Regional specification of stomatal production by the putative ligand CHALLAH

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SUMMARY
The problem of modulating cell fate programs to create distinct patterns and distributions of specialized cell types in different tissues is common to complex multicellular organisms. Here, we describe the previously uncharacterized CHALLAH (CHAL) gene, which acts as a tissue-specific regulator of epidermal pattern in Arabidopsis thaliana. Arabidopsis plants produce stomata, the cellular valves required for gas exchange, in virtually all aerial organs, but stomatal density and distribution differ among organs and along organ axes. Such regional regulation is particularly evident in plants mutant for the putative receptor TOO MANY MOUTHS (TMM), which produce excess stomata in leaves but no stomata in stems. Mutations in CHAL suppress tmm phenotypes in a tissue-specific manner, restoring stomatal production in stems while minimally affecting leaves. CHAL is similar in sequence to the putative stomatal ligands EPF1 and EPF2 and, like the EPFs, can reduce or eliminate stomatal production when overexpressed. However, CHAL and the EPFs have different relationships to TMM and the ERECTA (ER) family receptors. We propose a model in which CHAL and the EPFs both act through ER family receptors to repress stomatal production, but are subject to opposite regulation by TMM. The existence of two such ligand classes provides an explanation for TMM dual functionality and tissue-specific phenotypes.

KEY WORDS: ERECTA, TOO MANY MOUTHS, Cell-cell communication, Ligand-receptor interaction, Stomata, Arabidopsis

INTRODUCTION
To generate and maintain a complex body plan, multicellular organisms must selectively regulate cell fate programs to generate distinct classes, patterns and distributions of cell types in different tissues. Although regional regulation is apparent in the broad brushstrokes of patterning – in the definition of major body axes and establishment of organ domains – it also manifests itself in more subtle cellular patterning events. Regional regulatory cues can influence the production and progression of specialized cell lineages, modulating how many cells enter the lineage, how frequently they divide, and what fate they ultimately adopt. In some instances, regional information simply alters the frequency with which a particular cell type is produced. For example, in Arabidopsis thaliana numerous leaf hair cells are found on the adaxial (top) surface of rosette leaves, but few or no such cells are generated on the abaxial (bottom) surface (reviewed by Hulskamp and Schnittger, 1998). In other cases, regional information alters the division potential of precursors or the ultimate identity of their progeny. For instance, in the arthropod nervous system, segmentally repeated neural precursors give rise to lineages that differ in size, cell type composition and morphology depending on their anterior-posterior position (Prokop et al., 1998; Udolph et al., 1993). Such tissue specificity of cellular behavior implies that there must be regional regulatory factors that can impinge on the core networks that control the production and progression of specialized cell lineages.

In plants, the patterning of the epidermal structures called stomata provides an excellent system in which to study tissue-specific control of specialized cell type behavior. Stomata consist of paired guard cells surrounding a central pore, and these guard cells contract and relax via changes in turgor pressure to modulate gas exchange. In Arabidopsis thaliana, stomata represent the terminal product of a series of stereotyped, yet flexible, asymmetric divisions (Fig. 1A). This relatively sophisticated developmental program provides numerous control points at which regional information can influence stomatal pattern. Initially, naïve epidermal cells known as meristemoid mother cells (MMCs) undergo asymmetric entry divisions to produce a small, triangular meristemoid and a larger sister cell. The meristemoid then undergoes one to three rounds of amplifying divisions, regenerating itself and producing a larger stomatal lineage ground cell (SLGC) each time, much like a mammalian stem cell (Bergmann and Sack, 2007). Eventually, the meristemoid differentiates into a guard mother cell (GMC), which undergoes a symmetric division to produce the paired guard cells of the stoma. Importantly, cells adjacent to an existing stoma or precursor undergo specialized entry divisions known as spacing divisions, which are oriented such that the new precursor forms away from the existing stomatal cell. Spacing divisions prevent stomata from forming in contact and are the primary basis of the ‘one-cell spacing rule’ of stomatal pattern (Geisler et al., 2000). Although this rule is obeyed in all tissues that produce stomata, other aspects of stomatal lineage progression, including the number of entry divisions, the duration of meristemoid self-renewal in the amplifying phase and the incidence of spacing divisions, are more plastic and differ among organs and along organ axes (Bhave et al., 2009; Geisler et al., 1998).

A number of genes encoding elements of classical signal transduction systems have been implicated in the establishment of Arabidopsis stomatal pattern, including plasma membrane receptors, putative ligands and cytoplasmic kinases. Key stomatal receptors include the leucine-rich repeat receptor-like protein TOO MANY MOUTHS (TMM) and the ERECTA (ER) family of leucine-rich repeat receptor-like kinases (Nadeau and Sack, 2002; Shpak et al., 2005). The ER family includes three genes of overlapping function, ERECTA (ER), ERECTA-LIKE1 (ERL1) and

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Accepted 22 November 2009
ERECTA-LIKE2 (ERL2), and triple mutants produce dense guard cell clusters in all organs that generate stomata (Shpak et al., 2005). Mutations in TOO MANY MOUTHS, although best known for their eponymous production of excess clustered stomata in rosette leaves, confer a suite of other regional phenotypes that highlight tissue-specific rules for stomatal patterning. Most striking among these phenotypes is the elimination of stomata from stems (Bhave et al., 2009; Geisler et al., 1998).

