Plant Mol Biol (2011) 75:67–75
DOI 10.1007/s11103-010-9708-2

Characterization of a CLE processing activity

Jun Ni · Yongfeng Guo · Huiyan Jin · Jennifer Hartsell · Steven E. Clark

Received: 16 April 2010 / Accepted: 19 October 2010 / Published online: 4 November 2010

Abstract Proteins containing a conserved motif known as the CLE domain are found widely distributed across land plants. While the functions of most CLE proteins are unknown, specific CLE proteins have been shown to control shoot meristem, root and vascular development. This has been best studied for CLV3 which is required for stem cell differentiation at shoot and flower meristems. In vivo evidence indicates that the CLE domain is the functional region for CLV3, and that it is proteolytically processed from the CLV3 precursor protein. But the mechanism and activity responsible for this processing is poorly understood. Here we extend analysis of an in vitro CLE processing activity and show that in vitro cleavage occurs at Arg70, exactly matching in vivo maturation. We provide evidence that related processing activities are present in multiple tissues and species. We show that efficient protease recognition can occur with as little as four residues upstream of the CLE domain, and that the conserved arginine at position +1 and conserved acidic residues at positions −2 and/or −3 are required for efficient cleavage. Finally, we provide evidence that the N-terminal processing enzyme is a secreted serine protease while C-terminal processing may occur via a progressive carboxypeptidase.

Keywords Meristem · CLAVATA · Proteolysis · Signaling · Ligand

Electronic supplementary material The online version of this article (doi:10.1007/s11103-010-9708-2) contains supplementary material, which is available to authorized users.

J. Ni · Y. Guo · H. Jin · J. Hartsell · S. E. Clark (✉)
Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA
e-mail: clarks@umich.edu

Present Address:
J. Ni
Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA 94305, USA

Present Address:
H. Jin
School of Life Sciences, Tsinghua University, 100084 Beijing, People's Republic of China

Introduction

CLAVATA3 (*CLV3*) functions to regulate the development of shoot and flower meristems in Arabidopsis (Clark et al. 1995; Fletcher et al. 1999). *CLV3* is required to promote differentiation and ectopic *CLV3* expression is sufficient to drive differentiation of shoot and flower meristems (Brand et al. 2000). CLV3 and the receptors CLAVATA1 (CLV1) and CLAVATA2 (CLV2) act to limit the expression of the stem cell-promoting transcription factor WUS (Schoof et al. 2000). *CLV3* is expressed in the apical layers of the shoot meristem where CLV3 protein is secreted to the apoplast (Rojo et al. 2002). Secreted CLV3 is proposed to act as a short distance signal to activate the putative receptors (CLV1 and CLV2) in underlying L3 cell layer to repress *WUS* expression (Clark 1997; Kayes and Clark 1998; Jeong et al. 1999).

CLV3 contains an N-terminal signal peptide and a short C-terminal region referred to as the CLE domain (Cock and McCormick 2001; DeYoung and Clark 2001). This domain is found in a large number of unknown and hypothetic proteins across all land plants and is nearly always found in the same organization as CLV3: N-terminal signal peptide, variable domain, C-terminal CLE domain (Oelkers et al. 2008). The CLE domain is the functional region of CLV3—even if the entire CLV3 sequence upstream of the

CLE domain is replaced with unrelated sequences, the resulting chimeric protein is still fully functional in vivo (Ni and Clark 2006).

A number of lines of evidence suggest that the CLE domain is proteolytically processed from the mature CLV3 protein in vivo prior to its activity. The first evidence for this came from experiments demonstrating that synthetic peptides corresponding to the CLV3 CLE domain were active when added exogenously to Arabidopsis roots or shoots, and, indeed, could rescue the *clv3* mutant phenotype (Fiers et al. 2005, 2006). In addition, over-expression of *CLV3* in calli or secreted into media from submerged seedlings led to the identification of secreted peptides corresponding to 12-mers or 13-mers of the CLE domain (Ito et al. 2006; Ohyama et al. 2009). Furthermore, CLV3 and the related CLE1 are both C-terminally processed at or near the CLE domain when incubated with cauliflower extracts (Ni and Clark 2006). Finally, synthetic peptides corresponding to the CLE domain can bind to the extracellular domains of the CLV1, CLV2 and BAM receptors (Ogawa et al. 2008; Guo et al. 2010).

Here we report the presence of CLE-processing activity across different plant species. We demonstrate that the upstream processing that occurs in vitro is identical to that identified in vivo. We provide an analysis of residues critical for protease recognition in vitro. Furthermore we show that the processing activity is secreted, and likely carried out by a serine protease.

Results

In vitro processing occurs at Arg70

We have shown previously that *E. coli* expressed glutathione S-transferase (GST)-tagged mature CLV3 and CLE1 (GST-mCLV3; GST-mCLE1) undergo C-terminal proteolytic processing when incubated with cauliflower (*Brassica oleracea*) protein extracts (Ni and Clark 2006). Among the two processed forms of CLV3 (p1 and p2, (Ni and Clark 2006)), time-of-flight (TOF)-mass spectrometry (MS) analysis suggested that p1 processing occurred at the conserved arginine at the beginning of the CLE motif [matching processing indicated by peptide analysis in Arabidopsis calli and seedlings over-expressing CLV3 (Ito et al. 2006; Ohyama et al. 2009)], while p2 processing occurred at a string of methionines in the variable domain. However, this data was approximate, because the mass analysis was performed on the remaining N-terminal fusion protein, and the assignment of the in vitro processing site upstream or downstream of the conserved arginine was uncertain.

