end). Fertilisation frequency was determined from siliques with non-zero seed set.

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Figure 4. *CEPR1* determines the extent of seed filling. The resource availability per siliques was increased by thinning the number of reproductive sinks. (A) Images of No-0 (WT) and *cepr1-1* before (left) and after (right) thinning. Three to four open flowers or early stage siliques were left at the apex of the main stem. (B) Relative seed area for thinned and untreated plants. ANOVA followed by Tukey HSD test (alpha = 0.05), n=3 plants, 100-200 seeds per plant. Error bars show SE.
Figure 5. Seed size depends upon CEPR1 activity in the bolt. Young No-0 (WT) and cepr1-1 bolts were excised approximately 2 cm from the base and grafted to a recipient stock, which provided vegetative rosette and root tissues to the transplanted reproductive tissue. Secondary branches that formed from the stock post grafting were continuously removed so that the final reproductive organs were derived from the donor bolt only. The bolt and stock genotypes are labelled above and below the horizontal line, respectively. (A,B) Reciprocal grafting of WT and cepr1-1 plants. Representative images of plants (A) 0 and (B) 26 days after grafting (bolt/stock genotype). Arrow heads in A indicate the graft junction. Scale bars = 5 cm. (C) Representative images of inflorescences from hetero-grafted plants. Note the accumulation of anthocyanin in the siliques of cepr1-1 bolt-grafts. (D) Quantification of single seed mass for grafted plants. Statistically significant differences determined by ANOVA followed by Tukey HSD test (alpha = 0.05), n = 6-9. (E) Distribution of seed size. n = 6-9 plants, 100-200 seeds per plant. Error bars show SE.
Figure 6. Yield is primarily determined by CEPR1 activity in the reproductive bolt. (A-C) Analysis of yield on a per plant basis for reciprocal WT and cepr1-1 bolt grafted plants. Quantification of (A) total seed yield, (B) bolt dry mass (minus seed), and (C) bolt harvest index (the ratio of seed yield to total bolt biomass). Significant differences determined by ANOVA followed by Tukey HSD test (alpha=0.05). Error bars show SE. n = 6-9 plants.
**Figure 7. CEPR1 expresses in the vasculature of reproductive organs.** \( pCEPR1:GUS \) expression in the reproductive organs. (A) whole inflorescence, (B) buds, (C) stage 14 flower, (D) stage 15 gynoecium and stamens, (E) stage 12 gynoecium, (F) stage 14 gynoecium (s.v = style vasculature, m.v = medial vasculature, l.v = lateral vasculature), (G) mature silique, (H) funiculus and chalazal seed coat (CZSC = chalazal seed coat, fun. = funiculus), and (I) mature silique funiculus. Scale bars = 1 mm (A-C,G) or 0.1 mm (B,H,I). Staining carried out for 18 h (A,D,G,I), 24 h (H) or 72 h (B,C,E,F). (J) Microarray expression of CEPR1 in the developing seed from the Arabidopsis eFP browser: https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Belmonte et al., (2013).
Figure 8. Complementation of cep1 mutants using a CEPR1 genomic fragment. Reproductive development phenotypes were assessed for independent complementation (comp) lines in the No-0 cep1-1 (#1 and #2) and Col-0 cep1-3 (#3 and #4) backgrounds compared to their respective cep1 mutant and WT lines. (A, B) Representative images showing a side view of the inflorescence (upper panels) and dissected post-anthesis flowers (lower panels) for lines in the (A) No-0, and (B) Col-0 ecotypes. (C, G) Quantification of ovule number for siliques with non-zero fecundity for (C) No-0 (n=4-10, 2 siliques per plant), and (G) Col-0 ecotype lines (n=3-11, 1-4 siliques per plant). (D, H) Measurement of seed set for (D) No-0 (n=4-10) and (H) Col-0 ecotype lines (n=3-11). Two siliques were examined per plant from positions on the stem corresponding to the phase of WT maximal seed set (see Fig. 3C,D). (E, I) Frequency of aborted seeds observed at fertilised ovule positions for lines in the (E) No-0 (n=4-10, 2 siliques per plant), and (I) Col-0 ecotypes (n=3-11, 1-4 siliques per plant). (F, J) Seed area for (F) No-0 (n=6-11 plants) and (J) Col-0 ecotype lines (n=3-9 plants) determined from 60-120 seeds per plant. Significant differences determined by ANOVA followed by Fisher’s Least Significant Difference test (alpha = 0.05).
Figure 9. Loss of CEPR1 function affects the expression of genes involved in nitrogen homeostasis in bolt tissue. For RT-qPCR analyses, the primary inflorescence tissue was harvested from WT and cepr1 plants upon first flower opening. The fold change in expression (log2) was determined for cepr1-1 and cepr1-3 relative to WT (N0-0 and Col-0, respectively) for a selection of (A) CEP-CEPR1 pathway genes, (B) genes related to nitrogen (N) homeostasis, and (C) those involved in response to low nitrogen. Statistically significant differences were determined by a two-sample t-test: p < 0.05, ** p < 0.01, *** p < 0.001. Error bars show 1SE, n=3 biological replicates each consisting of two pooled plants. n.d.; not detected.
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