Although upstream components of the TMM and ER pathways remain largely unknown, the peptides EPIDERMAL PATTERNING FACTOR1 (EPF1) and EPF2 are required for correct stomatal patterning and might act as ligands for these receptors (Hara et al., 2009; Hara et al., 2007; Hunt and Gray, 2009). Similarly, although the cytoplasmic targets of TMM and the ER family have not been identified, several mitogen-activated protein kinase (MAP kinase) signaling components, including the MAPKKK YODA (YDA), are required for correct stomatal patterning and display genetic interactions consistent with a function downstream of these receptors (Bergmann et al., 2004; Wang et al., 2007). Unlike tmm, mutations in these other stomatal regulators confer patterning defects that are relatively constant across organs and regions.

To identify tissue-specific regulators of the stomatal development program, we screened for modifiers of tmm-1 tissue-specific phenotypes and identified CHALLAH (CHAL) as a recessive suppressor of stomatal elimination from stems. Here, we show that CHAL represses stomatal production in a subset of tissues and that its expression defines organ regions rather than marking the stomatal lineage. Both the protein sequence and behavior of CHAL suggest that it might act as a ligand for ER family receptors, and, indeed, CHAL is related to putative ligands EPF1 and EPF2. The functional differences between CHAL and the EPFs suggest that these factors belong to a single ligand superfamily that has diversified to include both universal and regional regulators of stomatal development.

MATERIALS AND METHODS

Screen- and map-based cloning of chal-1

Columbia (Col) plants homozygous for tmm-1 and for guard cell-expressed GFP enhancer trap E1728 (Gardner et al., 2008) were mutagenized with ethylmethane sulfonate (EMS). M2 seedlings were screened for enhancement or suppression of tmm-1 stomatal phenotypes. A chal-1 mapping population was generated by outcrossing the original mutant to tmm-3 (C24 ecotype), and a PCR-based approach was used to trace chal-1 to a 106 kb region spanning BACs T9D9 and T6B20 (flanked by CER461306 and CER460870, http://www.arabidopsis.org/browser/Cereon/index.jsp). Genes in this region were sequenced and a C-to-T point mutation was found at position 364 in DQ446581 (numbering derived from cDNA clone DQ446581), which is predicted to confer an amino acid substitution of P122S.

Phenotypic characterization

For stomatal counts, tissues were collected from healthy, growing plants, cleared and stored in 70% ethanol, then rehydrated and mounted in Hoyer’s medium. Statistical analysis was performed using R statistic software (http://www.R-project.org). Because meristic (count) data are typically non-normal (Sokal and Rohlf, 1995), the Wilcoxon two-sample test, a nonparametric analog of the t-test, was used for analysis. All comparisons were performed as isolated two-sample tests without adjustment of α for multiple comparisons. E1728::GFP, N7::GFP (Cutler et al., 2000) and 3SS::YFP-CHAL, as well as select overexpression lines, were imaged on a Leica SP5 confocal microscope with propidium iodide counterstaining to visualize cell outlines.

Expression analyses

To assay β-glucuronidase (GUS) expression in CHALpro::GUS lines and enhancer trap line CS100155, tissues (except ovules) were permeabilized in acetone at -20°C and washed twice in PBS, then incubated in GUS staining solution as described (Sessions et al., 1999) with modifications. Following staining, tissues were either fixed in 3:1 ethanol/acetic acid and stored in 70% ethanol or cleared directly in 70% ethanol. For cellular-level analyses, tissue samples were rehydrated and mounted in Hoyer’s medium. Reported expression patterns were observed in at least four independent lines unless otherwise noted. For analyses of mRNA expression level in Col, chal-2, and 3SS::CHAL, RNA was isolated from seedlings using the RNeasy Plant Mini Kit (Qiagen) with on-column DNase digestion. Total RNA was subjected to additional DNase I (Invitrogen) treatment if necessary and used in a SuperScript III first-strand synthesis reaction with oligo(dT) primers (Invitrogen). In 3SS::CHAL analysis, fast genomic contamination was not wholly eliminated, but was comparable among samples. Gene-specific transcripts were amplified from the resulting cDNA using Accuprime PfX DNA polymerase (Invitrogen). CHAL was amplified for 35 cycles (chal-2 analysis) or 30 cycles (3SS::CHAL analysis) with annealing at 54°C. Actin (ACTIN1) was amplified for 35 cycles with annealing at 54°C [primers were either as described previously (Ohashi-Ito and Bergmann, 2006) or primers were based on published sequences (Ohashi-Ito and Bergmann, 2006, 2008)]

