

A misannotated locus positively influencing *Arabidopsis* seed germination is deconvoluted using multiple methods, including surrogate splicing

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ABSTRACT

A screen of activation tagged lines of *Arabidopsis thaliana* retrieved *COLD TEMPERATURE GERMINATING10-D_(tag)* (*CTG10-D_(tag)*) seeds, capable of radicle protrusion in advance of wild type (WT) at suboptimal- and optimal-temperatures. Genomic walking revealed T-DNA in the intragenic region that upregulates expression of the At4g19330 locus previously predicted to encode an F-BOX protein. A combination of surrogate splicing, primer scanning, RACE, and Illumina PolyAdenylation Tag (PAT) sequencing in petunia (*Petunia X hybrida*) and *Arabidopsis* were required to demonstrate that the region around At4g19330 was misannotated and is actually compromised of two separate genes. Even though homologous regions nearby and elsewhere on chromosome 4 are confounding elements in the molecular characterization of the locus, we could determine that the 5' entity encodes a ribonucleoprotein of unknown function whereas the 3' gene includes the promoter and full coding region of an F-BOX protein. Although both genes were upregulated, only independently-transformed lines over-expressing the F-Box exhibited enhanced completion of seed germination. We named it *CTG10*, and a single, poorly penetrant, mutant line of *ctg10* manifested the expected reduced completion of seed germination. Whereas *CTG10-OE* lines are hypersensitive to the gibberellin biosynthetic inhibitor paclobutrazol, the *ctg10* mutant line is hypersensitive; a phenotype which could be alleviated when transgenically rescued with *CTG10*. The F-Box moiety promoted association of *CTG10* with ASK proteins in yeast two hybrid assays, indicating that it likely assembles into an SCF-type ubiquitin ligase to promote the ubiquitination of one or more substrates. In this capacity *CTG10* might target a protein repressing seed germination for polyubiquitination and subsequent proteasomal degradation.

1. Introduction

Seed germination is a critical control point in plant development; the culmination of which demarcates the switch from the stage most impervious to that most susceptible to stress. Crop production, weed management, and the establishment and range of native and invasive species all depend upon whether, when, and how many seeds in a

population are stimulated to complete germination (Bradford, 2002; Bais et al., 2003; Donohue, 2009; Cochrane et al., 2014; Hoyle et al., 2015; Finch-Savage and Bassel, 2016). Conversely, the production of high quality seeds as foodstuffs or as germplasm repositories depends on the inhibition of precocious germination to allow the seeds to attain maturity, the prevention of preharvest sprouting once mature, and the maintenance of defense systems during seed storage (White et al., 2000;

Abbreviations: (bps), base pairs; (FBPs), F-BOX PROTEINS; (nts), nucleotides; (RT-PCR), reverse transcription-polymerase chain reaction; (WT), wild type

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Kulwal et al., 2004; Xu et al., 2004; Boudet et al., 2006; Tolleter et al., 2007; Chen et al., 2010; Nayak et al., 2013; Schramm et al., 2013; Verma et al., 2013; Gu et al., 2016). Knowledge of the mechanisms by which germination and subsequent seedling development are regulated would lead to immediate applications improving establishment speed and uniformity of crop plants in the field, as well as preventing preharvest sprouting (Gu et al., 2003). Such knowledge could also provide new strategies for controlling weedy- and invasive-species (Weller et al., 2001) or stimulating faster, more uniform germination, synchronizing its completion. Understanding the processes underlying seed germination is vital because our reliance on seeds is so absolute (TeKrony, 2006; Bewley et al., 2013).

There is a voluminous literature describing alterations in the transcriptome following imbibition as the cell progresses from seed developmental programs to embark on those conducive to seed germination (Nakabayashi et al., 2005; Preston et al., 2009; Narsai et al., 2011; Dekkers et al., 2013). In addition to these transcriptomic changes, profound proteomic alterations have been documented that are responsible for orchestrating the amalgam of physiological modifications required at each stage of germination (Gallardo et al., 2001; Galland et al., 2014). The cessation of transcription supporting seed development upon imbibition is coupled with the decay of mRNA species from genes inappropriate to this phase of the life cycle (Downie et al., 2004) and with the degradation of proteins whose function is similarly ill-suited for this stage (Galland et al., 2012). In addition to a general reduction of developmental-stage specific proteins, environmental cues, to which the mature hydrated seed is sensitive, can also orchestrate selective removal or stabilization of proteins during germination that controls protrusion of the embryo from the seed (Oh et al., 2006; Holman et al., 2009; Ariizumi et al., 2011; Albertos et al., 2015). One mechanism by which proteins are selectively eliminated involves their delivery to the 26S proteasome in a state conducive to their entry into the catalytic core of the particle (Vierstra, 2009).