To determine more precisely if the in vitro processing occurs at the same position as in vivo processing, we analyzed the smaller mass C-terminal fragments from unpurified processing reactions with GST-mCLV3, and compared the results to cauliflower extract alone. We observed four different fragments specific to GST-mCLV3 processing: 2,239 daltons, 2,395 daltons, 2,623 daltons and 3,108 daltons (Fig. 1a). None of these species were observed in cauliflower or GST-mCLV3 alone (Fig. 1b, c). These fragments match exactly the predicted masses of peptides extending from Arg70 to Pro89, Arg 70 to Arg90, Arg70 to Asn92, and Arg70 to Pro96, respectively. To determine if these assignments were correct, we subjected each of the four processed fragments to MS/MS analysis. In each case, the fragmentation pattern matched that of the predicted peptide (Fig. 1d–g). These results confirm that p1 processing corresponds to the N-terminal cleavage of the in vivo production of the CLE peptide. Interestingly, the C-terminal trimming of CLV3 that occurs in vivo is partially replicated in the in vitro assay, in that successively smaller C-terminal tails were detected, suggesting that the trimming may occur by action of a progressive carboxypeptidase (Fig. 1h).

Secreted CLE processing activity is present in multiple plant species and in different tissues

We tested which tissues contained CLE processing activity by isolating extracts from Arabidopsis inflorescences and leaves. In both extracts, p1 processing of CLV3 occurred, but at lower levels than the meristem-rich cauliflower extracts (Fig. 2a). Arabidopsis extracts also C-terminally processed the related CLE1 protein (Fig. 2c). We also tested tobacco BY-2 cell culture to determine if more distantly related plants contained a related activity. Interestingly, cultured media, but not cell extracts, from BY-2 cultures contained apparent p1 processing activity, suggesting that the protease(s) responsible is only active when secreted (Fig. 2b). This would fit well with the apoplastic function of CLV3. While tobacco cell culture contained robust processing activity, tobacco leaves and roots have very little processing activity (Y. Guo and S. Clark, unpublished).

We have previously inferred from time course analysis that the p1 and p2 processing events are not sequentially dependent events (Ni and Clark 2006). However, it was possible that both processing events were carried out by a single enzyme. Evidence to the contrary came from extracts isolated from BY-2 cultures each day following inoculation at low cell density. p1 processing activity was dominant in exponential growth phase, usually during 2–4 days post inoculation (Fig. 3a), while p2 processing was dominant during stationary phase. This indicates the two processing events are likely carried out by different proteases. Both BY-2 cultured media and cauliflower