DNA manipulations

All constructs were generated using the Gateway Cloning System (Invitrogen). To determine the expression pattern of CHAL, a 2.9 kb region of the translational start site was amplified with Accuprime PfX using primers 30370pro-ea1F (5’-CAGAGTCGAGCACAATACCG-3’), 30370pro-ea1R (5’ACCGCGGCAGCTTACCGCTGACGACCC-3’) and 30370pro-ea1R (5’-CGCCCGCTTCGTAACACGTGAGCGGC-3’). The amplicon was cloned into pENTR (Invitrogen), then transferred into pBGGUS (Kubo et al., 2005) to generate CHALpro::GUS. For overexpression, CHAL cDNA clone DQ446581 [Arabidopsis Biological Resource Center (ABRC)] was inserted downstream of the 35S promoter in pH35GS (Kubo et al., 2005) to generate 35S::CHAL. A mock artificial microRNA, amiR-mock, cloned into pH35GS was used as a negative control for overexpression experiments. For overexpression of the chal-1 cDNA, the chal-1 mutation was introduced into DQ446581 using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene), and the resulting sequence was cloned into pH35GS to generate 35S::CHALpro::amiR. For overexpression of N-terminal YFP-tagged CHAL, the DQ446581 cDNA was cloned into pH35YG (Kubo et al., 2005). Constructs were transformed into plants via Agrobacterium-mediated floral dip (Clough and Bent, 1998), and T1s were selected on soil using Finale (1:2000) or on agar plates supplemented with hygromycin (50 µg/ml) as appropriate.

Plant material and growth conditions

Columbia (Col) ecotype Arabidopsis and E1728 (Col) were treated interchangeably as wild type in all experiments. chal-2 (SALK_072522) was obtained from the ABRC. tmm-1 has been described previously (Nadeau and Sack, 2002). tmm;er;erl1, tmm;er;erl2, tmm;erl1;erl2, tmm;erl1 and er;erl1/+;erl2 (Shpak et al., 2005) were gifts of Dr Keiko Torii. As some lines were in a gl1 (glabrous) background, stomatal phenotypes were scored quantitatively only in tissues lacking trichomes. Multiple mutants were generated by crossing and were identified based on stomatal phenotypes, whole-plant phenotypes (er), sequencing and PCR genotyping. Genotyping primers included Mr_30370-ea1F (5’-TACCGTGGAAGTGTCCTGACGACCCC-3’) and Mr_30370-ea1R (5’-CTCCAAGTGGAAGATGCTC-3’), which were designed to amplify a derived chlorophyll a/b-binding protein sequence (dCAP) when digested with TasI, or digestion of the Arabidopsis gene [Materials and Methods].
Regional stomatal ligand CHALLAH

RESULTS

Isolation and phenotypic analysis of the chal-1 mutation

The challah-1 (chal-1) mutation was recovered in a suppressor screen performed in a tmm-1 background. Although initially identified for their production of hypocotyl stomata, tmm;chal-1 plants also produce small groups of hypocotyl cells in an arrangement that resembles braided ‘challah’ bread (Fig. 1B,C,G). During characterization and cloning, chal-1 behaved as a recessive allele with incomplete penetrance: we observed hypocotyl stomata in less than a quarter of progeny from tmm;chal-1/+ populations and in approximately two-thirds of tmm;chal-1 homozygotes \( n=23/32 \) at 12 days post-germination (dpg). ‘Challah’ clusters of small cells were typically present in homozygous plants that failed to differentiate stomata. As the hypocotyl grows, these cells elongate and may take on a morphology reminiscent of the excess SLGCs observed in \( er \) mutants (Fig. 1D) (Shpak et al., 2005). Similar cells are produced in tmm hypocotyls, although in smaller numbers and unaccompanied by stomata, and have been shown to represent arrested, dedifferentiated meristemoids (Bhave et al., 2009).

We next investigated whether the chal-1 mutation affected tmm phenotypes in organs other than the hypocotyl. In the seedling, chal-1 did not significantly alter stomatal density in the adaxial cotyledon (although a subsequently identified stronger allele, chal-2, had a slight effect in this organ) (Fig. 1G and see Fig. S1G in the supplementary material; see below). In the inflorescence stem, by contrast, chal-1 increased stomatal density more than 20-fold from near-zero starting levels, much as it did in the hypocotyl (Fig. 1E-G). The chal-1 mutation thus appears to derepress stomatal production in a specific subset of tissues, conferring little or no effect in other regions.

**CHAL encodes a putative ligand**

Using a PCR-based positional cloning approach (see Materials and methods), we found that chal-1 was a missense mutation in At2g30370, an uncharacterized gene predicted to encode a small, allergen-like protein (TAIR, http://www.arabidopsis.org). At2g30370 will subsequently be referred to as CHAL. Although the annotation indicates a gene structure encoding four exons and yielding a 593 bp mRNA coding sequence, we did not detect this splice variant in seedling tissues. Instead, we observed a transcript consistent in size with a 471 bp cDNA sequence deposited in GenBank (DQ446581), which corresponds to a gene structure with three exons and a large second intron (Fig. 2A). This mRNA encodes a 156 amino acid protein with a series of hydrophobic residues near the N-terminus that is predicted to act as a transmembrane domain or signal peptide (SignaLP 3.0, http://www.cbs.dtu.dk/services/SignaLP/; Aramemnon, http://aramemnon.botanik.uni-koeln.de/).