F-BOX PROTEINS (FBPs) are a component imparting substrate specificity to the Skp1-Cdc53/Cullin-F-Box (SCF) family of Ubiquitin (Ub) ligase (E3) complexes involved in protein degradation through the 26S proteasome (Smalle and Vierstra, 2004). This ancient, highly conserved protein degradation pathway is present throughout eukaryotes. It is particularly diverse in plants playing a key role in many cellular processes including cell cycle control, circadian rhythm, flower development, and hormone signal transduction (Xie et al., 1998; Samach et al., 1999; Zhao et al., 1999; Gray and Estelle, 2000). There are approximately 700 FBPs in *Arabidopsis* (Gagne et al., 2002) which are thought to recognize and bind to a plethora of target proteins through a variety of protein interacting domains (e.g. kelch β -propellers). Target proteins are introduced to Ub ligase complexes through the interaction of the F-BOX domain with Skp1-like proteins, associated in turn with CULLIN and RING proteins. The target protein is then polyubiquitinated causing it to become a candidate for 26S proteasome-mediated destruction (Smalle and Vierstra, 2004). In *Arabidopsis*, an aberrant member of the base of the regulatory particle component of the proteasome (*rpn10-1*), while viable, leads to a plethora of perturbations in all aspects of plant growth, including seed germination (Smalle et al., 2003), implicating proteasomal-mediated protein degradation in the normal completion of germination. A more defined role for the 26S proteasome in seed germination was acquired from work with the FBP SLEEPY1 (SLY1) responsible for the degradation of members of the GRAS family of transcription factors (DELLA proteins) inhibitory to the GA response (McGinnis et al., 2003; Sasaki et al., 2003; Dill et al., 2004; Griffiths et al., 2006; Sun, 2010).

In this study we have identified, by activation tagging, an *Arabidopsis* locus that can speed radicle protrusion at suboptimal germination temperatures upon overexpression. The affected locus, designated *COLD TEMPERATURE GERMINATING10* (*CTG10*) (Salaita et al., 2005), was discovered by genomic walking but was misannotated, comprising two genes, only one of which encodes an FBP capable

of influencing seed germination by presumably directing the polyubiquitination, leading to the degradation, of a repressor of seed germination.

2. Materials and methods

2.1. Plant material

In all experiments, unless otherwise stated the *Arabidopsis thaliana* Col-0 ecotype was used. Plants were grown, and seeds germinated, under constant light ($135 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 25°C . In each experiment, seeds from plants grown under the same conditions, planted and harvested at the same time were compared. *Petunia X hybrida* plants were grown from seeds (W. Atlee Burpee & Co., Warminster, PA, USA) in a greenhouse with supplemental light (14/10-h light/dark cycle). After 4 weeks, young, healthy leaves were infiltrated and used as a source of mRNA (see section 2.4. *Surrogate splicing*).

2.2. Germination assay

Seed germination assays comprised 3 replications of 50 seeds each placed on two layers of Whatman No. 1 filter paper (Whatman, Florham Park, NJ, USA) moistened with 2 mL distilled, deionized water or varying concentrations of paclobutrazol. Seed germination was determined to be completed as soon as the radicle protruded beyond the testa. Seeds that had or had not been afterripened were used, depending on the objectives of the germination assessment. In no instance were the seeds moist chilled.

2.3. Genome walk

A genome walking strategy (Universal GenomeWalker™ Kit, Clontech, Mountain View, CA, USA) was used to identify the insertion site of the T-DNA in the dominant, activation-tagged (Salaita et al., 2005), *COLD TEMPERATURE GERMINATING10-D_(tag)* (*CTG10-D_(tag)*) mutant. Aliquots of purified genomic DNA from *CTG10-D_(tag)* leaves were digested with one of four, blunt-cutting restriction enzymes (*Dra*I, *Stu*I, *Eco*RV, and *Pvu*II) to create libraries. The digested genomic DNA was ligated to adaptor arms and two successive rounds of PCR per library were performed using nested T-DNA-specific and adaptor-specific primers (Table 1) according to the directions of the manufacturer (Clontech). Amplicons were size fractionated, recovered from the gel, cloned, and sequenced.