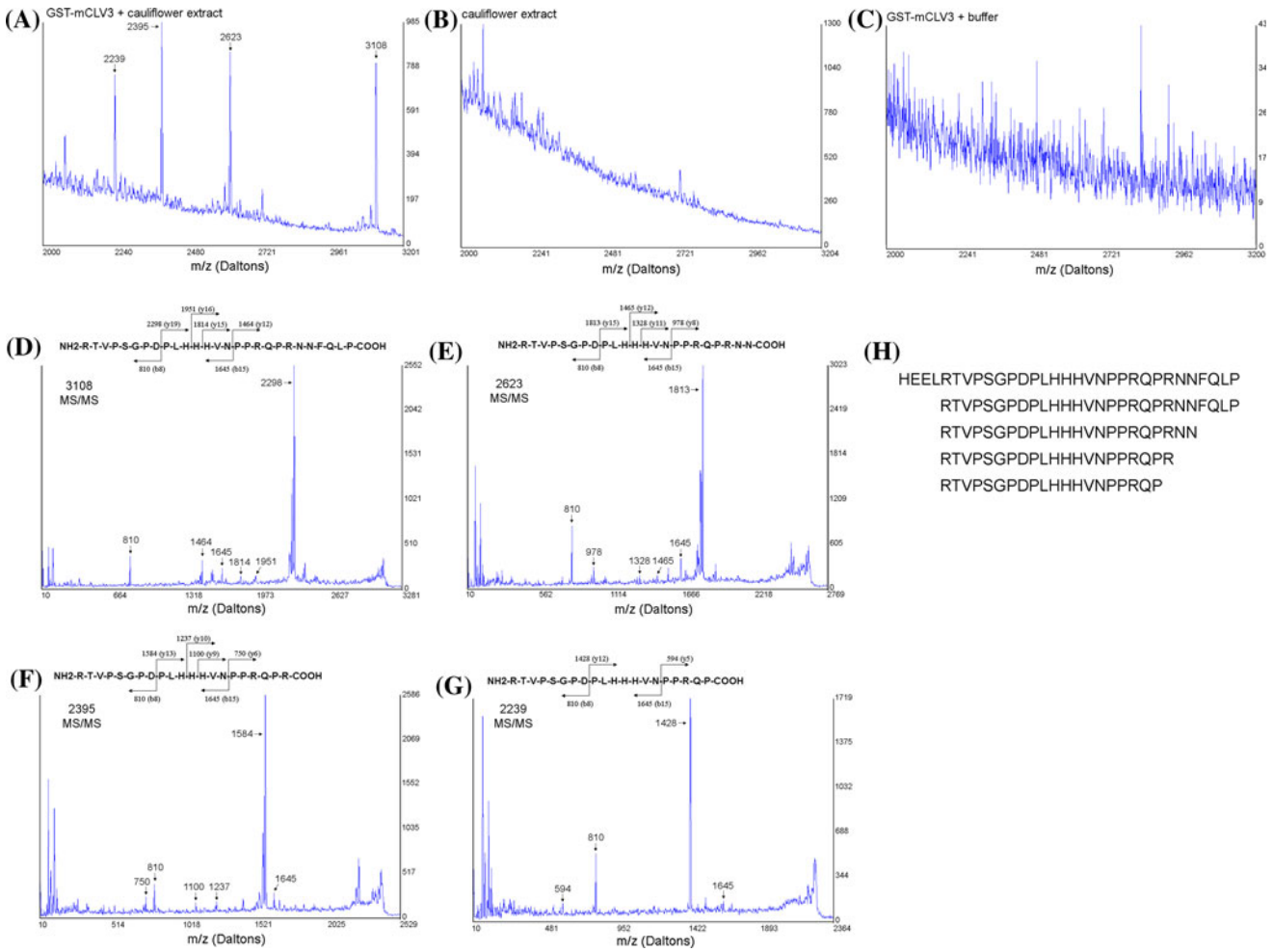

Fig. 1 In vitro cleavage occurs at Arginine 70. (**a**) MS analysis of purified GST-mCLV3 after incubation with cauliflower extracts in an in vitro processing reaction. For all such analysis, the y-axis is a measure of signal intensity. The mass of major peptide fragments are indicated. (**b**) MS analysis of cauliflower extracts. (**c**) MS analysis of purified GST-mCLV3 incubated with buffer in a control reaction. Note the intensity scale is much different than in *panels* (**a**) or (**b**). (**d**–**g**) MS/MS analysis of the four major peptides identified in (**a**). At the *top* is the peptide sequence of the peak based on total mass and the mass of fragmented species. b- and y-fragments from the MS/MS assay are indicated in the peptide sequence and peaks are marked. (**h**) The CLV3 C-terminal sequence from the −4 position to the C-terminus. Also shown are the four processed species identified from (**a**–**g**)

extracts showed a preference for p1 processing in the pH 6 range (Fig. 3b, c).

We also generated and tested a CLE domain protein from soybean (GmCLE23) to further test cross-species processing. GST-GmCLE23 was efficiently processed at the C-terminus by cauliflower extracts (Fig. 2d). The reaction was inhibited by excess His-CLV3 (Stenvik et al. 2008), suggesting that the two proteins are processed by the same enzyme (Fig. 2e).

Cleavage recognition motif

To determine what sequences in CLV3 are required for recognition by the processing protease, we used the previously described chimeric protein ERC3, which can fully replace CLV3 function in vivo (Ni and Clark 2006). To generate ERC3, the variable domain of CLV3 was replaced by unrelated sequences from the receptor-kinase ERECTA and only four residues upstream of the CLE domain (starting at His66) were included (Fig. 1h). This chimeric protein was fully processed in in vitro reactions and ERC3 processing was also inhibited by the same agent that inhibits CLV3 processing (Fig. 4a, b; see below). The efficient recognition of this substrate suggests that protease recognition requires only the CLE domain plus a few upstream residues (Fig. 1h).

The arginine at the beginning of the CLE motif (Arg70) of CLV3 protein is the +1 site of in vitro and in vivo processing and it is absolutely conserved among CLE-containing proteins (Oelkers et al. 2008; Fig. 1h). To test the relevance and importance of Arg70 to CLV3 proteolysis, an alanine substitution of Arg70 (R70A) was generated

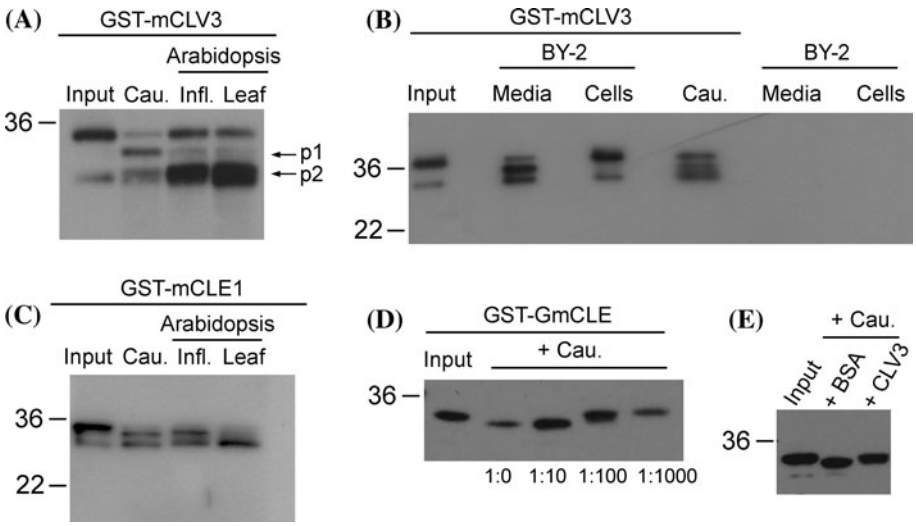

Fig. 2 CLE processing activity from different tissues and species. (**a**) GST-mCLV3 input incubated with cauliflower extracts and Arabidopsis extracts from inflorescences and leaves. p1 and p2 processed fragments are indicated. (**b**) GST-mCLV3 incubated with cauliflower extracts and extracts from BY-2 cultured media and BY-2 cells. The BY-2 extracts alone show no cross-reacting species with the anti-GST antibodies. (**c**) GST-mCLE1 incubated with cauliflower and Arabidopsis extracts. (**d**) GST-GmCLE from soybean incubated with a dilution series of cauliflower extracts. (**e**) GST-GmCLE incubated with cauliflower extracts in the presence of 20-fold excess His-mCLV3 (Ni and Clark 2006) or BSA). All protein gel blots were probed with anti-GST antibodies