As CHAL contains no known functional domains, we performed BLAST searches to identify related molecules. Putative orthologs of CHAL are found in diverse plant species, including grape, rice, poplar and Physcomitrella patens (GI:157336978, GI:57900264, GI:169118872, GI:168054544), and the gene family appears to be plant specific. In the Arabidopsis genome, eleven loci are predicted to encode small proteins homologous to CHAL (Fig. 2B,C).

Intriguingly, these homologous genes include EPF1 and EPF2, which act as negative regulators of stomatal production and have been proposed to encode ligands for TMM and/or ER family receptors (Fig. 2B,C) (Hara et al., 2009; Hara et al., 2007; Hunt and Gray, 2009). CHAL and its relatives display sequence similarity...
primarily in a C-terminal region that is characterized by six cysteine residues with conserved spacing (Fig. 2C). In the \textit{chal-1} mutation, the proline adjacent to the fourth cysteine, which is common to CHAL and the EPFs and is generally conserved among CHAL homologs (9/11), is converted to a serine (P122S; Fig. 2A,C, arrow).

The replacement of proline, a sterically constrained amino acid that generates turns and kinks, with serine, a more labile and less specialized residue, would tend to cause disruptions in secondary structure and might prevent or perturb the formation of disulfide bonds. Given the molecular nature of CHAL, we hypothesized that this allele might encode either a defective protein or a protein with novel functions, perhaps similar to the stabilizing proline-to-serine alleles of IAA proteins (e.g. \textit{bodenlos-1}) (Hamann et al., 2002; Nagpal et al., 2000; Tian and Reed, 1999). To confirm that the lesion identified in \textit{chal-1} mutants was responsible for the \textit{chal} phenotype and to determine whether \textit{chal-1} was a loss-of-function allele, we obtained and characterized a line with a T-DNA insertion in the first intron of \textit{CHAL} locus (\textit{chal-2}, SALK_072522). In RT-PCR analysis, no wild-type full-length \textit{CHAL} transcript could be detected in \textit{chal-2} homozygous plants (Fig. 2D). When introgressed into a \textit{tmn;chal-1} background, this mutation appeared to be allelic to \textit{chal-1}: 17/21 F2 plants produced hypocotyl stomata in a \textit{tmn} background, including both \textit{chal-1/chal-2} and \textit{chal-2} homozygous individuals. The two alleles displayed qualitatively similar behavior in a \textit{tmn} background, restoring stomata to hypocotyls and generating clusters of small cells, but \textit{chal-2} restored stomata at higher penetrance \((n=24/25)\) and in larger numbers (Fig. 2D,F). These results indicate that \textit{chal-1} is a reduction-of-function allele, largely abolishing CHAL activity rather than producing a protein with novel activities. This conclusion was confirmed by overexpression analysis of the defective \textit{chal-1} cDNA (see below). Notably, the phenotypes associated with loss of CHAL function are dependent on the absence of functional TMM. In a wild-type background, \textit{chal} does not appear to alter stomatal density in the hypocotyl or cotyledons, nor does it confer production of ‘challah’ clusters of small cells (see Fig. S1A-F in the supplementary material).

\textbf{The CHAL expression pattern defines regions rather than stomatal cell types}

Because \textit{chal} mutations primarily affect the hypocotyl and inflorescence stem, we hypothesized that \textit{CHAL} expression might be restricted to, or elevated in, these organs. Furthermore, we anticipated that \textit{CHAL}, like \textit{EPF1} and \textit{EPF2}, might be expressed specifically in the developing stomatal lineage (Hara et al., 2009; Hara et al., 2007; Hunt and Gray, 2009). To visualize \textit{CHAL} expression, we generated a transcriptional reporter comprising 2.9 kb of \textit{CHAL} upstream sequence fused to the \(\beta\)-glucuronidase gene (\textit{CHALpro::GUS}) and assessed its activity in seedlings and the developing inflorescence. To confirm that the activity of \textit{CHALpro::GUS} accurately reflected expression of the \textit{CHAL} gene, we also obtained and characterized a \textit{GUS} enhancer trap line (CS100155) bearing an insertion in the first exon of \textit{CHAL} (see Fig. S2I-M in the supplementary material).

We found that \textit{CHAL} expression is regionally specific, consistent with observed \textit{chal} phenotypes, but that it is not specific to the stomatal lineage or epidermis. At 36 hours post-germination (hpg), before hypocotyl stomata are present, we observed moderate but distinct expression of the \textit{CHALpro::GUS} reporter in the internal layers of the root and hypocotyl, with staining typically stronger in the basal hypocotyl region (Fig. 3A). At a cellular level, signal was strongest in a ring of cells surrounding the vascular elements and was sometimes observed in a subset of cells above the root meristem (see Fig. S2A,B in the supplementary material). At 3 dpg, when both stomata and precursors are present in the hypocotyl, \textit{CHALpro::GUS} activity continued in the internal tissues, with a maximum at the top of the hypocotyl (Fig. 3B). Expression of \textit{CHALpro::GUS} was not typically observed in cotyledons at either 36 hpg or 3 dpg (Fig. 3A,B), but strong staining was detected in the midrib of developing rosette leaves in older (14 dpg) plants (Fig. 3D and see Fig. S2C,D in the supplementary material). Interestingly, we did not observe elevated expression of \textit{CHALpro::GUS} in stomatal precursors or stomata at any stage, even in plants with strong subepidermal \textit{GUS} signal (Fig. 3C).
In the inflorescence, CHALpro::GUS displayed a more complex pattern of activity, a predominant feature of which was strong expression in young stem tissue. We observed particularly high activity in immature axillary stems (Fig. 3E) and in young tissue near the apex of more mature stems (e.g. Fig. 3F, bracket). As in the hypocotyl, CHALpro::GUS was expressed strongly in the internal tissues of the inflorescence stem, with expression strongest in (but not exclusive to) a ring of cells subtending the cortex and encircling the vascular core (see Fig. S2E-G in the supplementary material). Expression was concurrent with stomatal production, and a faint epidermal signal was detectable in the strongest lines, but we did not observe specific GUS signal in either precursors or guard cells during stomatal lineage progression (see Fig. S2H in the supplementary material). Strong staining was also present in a ring of cells at the base of the developing siliques, at the point of floral organ attachment (Fig. 3F, arrow), and in the chalazal endosperm of developing seeds (Fig. 3G). Although no expression was observed in early embryos, essentially mature embryos (as found in ovules with browning seed coats) showed clear GUS activity in the hypocotyl and root, with the signal highest in, but not exclusive to, the internal tissues (Fig. 3H; faint expression is observed in the hypocotyl epidermis and in the cotyledons of the strongest line).

The CS100155 enhancer trap line recapitulated the primary features of the CHALpro::GUS expression pattern, displaying strong, consistent activity in the embryonic hypocotyl (see Fig. S2L in the supplementary material) and in growing stems (see Fig. S2J,K,M in the supplementary material). Interestingly, signal was observed more broadly in cotyledons, leaves and floral organs of CS100155 (see Fig. S2I-L in the supplementary material), although strong stem activity remained the most striking feature of the expression pattern. The elevated transcriptional activity in young hypocotyls and stems observed in both CHAL reporter lines is consistent with the stomatal phenotypes observed in chal mutants.

**Ubiquitous overexpression of CHAL can inhibit stomatal production**

CHAL is expressed in a relatively limited domain, and its domain of expression correlates with its loss-of-function stomatal lineage phenotypes. We thus hypothesized that spatially constrained expression of CHAL, rather than limited availability of a receptor or modifier, might underlie its regionally specific effects. To assess whether widespread expression of CHAL could confer ubiquitous stomatal phenotypes, we generated stable transgenic lines expressing CHAL cDNA under control of the constitutive 35S promoter (35S::CHAL). Consistent with a failure to identify CHAL in a previous large-scale screen for stomatal-regulatory ligands (Hara et al., 2009), most 35S::CHAL T1 plants did not display obvious stomatal phenotypes, and stomatal density in T1s did not consistently differ overall from that in controls (Fig. 5A). In analyzing larger numbers of T1s, however, we repeatedly observed a fraction of plants that displayed distinct reductions in stomatal number (n=8; Fig. 4B,C). Moderately affected individuals produced some normal stomata in the hypocotyl and cotyledons but also displayed arrested stomatal lineage cells or small pavement cells suggestive of stomatal

![Fig. 3. CHAL expression pattern. (A-D) CHALpro::GUS expression in Arabidopsis seedlings. (A,B) GUS activity in 36 hpg (A) and 3 dpg (B) hypocotyls. Bracket indicates apical staining. (C) Magnified image of 3 dpg hypocotyl, showing lack of expression in the stomatal lineage. Asterisks, stoma; arrow, meristemoid. (D) GUS activity in midrib of developing leaves (14 dpf). (E-G) CHALpro::GUS expression in the inflorescence. (E,F) GUS activity in immature axillaries (E) and near the apex of mature stems (F, bracket). Arrowhead, base of siliques. (G) Ovule with chalazal staining (arrowhead). (H) GUS activity in the late embryonic hypocotyl and root (bracket). Scale bars: 100 μm in A,B,D; 50 μm in C.

![Fig. 4. Overexpression of CHAL. (A-C) Adaxial cotyledon of wild-type (A), moderate 35S::CHAL (B), and strong 35S::CHAL (C) T2 Arabidopsis plants (8 dpf, PI stained). Arrows indicate probable transdifferentiated stomatal lineage cells. (D,E) Inverse correlation between CHAL expression level (D) and adaxial cotyledon stomatal density (E) in 35S::CHAL plants. Counting and RT-PCR were performed on bulk 12 dpf T3 plants selected for phenotype (see text). Counts are per 0.250 mm² field. ***, P<0.001 as compared with wild type, Wilcoxon two-sample test. Error bars indicate s.e.m. Scale bar: 50 μm.
lineage transdifferentiation (Fig. 4B, arrows), and some also produced paired stomata at elevated frequency (see Fig. S3A in the supplementary material). Severely affected plants produced few or no stomata, with the most dramatic individuals resembling speechless (spch) mutants, although a few entry divisions were typically observed (Fig. 4C) (MacAlister et al., 2007). Although these phenotypes were scored primarily in T1 seedlings, similar effects were apparent in mature tissues (see Fig. S3B in the supplementary material) and in the T2 and T3 generations.