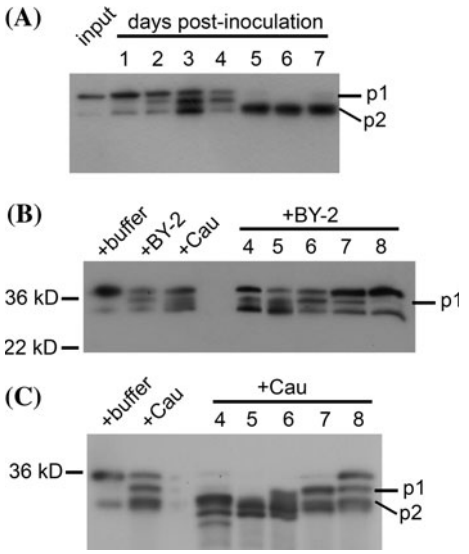

Fig. 3 CLE processing activity at various pH levels and from BY-2 cells at different growth stages. (**a**) BY-2 cells were inoculated at low density into fresh media. Aliquots of the media were collected at 1–7 days post-inoculation as the cells moved from exponential to stationary phase growth and were used in vitro processing reactions with GST-mCLV3. (**b**) Purified GST-mCLV3 was incubated with buffer, BY-2 media and cauliflower extracts, and then compared to BY-2 cell media in which the pH was adjusted as shown. (**c**) Cauliflower extracts were adjusted to various pH levels and used for in vitro processing reactions with GST-mCLV3. All protein gel blots were probed with anti-GST antibodies. The p1 and p2 processed peptides are indicated

by site-directed mutagenesis (GST-mCLV3-R70A). This substitution eliminated detectable p1 processing with both cauliflower and BY-2 cultured media extracts, while the p2 processing was unaffected (Fig. 4c, d), indicating this residue is essential for p1 protease recognition in vitro.

CLE protein processing in vivo is thought to be required to generate active CLE peptide. We hypothesized that if proteolytic processing of CLV3 observed in vitro has biological significance, the R70A substitution should affect CLV3 function in vivo. We transformed Arabidopsis *clv3-1* mutants with constructs in which the *CLV3* cis elements (P_{CLV3}) drove expression of CLV3 in which the variable domain was replaced by the MYC epitope tag (Fig. 4e, f). While P_{CLV3}*:MYC-CLV3* fully complemented *clv3-1*, P_{CLV3}*:MYC-CLV3-R70A* did not complement the mutant fully. Thus, this residue is essential for processing and/or ligand binding. Perhaps the most interesting aspect of these observations is that while ligand competition assays indicate no detectable function for the CLE-R70A peptide (Kondo et al. 2008), the CLV3-R70A protein retains some activity in vivo, based on the partial suppression it provides of the *clv3-1* phenotype. This suggests that in vivo complementation under the native promoter is a more sensitive assay for CLE function than other assays. One key note is that we have found that the lesion in *clv3-1* is different than reported previously. We have sequenced this allele and found that the GGA codon for Gly75 is altered to AGA leading to an Arg missense codon.

Using the same approach we assessed the role of acidic residues immediately upstream of the arginine at the CLE processing site, in enzyme recognition and processing (Fig. 1h). An acidic residue at one or both of these