Reduced stomatal production in 35S::CHAL plants is consistent with the tmm;chal phenotype, which indicates an endogenous role for CHAL as a stomatal inhibitor. To correlate stomatal phenotypes with construct expression level, we screened T3 seedlings from multiple lines and collected individuals that fell into either of two phenotypic categories: moderate (arrested cells and at least ten stomata in the adaxial cotyledon) and severe (fewer than five stomata in the adaxial cotyledon). Bulked individuals from each category were split into two groups, one of which was used for quantification and the other for RT-PCR analysis. As expected, both overexpression lines produced more CHAL mRNA than wild-type plants, and CHAL expression was negatively correlated with cotyledon stomatal production (Fig. 4D,E). To confirm that high levels of CHAL protein conferred stomatal underproduction phenotypes, we also generated transformants overexpressing a YFP-tagged CHAL variant (35S::YFP-CHAL). T2 transformants with bright fluorescence (n=4 independent lines) frequently showed phenotypes similar to those of 35S::CHAL plants, including transdifferentiated or arrested stomatal lineage cells (see Fig. S3C in the supplementary material). These results confirm that high levels of CHAL protein confer reduced stomatal production.

**chal-1 is a reduction-of-function allele**

Taking advantage of the stomatal reduction phenotypes conferred by CHAL overexpression, we analyzed the chal-1 missense allele and the functionality of its protein product in greater detail. If chal-1 were a reduction-of-function allele, its overexpression would be likely to confer a weak phenotype resembling that of 35S::CHAL. If it were a gain-of-function allele, overexpression would be expected to generate a chal-like phenotype, perhaps restoring stomata to hypocotyls or ubiquitously increasing stomatal production in *tmm*. To distinguish between these possibilities, we overexpressed the chal-1 mutant cDNA under control of the 35S promoter (35S::CHAL<sup>Pro</sup><sup>-Ser</sup>) in a *tmm* background. T1 transformants displayed stomatal underproduction phenotypes resembling those of 35S::CHAL in *tmm* (see Fig. S3E in the supplementary material; see below), with some plants failing to produce stomata in the cotyledons (n=4) and others producing markedly reduced numbers (n=4). 35S::CHAL<sup>Pro</sup><sup>-Ser</sup> phenotypes were less severe than those conferred by wild-type CHAL overexpression in a *tmm* background, indicating that the chal-1 protein product retains some activity but acts less efficiently than wild-type CHAL.

**CHAL and EPF1/2 overexpression phenotypes diverge in tmm**

The molecular features of CHAL, together with its ability to confer overexpression phenotypes in diverse tissues, suggest that it, like EPF1/2, might serve as a ligand for a broadly expressed stomatal receptor. CHAL signaling, therefore, might feed into known stomatal-inhibitory pathways, perhaps acting through TMM or ER family receptors. The identification of chal as a genetic suppressor of *tmm*, however, argues against it being a ligand for TMM itself. To test the relationships between CHAL and its potential receptors, we first overexpressed CHAL in *tmm*, a background that blocks both EPF1 and EPF2 overexpression phenotypes (Hara et al., 2009; Hara et al., 2007). In striking contrast to the EPF1/2 results, the CHAL overexpression phenotype was dramatically enhanced in *tmm*, such that virtually all T1 transformants failed to produce stomata and resembled spch mutants (Fig. 5B,D). This finding is consistent with our hypothesis that CHAL is unlikely to be a TMM ligand.

**Loss of ER family receptors mitigates CHAL overexpression phenotypes**

We then examined whether one or more of the ER family receptors might mediate CHAL activity. For this assay, we used the unequivocal overexpression phenotype observed in *tmm* as a baseline and assayed the mitigating effects of *ER* family mutations, overexpressing CHAL in *tmm* plants bearing *ER* family double-mutant combinations (*tmm;er;erl1* and *tmm;erl1;erl2*). Substantial suppression of the 35S::CHAL phenotype was observed in all three backgrounds, such that more than 20% of transformants in each background produced one or more stomata in the cotyledon.

**Fig. 5. CHAL overexpression in receptor mutant backgrounds.**

(A, B) Quantification of 35S::CHAL T1 phenotypes (axial cotyledon, 10 dpg) in Col (A) and *tmm*-1 (B) Arabidopsis plants. The median is marked as a bold horizontal line, and upper and lower quartiles are indicated by the top and bottom of the box, respectively. Dashed lines denote the largest and smallest observations within 1.5 interquartile ranges of the box. Circles indicate outliers. Counts are number of stomata per 0.250 mm<sup>2</sup> field. ***, P<0.001 by Wilcoxon two-sample test. (C) Phenotypic categorization of 35S::CHAL T1s in multiple mutant backgrounds, showing mitigated phenotypes from ER family mutations but not from *sdd1*. All plants were scored at 10 dpg. (D-H) Representative images of 35S::CHAL in *tmm*-1 (D), *tmm;erl1* (E), *tmm;erl2* (F), *tmm;erl1;erl2* (G) and *tmm;sd1* (H). Images depict PI-stained 8-9 dpg abaxial cotyledons. Scale bar: 50 μm.
In *tmm;er;erl1* and *tmm;er;erl2* backgrounds, stomatal differentiation was not extensively rescued (typically, stomata were not produced or were produced only in the hydathode; Fig. 5C), but asymmetric divisions were observed in the cotyledons of transformants at high frequency (Fig. 5E,F). In *tmm;erl1;erl2*, stomatal differentiation was more broadly restored, with more than 90% of transformants producing a stoma and more than 20% displaying stomata in the peripheral or central cotyledon (Fig. 5C,G). Importantly, although suppression of 35S:*CHAL* phenotypes was observed in each *ER* family double-mutant background, the majority of transformants in all backgrounds still produced stomata at greatly reduced density, suggesting that any individual *ER* family receptor is sufficient to transduce *CHAL* activity.

Using a similar approach, we examined the relationship between *CHAL* and the protease *STOMATAL DENSITY AND DISTRIBUTION1* (*SDD1*) (Berger and Altmann, 2000). As with the *ER* family receptors, *SDD1* prevents stomatal overproduction and clustering, but is believed to act independently of the *TMM*/*ER* family pathway (Hara et al., 2007; Lampard et al., 2008). When the *CHAL* overexpression assay was repeated in a *tmm;sdd1* background, almost all transformants failed to produce stomata in the cotyledon (44/45 T1s), much as in a *tmm* background (Fig. 5C,H). Although asymmetric divisions were observed in many T1s, such divisions were relatively uncommon (as compared with *tmm;er;erl1* or *tmm;er;erl2* T1s) and only rarely gave rise to small, precursor-like cells. These observations indicate that *CHAL* is unlikely to be processed by *SDD1*, but, equally importantly, they suggest that suppression of 35S:*CHAL* phenotypes by *ER* family mutations might be specific to this family (rather than a non-specific, additive effect conferred by the loss of any stomatal inhibitor). Together, the overexpression assays indicate that *TMM* acts to restrict the stomatal-inhibitory effects of *CHAL*, and that *ER* family receptors are likely to mediate these inhibitory effects.

### *chal* phenotypes in *ER* family mutant backgrounds

Because the results of our overexpression assays suggested that *ER* family receptors might mediate *CHAL* activity, we generated multiple mutant combinations to assess the genetic relationships between *CHAL* and *ER* family members. As the *chal* phenotype is detectable only in a *tmm* background, we generated triple mutants bearing *tmm;chal-1*, and an additional *er* or *erl2* mutation and assessed the effect of each on hypocotyl stomatal production. The two mutations had distinct effects on the *chal-1* phenotype, perhaps reflecting ligand-receptor specificity or complex combinatorial interactions among *ER* family receptors. An *erl2* mutation moderately, but significantly, enhanced hypocotyl stomatal production relative to *tmm;chal-1* (Fig. 6D,F). An *er* mutation, on the contrary, did not enhance *chal-1* restoration of stomata to hypocotyls, but instead suppressed this phenotype, such that *tmm;er;chal-1* plants seldom produced hypocotyl stomata (n=2/29; Fig. 6E,F). A similar epistatic relationship was recently reported between *er* and *epf2* in control of stomatal density (Hunt and Gray, 2009). Because *erl1*, like *chal*, can restore stomata to *tmm* stems (Shpak et al., 2005), we also compared hypocotyl stomatal production in *tmm;chal-1* and *tmm;erl1* plants. *tmm;erl1* produced more hypocotyl stomata than *tmm;chal-1*, consistent with a role for *CHAL* as one of several partially redundant *ERL1* ligands (Fig. 6C,F).

### DISCUSSION

*CHAL* encodes a small, potentially secreted protein that is homologous to the known stomatal regulators *EPF1* and *EPF2* (Hara et al., 2009; Hara et al., 2007; Hunt and Gray, 2009). Consistent with their shared sequence motifs, both *CHAL* and the *EPFs* regulate stomatal production, and both act as repressors in this process. Nonetheless, the distinct loss-of-function phenotypes and genetic interactions of these factors suggest fundamental differences in their endogenous functions. First, whereas *epf1* and *epf2* confer similar patterning defects in all tissues examined (Hara et al., 2007) (E.B.A., unpublished observations), *chal* confers strong phenotypes only in select organs, notably the hypocotyl and inflorescence stem. Thus, unlike *EPF1* and *EPF2*, which universally regulate specific stages of stomatal development, *CHAL* displays regional specificity. Second, and perhaps more intriguingly, *CHAL* and *EPF1/2* display fundamentally divergent genetic interactions with *TMM*. Whereas a *tmm* mutation is fully epistatic to *epf1* and can block overexpression phenotypes of both *EPF1* and *EPF2* (Hara et al., 2009; Hara et al., 2007), *chal* was isolated as a regional suppressor of *tmm* and its phenotypes are apparent only in a *tmm* background. The relationship between *TMM* and *CHAL*, unlike that between *TMM* and *EPF1/2*, does not suggest a stimulatory ligand-receptor interaction, but rather indicates that *CHAL* must act through one or more receptors other than *TMM*.