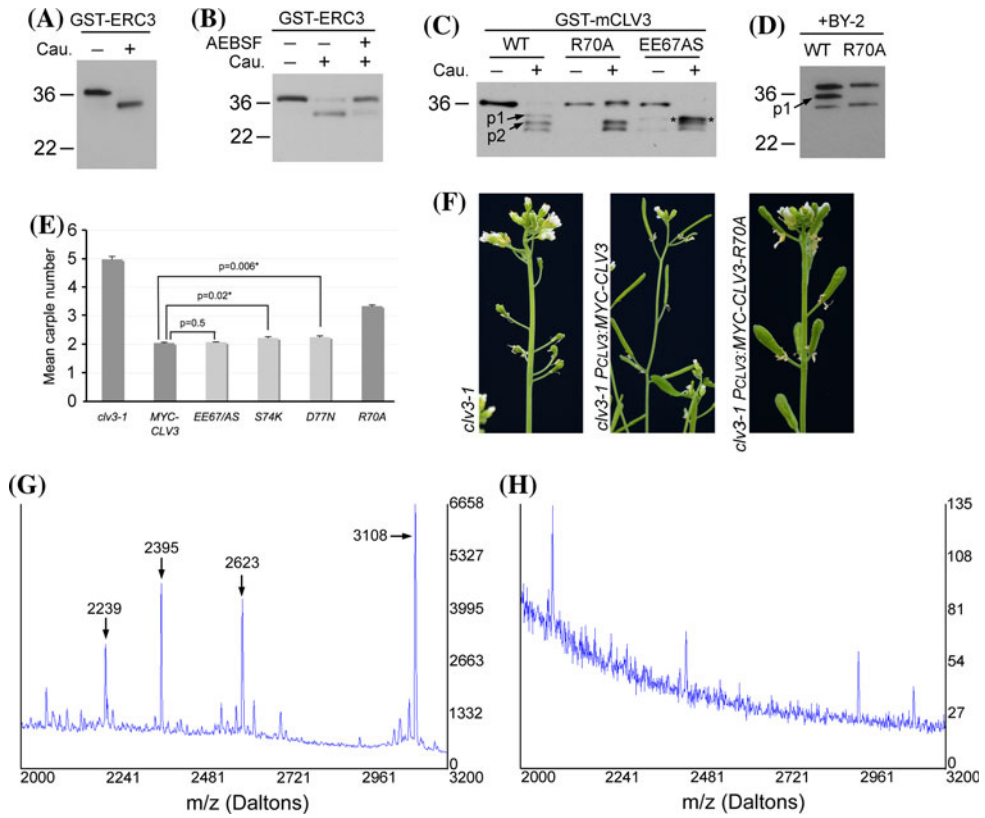

Fig. 4 Arginine at +1 and glumatic acids at −2 and −3 are required for efficient processing. (**a**) The ER-CLV3 chimeric protein previously described (ERC3—Ni and Clark 2006) was processed by cauliflower extracts. Note that the only CLV3 sequences in this construct are those indicated at the *top* of Fig. 1h. (**b**) ERC3 processing was inhibited by AEBSF, which inhibits GST-mCLV3 processing (see Fig. 5). (**c**) Site-directed mutants R70A and EE67AS were incubated with cauliflower extracts alongside wild-type GST-mCLV3. R70A processed at the p2, but not p1 site. EE67AS did not produce normal amounts of p1 processed fragments. Note, EE67AS input contained a novel background band (*asterisks*). (**d**) The R70A mutant protein was incubated with BY-2 cell media, but did not display any of the p1 processing that wild-type GST-mCLV3 did. (**e**) The ability of MYC-CLV3 and site-directed mutants R70A, EE67AS, D77 N and S74K mutants driven by the *CLV3* cis elements to complement *clv3-1* was measured by the mean number of carpels/flower for the resulting transgenic lines. The complementation defects for S74K, D77N and R70A were significant based on student *t* tests. (**f**) Inflorescence and flower phenotypes for *clv3-1*, *clv3-1* P_{CLV3}*:MYC-CLV3* and *clv3-1* P_{CLV3}*:MYC-CLV3-R70A* plants are shown. (**g**) MS analysis of EE67AS indicates that even though p1 processing was difficult to detect in protein gel blots, some correct processing still occurred. (**h**) Control EE67AS protein incubated with buffer was analyzed by MS. For (**g**) and (**h**), the y-axis is signal intensity. All protein gel blots were probed with anti-GST antibodies. The p1 and p2 processed peptides are indicated

locations is relatively conserved among CLE-containing proteins (Oelkers et al. 2008). The paired aspartic acid residues 67 and 68 in CLV3 were simultaneously replaced by alanine and serine (corresponding to the residues found in the non-functional CLE8 (Ni and Clark 2006) to form MYC-CLV3-EE67AS. The EEAS substitutions blocked most p1 processing in vitro, based on protein gel blot analysis (Fig. 4c). While no obvious p1 processing band was observed, an EE67AS-specific background band made it difficult to rule out some low level of p1 processing. Indeed, MS analysis revealed some accumulation of the same processed fragments identified from wild-type CLV3 protein (Fig. 4g, h). Consistent with the sensitive nature of the endogenous rescue, attempts to complement *clv3-1* with P_{CLV3}*:MYC-CLV3-EEAS*, lead to nearly full rescue with phenotypes much closer to wild-type than observed in P_{CLV3}*:MYC-CLV3-R70A clv3-1* transformants (Fig. 4e, f).

Other site-directed mutants targeting suspected key residues within the CLE domain (MYC-CLV3-S74 and MYC-CLV3-D77) caused minor adverse effects in vivo (Fig. 4e) that may be related to ligand binding.

CLE cleavage is likely catalyzed by a serine protease

To identify chemical inhibitors of the processing protease that might reveal the nature of the enzyme, we tested a suite of protease inhibitors in the in vitro proteolytic reaction (Table 1). The majority of tested compounds did not inhibit any of the processing events (Table 1; Supplemental Figure 1). However, two serine protease inhibitors (AEBSF and Bestatin) demonstrated specific inhibitory

Table 1 Protease inhibitor panel

Protease inhibitor	Function	Concentration	Inhibition effect	
			p1	p2
Antipain	Serine/cysteine protease inhibitor	800 μM	+	+
Bestatin	Leucine aminopeptidase, aminopeptidase B, triamino pepidase inhibitor	2.88 mM	+	−
AEBSF	Serine protease inhibitor	20 mM	+	−
Chymostatin	Strong inhibitor of many proteases	400 μM	−	+
EACA	Inhibits chymotypsin, factor VIIa, lysine carboxypeptidase, plasmin and plasminogen activator	10 mg/ml	−	−
Aprotinin	Serine protease inhibitor	35 μM	−	−
E-64	Cysteine protease inhibitor	1.71 mM	−	−
Leupeptin	Serine/thiol protease inhibitor	1.25 mM	−	−
Phosphoramidon	Metalloendoproteinases, thermolysis, elastase inhibitor	500 μM	−	−
Trypsin inhibitor	Trypsin inhibitor	0.5 mg/ml	−	−
NEM	Cysteine protease inhibitor	20 mM	−	−
Pepstatin A	Aspartyl peptidases inhibitor	250 μg/ml	−	−
EST	Cysteine protease inhibitor	500 μM	−	−
TLCK	Trypsin-like serine protease inhibitor	500 μM	−	−
TPCK	Chymotrypsin inhibitor	500 μM	−	−
PMSF	Serine protease inhibitor	5 mM	−	−