The distinct properties of *CHAL* and *EPF1/2* may be ascribed to differences in both expression pattern and protein activity. Specifically, the transcriptional patterns of these factors can explain their respective regional and general effects, whereas differences in biochemical activity appear to underlie their opposite interactions with *TMM*. Unlike *EPF1* and *EPF2*, which are expressed specifically and universally in particular stomatal precursors (Hara et al., 2009; Hara et al., 2007; Hunt and Gray, 2009), *CHAL* does not show elevated expression in the stomatal lineage and is instead expressed in a region-specific manner consistent with its loss-of-function phenotypes. Differences in expression, however, cannot account for the opposite interactions of these factors with *TMM*, as these interactions are observable in constitutive expression assays. *CHAL*, *EPF1* and *EPF2* all reduce stomatal production when overexpressed in a wild-type background, yet their effects diverge in *tmm*:*CHAL* overexpression confers an enhanced phenotype, such that plants almost invariably fail to produce stomata, whereas *EPF1/2* overexpression no longer reduces stomatal number (Hara et
al., 2009; Hara et al., 2007). The opposing genetic interactions of these factors with TMM are thus inherent in their respective coding sequences, pointing to differences in protein activity that might reflect distinct ligand-receptor interactions.

A model for chal suppression of tmm region-specific phenotypes

Jointly, the expression pattern and genetic interactions of CHAL offer a straightforward explanation for one of the more striking regional phenotypes of tmm: the loss of stomata from stems. CHAL not only shows elevated expression in young stem tissue (Fig. 7A), but also displays dramatically enhanced stomatal repressor activity in a tmm background. Thus, elimination of stomata from tmm stems is likely to reflect overactivity of endogenous CHAL due to loss of a TMM-dependent buffering mechanism, with overactivity conferring meristemoid arrest and dedifferentiation (Bhave et al., 2009). This explanation raises the intriguing question of how such a mechanism might function at the molecular level. In previous models of stem stomatal patterning, TMM has been proposed to dampen signaling through ERL1 and other ER family receptors via ligand titration or physical inhibition, such that loss of TMM confers ER family overactivity and eliminates stomatal production (Shpak et al., 2005). These models point to the existence of an ER family ligand, the effects of which, unlike those of EPF1 and EPF2, are suppressed rather than enhanced by TMM.

We propose that CHAL corresponds to this hypothesized ligand, repressing stomatal production through multiple ER family receptors in a pathway that is inhibited by TMM. Several lines of evidence support such a model. First, loss of ER family receptors can suppress CHAL overexpression phenotypes, suggesting that CHAL signaling passes through one or more members of this receptor family. Second, CHAL overexpression can still confer phenotypes in the absence of any two ER family receptors (in tmm;erl1, tmm;erl2 and tmm;erl1;erl2 backgrounds), indicating that each of the three receptors is individually capable of mediating CHAL activity. We thus propose a model in which endogenous CHAL interacts with multiple ER family members (Fig. 7B), such that the chal phenotype reflects reduced activity of multiple receptors. To explain the unexpected epistasis of er to chal-1, we further propose that ER may serve as both a mediator of CHAL activity and a buffer that protects its sister receptors from excess CHAL signal. The CHAL pathway appears to be independent of the protease SDD1, as an sdd1 mutation fails to mitigate CHAL overexpression phenotypes, adding to a growing body of evidence (Hara et al., 2009; Hara et al., 2007; Lampard et al., 2008) that places SDD1 in a pathway separate from TMM and the ER family.

The activity of CHAL and the EPFs alone cannot account for the full spectrum of tmm phenotypes, raising the intriguing possibility that other EPF/CHAL superfamily members might also participate in stomatal patterning. Such additional factors might universally modulate stomatal production, similar to EPF1/2, or provide a layer of regional refinement, similar to CHAL. Further analysis of this superfamily might not only identify new stomatal regulators, but also define the sequence motifs that confer EPF-like and CHAL-like behaviors, laying the groundwork for biochemical and mechanistic studies of the TMM/ER family system.

Acknowledgements

We thank members of our laboratory for helpful comments on the manuscript; the S. R. Long laboratory (Stanford) and Carnegie Institute, Department of Plant Biology, for the use of microscopes; and Dr Keiko Torii (University of Washington) and Dr Julie Gray (University of Sheffield) for discussion and communication of results prior to publication. This work was supported by NSF grants IOS-0544895 and IOS-0844521. E.B.A. was supported in part by an ASFB SURF Fellowship, by a Stanford VPUE Grant (Biology Department) and by a Stanford Major Grant.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.040931/-/DC1

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