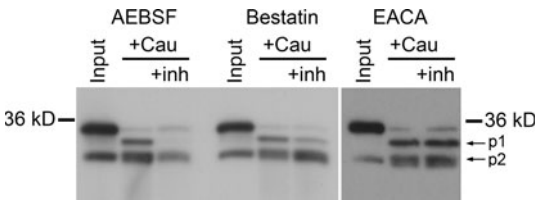

Fig. 5 AEBSF and bestatin are inhibitors of CLE processing in CLV3. GST-mCLV3 (*input*) was incubated with cauliflower extracts (+Caul) in the absence or presence (+inh) of the protease inhibitors AEBSF, bestatin or EACA (see Table 1). Note the strong reduction and moderate reduction in p1 accumulation upon AEBSF and bestatin incubation, respectively

affects on p1 processing, but had no effect on p2 processing (Table 1; Fig. 5, Supplemental Figure 1). One protease inhibitor (Chymostatin) demonstrated the opposite function, inhibiting the p2 proteolytic reaction while having no impact on p1 processing. This further demonstrates that the two reactions are catalyzed by different enzymes. Only one inhibitor, the serine/cysteine protease inhibitor antipain, blocked both p1 and p2 processing.

Discussion

We have characterized a processing activity in plants capable of proteolytic processing of the CLV3 CLE domain. We have observed a related activity expressed in multiple tissues, and in multiple plant species. Along with the conservation of the CLE domain across land plants, many CLE proteins may be processed by a conserved class of proteases.

Using chimeric constructs, as well as site-directed mutagenesis, our data suggests that the processing protease is capable of recognizing the CLE domain with as little as four upstream residues. Among the residues close to the processing site, the arginine at the +1 position is critical for processing, in addition to its role in receptor binding (Kondo et al. 2008). The acidic residues found in nearly all CLE proteins at the −2 and/or −3 position are also essential for efficient processing—only a small amount of processed form is detected from the mutant protein. Interestingly, when we previously compared the abilities of 14 different Arabidopsis CLEs to rescue the *clv3* mutants, we observed that all of those with detectable in vivo function contained acidic residue(s) at position −2 and/or −3, while all four non-functional CLEs lacked an acidic residue at either position (CLE8, CLE14, CLE25, CLE26). Thus, a critical feature for CLE proteins in vivo—at least those that function in a manner analogous to CLV3—may be acidic residues to promote efficient processing.

One observation that arises from comparing our in vitro and in vivo results, and the results that others have performed on both ligand binding and peptide addition to growing seedlings, is that our in vivo mutant

complementation under the native promoter is a much more sensitive test of function than any in vitro assay. For example, the CLV3-R70A mutant has no detectable ligand binding activity, no detectable in vitro processing, yet retains measurable, albeit weak, ability to rescue *clv3-1*. Furthermore, a mystery arises when ligand binding results are compared to complementation results for both the R70A mutation and the *clv3-1* mutation (G75R). R70A has no detectable ligand binding activity, while G75A and G75R mutants have measurable, if reduced, ligand binding activity (Kondo et al. 2008; Guo et al. 2010). Despite this, the only two *clv3* missense alleles identified to date are G75R substitutions (Fletcher et al. 1999). In addition, R70A can partially complement *clv3-1*. This discrepancy between in vivo function and in vitro assays suggests that some measure of in vivo function is not being assayed in vitro. One possibility is binding to different receptors, as some CLEs do not rely upon CLV1 for meristem function (Ni and Clark 2006). Another possibility is that ligand binding within the meristem is different than in the cell culture system utilized. Finally, processing in vivo may not be as affected by the R70A substitution as we observe in vitro. In a related note, we previously observed that R- > N and R- > K substitutions did not noticeably alter CLE1 processing in vitro. This could be due to differences between CLV3 and CLE1, or due to the fact the substitution in CLV3 that we tested is a more dramatic alteration of the arginine to alanine.

The enzyme responsible for CLE processing in our in vitro system is likely to be a serine protease, based on inhibitor profiles. Both inhibitors that specifically target p1 processing (bestatin and AEBSF) and the one that targets both p1 and p2 processing (antipain) are serine protease inhibitors. These inhibitors may prove useful as inhibitors of CLE processing in vitro and in vivo, as well as for identifying candidate proteases. The enzyme is likely to be secreted based on our analysis of BY-2 culture media, as well as the apoplastic function of CLV3. These characteristics should aid in identifying the enzyme(s) responsible in vivo.

One processing event for CLV3 and a subset of CLE proteins is the trimming of the C-terminal tails. CLV3 contains a 15 residue extension beyond the CLE domain, a number of functional and hypothetical CLE proteins also contain C-terminal extensions (Oelkers et al. 2008). How these are processed remains unclear. In the in vitro processing assays, C-terminal processing appears to be carried out by a progressive carboxypeptidase, based on the identification of progressively shorter C-terminal extensions among accumulated CLE fragments. Whether this reflects processing in vivo is currently unknown. However, it is interesting to note that mutations in the Arabidopsis carboxypeptidase SOL1 prevent CLE19 over-expression phenotypes (Casamitjana-Martinez et al. 2003).

Methods

Plant growth

Arabidopsis seeds were sown on a 1:1:1 ratio of Metromix 360 (Scott), medium vermiculite, and coarse perlite, supplemented with ~1 g Osmocote 14-14-14 fertilizer (Scott) per 3.5 inch pots and topped with a thin layer of Metromix 360. After 7-days cold treatment at 4°C, plants were grown at 22°C under constant cool white fluorescent light.

Construct generation

pGEX5-mCLV3

For *E. coli* expression of GST-CLV3. Mature *CLV3* sequence without the signal peptide was amplified from *CLV3* cDNA using the MCLV3Top and NewMCLV3bottom primers (see Table 2 for a list of oligonucleotide primers used in this study). PCR product was inserted into pGEX-5X-1 (Amersham) via Sma I and Xho I sites.

pGEX5-ERC3

For *E. coli* expression of GST-ERC3, pCB302-ERC3 (Ni and Clark 2006) was used as template to amplify a fusion fragment between *ERECTA* and *CLV3* sequences without the *ERECTA* signal sequence. The EcoR-mERC3 P5 and NewMCLV3bottom primers were used. PCR product was inserted into pGEX-5X-1 (Amersham) via Eco RI and Xho I sites.

pGEX5-mCLV3-R70A

GST-CLV3-R70A was generated according to manufacture protocol of QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the R70Aforward and R70Areverse primers. pGEX5-mCLV3 was used as amplification template.

P_{CLV3}*:MYC-CLV3*

pCB302-C3PT (Ni and Clark 2006) was used to amplify the N-terminal fragment of CLV3 with the primers newCLV5-p5Mlu and 3FCLV3sigP3. The C-terminal fragment was amplified with 3FCLV3C3BXP5 and cCLV3-3′Bam. The MYC-tag was amplified from pMyc (Clontech) using 3FmycP5 and 3FmycP3 primers. Fusion PCR was conducted

Table 2 Oligonucleotides used in this study

Name	Sequence
MCLV3Top	5′-ACATGATGCTTCTGATCTCACTCAAGCT
NewMCLV3bottom	5′-CCCTCGAGTTAAGGGAGCTGAAAGTTGTTTCTTGGC
EcoR-mERC3 P5	5′-CGAATTCACTGTGACTTCAGAGG
R70Aforward	5′-GGACTACATGAAGAGTTAGCGACTGTTCCTTCGGGACC
R70Areverse	5′- GGTCCCGAAGGAACAGTCGCTAACTCTTCATGTAGTCC
newCLV5-p5Mlu	5′-CCCCACGCGTCACTTTCTCTCTAAAAAATG
3FCLV3sigP3	5′-AGCTTTTGCTCCATCGGCCGAGCATGAGCTTGAGTGAGAT
3FCLV3C3BXP5	5′-ATTCGGTTCCCGGGAGATCTGAGAAGGCAAAGACGAAGGG
cCLV3-3′Bam	5′-CCCCGGATCCAAAGCAACAAGAGATTAGGTCA
3FmycP5	5′-ATCTCACTCAAGCTCATGCTCGGCCGATGGAGCAAAAGCT
3FmycP3	5′-CCCTTCGTCTTTGCCTTCTCAGATCTCCCGGGAACCGAAT
EEASforward	5′-GAAGGGTTTAGGACTACATGCGTCCTTAAGGACTGTTCCTTCGGG
EEASreverse	5′-CCCGAAGGAACAGTCCTTAAGGACGCATGTAGTCCTAAACCCTTC
S74Kforward	5′-GAAGAGTTAAGGACTGTTCCTAAGGGACCTGACCCGTTGCACC
S74Kreverse	5′-GGTGCAACGGGTCAGGTCCCTTAGGAACAGTCCTTAACTCTTC
D77Nforward	5′-GGACTGTTCCTTCGGGACCTAATCCGTTGCACCATCATGTGAAC
D77reverse	5′-GTTCACATGATGGTGCAACGGATTAGGTCCCGAAGGAACAGTCC

to ligate all three fragments and the fused fragment was inserted into pCB302-C3PT via Mlu I and Bam HI sites.

P_{CLV3}:MYC-CLV3-R70A; -EEAS; -S74; -D77

All constructs were generated according to manufacture protocol of QuikChange Site-Directed Mutagenesis kit (Stratagene) using P_{CLV3}:MYC-CLV3 as amplification template. Primers used to generate mutations were R70Aforward/R70Areverse, EEASforward/EEASreverse, S74Kforward/S74Kreverse, and D77Nforward/D77Nreverse.

Protein extraction from cauliflower and Arabidopsis

Cauliflower (*Brassica oleracea*) meristem protein extracts were prepared as described with or without 0.1% Triton X-100 (Ni and Clark 2006). Triton-containing extracts were used for all in vitro processing reactions except the large-scale reactions for MS/MS analysis. Note that 1 ml of protease inhibitor cocktail for use with plant cell extracts (P9599, Sigma, St. Louis) was added in the extraction buffer per 300 g of tissue. Before use, the extracts were centrifuged at 40,000*g* for 30 min at 4°C.

Inflorescence apices, including meristems and young flowers, or cauline leaves were collected from *Columbia* ecotype Arabidopsis. Tissues were grounded in liquid nitrogen and resuspended in 200 µl extraction buffer (50 mM Hepes, pH 7.4, 10 mM EDTA) per 0.1 g tissue, followed by 10 min centrifuge at 4°C. The supernatants were used for future analysis. Note that 1 ml of protease inhibitor cocktail for use with plant cell extracts (P9599, Sigma, St. Louis) was added in the extraction buffer per 300 g of tissue.

Proteolytic processing and detection

Proteins expressed in *E. coli* and purified were incubated with the indicated plant protein extracts or mock (ddH_2O or elution buffers for individual tagged proteins) for 2 h at room temperature with rotation. SDS loading buffers were added to end the reactions and samples were boiled at 100°C for 5 min before analysis.

Protein gel blots were performed as previously described (DeYoung et al. 2006). Chicken anti-GST antibodies were kindly provided by Ken Cadigan, and detected with HRP-conjugated rabbit anti-chicken secondary antibodies.

MS and MS/MS analysis

Approximately 20 µg of *E. coli* expressed and purified protein was incubated with 80 µl of cauliflower protein extracts for 2 h at room temperature. Samples were then subjected to centrifuge through Microcon YM-10 centrifugal filter (Millipore) at 14,000*g*, 4°C for 30 min. The flow-through fractions were collected for intact MS analysis at the Michigan Proteome Consortium (University of Michigan, Ann Arbor). Peaks of interest were further characterized by MS/MS analysis.

Tobacco BY-2 culture

Nicotiana tabacum BY-2 cells were grown in liquid Murashige and Skoog medium supplemented with sucrose (30 g/l), KH_2PO_4 (0.2 g/l), thiamine (1 mg/l) and 2,4-dichlorophenoxyaetic acid (0.2 mg/l). The suspension cultures (50 ml) were grown in 250 ml Erlenmeyer flasks at

28°C with shaking at 125 rpm. Subcultivation was performed after 7 days by transferring 1.5–2 ml of suspension culture into 50 ml of fresh medium.

Protease inhibitors

All the protease inhibitors were obtained from Sigma and diluted in suggested solvents. The –PI samples (Supplemental Figure 1) in each case included the solvents used for the corresponding protease inhibitor. Note that the inhibitor cocktail used to generate the original extracts contained AEBSF and bestatin, but at much lower concentrations than used for inhibitor profiling (0.3 and 0.02 mM, respectively).

Acknowledgments This work was support by grant USDA-2006-35304-17403 to S.E.C.

References

Brand U, Fletcher JC, Hobe M, Meyerowitz EM, Simon R (2000) Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. Science 289:617–619

Casamitjana-Martinez E, Hofhuis HF, Xu J, Liu CM, Heidstra R, Scheres B (2003) Root-specific CLE19 overexpression and the sol1/2 suppressors implicate a CLV-like pathway in the control of Arabidopsis root meristem maintenance. Curr Biol 13:1435–1441

Clark SE (1997) Organ formation at the vegetative shoot meristem. Plant Cell 9:1067–1076

Clark SE, Running MP, Meyerowitz EM (1995) *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. Development 121:2057–2067

Cock JM, McCormick S (2001) A large family of genes that share homology with CLAVATA3. Plant Physiol 126:939–942

DeYoung BJ, Clark SE (2001) Signaling through the CLAVATA1 receptor complex. Plant Mol Biol 46:505–513

DeYoung BJ, Bickle KL, Schrage KJ, Muskett P, Patel K, Clark SE (2006) The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in Arabidopsis. Plant J 45:1–16

Fiers M, Golemiec E, Xu J, van der Geest L, Heidstra R, Stiekema W, Liu CM (2005) The 14-amino acid CLV3, CLE19, and CLE40 peptides trigger consumption of the root meristem in Arabidopsis through a CLAVATA2-dependent pathway. Plant Cell 17:2542–2553

Fiers M, Golemiec E, van der Schors R, van der Geest L, Li KW, Stiekema WJ, Liu CM (2006) The CLAVATA3/ESR motif of CLAVATA3 is functionally independent from the nonconserved flanking sequences. Plant Physiol 141:1284–1292

Fletcher JC, Brand U, Running MP, Simon R, Meyerowitz EM (1999) Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. Science 283:1911–1914

Guo Y, Han L, Hymes M, Denver R, Clark SE (2010) CLAVATA2 forms a distinct CLE-binding receptor complex regulating Arabidopsis stem cell specification. Plant J 63:899–900

Ito Y, Nakanomyo I, Motose H, Iwamoto K, Sawa S, Dohmae N, Fukuda H (2006) Dodeca-CLE peptides as suppressors of plant stem cell differentiation. Science 313:842–845

Jeong S, Trotochaud AE, Clark SE (1999) The Arabidopsis *CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. Plant Cell 11:1925–1934

Kayes JM, Clark SE (1998) *CLAVATA2*, a regulator of meristem and organ development in *Arabidopsis*. Development 125:3843–3851

Kondo T, Nakamura T, Yokomine K, Sakagami Y (2008) Dual assay for MCLV3 activity reveals structure-activity relationship of CLE peptides. Biochem Biophys Res Commun 377:312–316

Ni J, Clark SE (2006) Evidence for functional conservation, sufficiency, and proteolytic processing of the CLAVATA3 CLE domain. Plant Physiol 140:1–8

Oelkers K, Goffard N, Weiller GF, Gresshoff PM, Mathesius U, Frickey T (2008) Bioinformatic analysis of the CLE signaling peptide family. BMC Plant Biol 8:1

Ogawa M, Shinohara H, Sakagami Y, Matsubayashi Y (2008) Arabidopsis CLV3 peptide directly binds CLV1 ectodomain. Science 319:294

Ohyama K, Shinohara H, Ogawa-Ohnishi M, Matsubayashi Y (2009) A glycopeptide regulating stem cell fate in Arabidopsis thaliana. Nat Chem Biol 5:878–880

Rojo E, Sharma VK, Kovaleva V, Raikhel NV, Fletcher JC (2002) CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. Plant Cell 14:969–977

Schoof H, Lenhard M, Haecker A, Mayer KF, Jurgens G, Laux T (2000) The stem cell population of *Arabidopsis* shoot meristems in maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. Cell 100:635–644

Stenvik GE, Tandstad NM, Guo Y, Shi CL, Kristiansen W, Holmgren A, Clark SE, Aalen RB, Butenko MA (2008) The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in arabidopsis through the receptor-like kinases HAESA and HAESA-LIKE2. Plant Cell 20:1805–1817