2.4. Surrogate splicing, 5'- and 3'-RACE define the *LIKE-SM RIBONUCLEOPROTEIN* and *KELCH REPEAT CONTAINING F-BOX* cDNAs

A surrogate splicing strategy was used to help to define the actual cDNA(s) produced by the region of the genome currently annotated as At4g19330. A 3895 bp genomic fragment downstream of the pSKI015 insertion site was cloned into a modified pRTL2 vector (double CaMV35S promoter, tobacco etch virus (TEV) translational enhancer, *Not*I sites introduced 5' and 3' to the cassette using site-directed mutagenesis (the kind gift of Gulvadee Chaiyaprasithi)). This was subcloned into the *Not*I site of pMLBART and subsequently transformed into *Agrobacterium tumefaciens* (strain GV3850). The At4g19330-carrying *Agrobacterium* were infiltrated into *Petunia X hybrida* leaves (Wu et al., 2005). After 4–6 days, the infiltration zones were collected and the RNA was extracted, DNase-I digested (Ambion®, Austin, TX, USA), the DNase inactivated and the RNA subjected to reverse transcription (RT) using SuperScript® III (Stratagene Corp., La Jolla, CA, USA) with oligo-dT_{18–22}. A variety of primer combinations was used to scan the cDNA thus produced for combinations capable of producing amplicons. The initial surrogate splicing amplicons determined that there were two genes comprising At4g19330, namely a *SM-LIKE RIBONUCLEOPRO-*

Table 1
Primers used in this study.

Operation and cDNA identity	Primer	Sequence
Genome Walker™ primers	Adaptor primer (F)	GTAATACGACTCACTATAGGGC
	Nested adaptor primer (F)	ACTATAGGGCACCGCTGGT
<i>pSKI015</i> left border primers	T-DNA specific	CTCATCTAACGCCCATGGACGT
	Nested T-DNA specific	CGCCATATAATACGACGGATCGTAATT
Surrogate splicing	pRTL2_SSP_NcoI (F) "A"	accATGGAGCGTGAGACTTCGTCTAC
	pRTL2_SSP_XbaI (R) "B"	<u>atctaga</u> GAGAGCTACTTAAGCGTCGTC
Gene Racer® 5' RACE	Adaptor primer (F)	CGACTGGAGCACGAGGACACTGA
	Nested adaptor primer (F)	GGACACTGACATGGACTGAAGGAGTA
Gene Racer® 3' RACE	Adaptor primer (R)	GCTGTCAACGATACGCTACGTAACG
	Nested adaptor primer (R)	CGCTACGTAACGGCATGACAGTG
<i>RNP</i> 5' RACE	GSP (R)	CCTGGAACACTCGTGTGCTATTATC
	Nested GSP (R)	CTCAACCTCTTCATAAACCTAAC
<i>F-BOX</i> 5' RACE	GSP (R)	GGAGAGGGCATCGTAGCAACCAATGT
	Nested GSP (R)	GGTACACTTGATGTCGATCGTCCAATG
<i>RNP</i> 3' RACE	GSP (F)	ATGAGCGTGAGACTTCGTCTTC
	Nested GSP (F)	GCCACCAAGCTAGGCATAATTCTGCTA
<i>F-BOX</i> 3' RACE	GSP (F)	ACTTGGTCTTTGGACCGTGGCG
	Nested GSP (F)	ATCTGGGTACATCGAATGGCTTGAT
Full length <i>RNP</i> cDNA	Forward primer	<u>acc</u> ATGGAGCGTGAGACTTCGTCTAC
	Reverse primer	<u>atctaga</u> TCAGACGCCACCTGGCGATG
<i>F-BOX</i> cDNA coding region	Forward primer	<u>acc</u> ATGGCATACTTAAGCTTCAAGTC
	Reverse primer	<u>atctaga</u> TTATTGCAAGAAGTCCATAC
<i>ctg10</i> knockout primers	Forward primer	TCACACTTGTGCGTCATACAC
	Reverse primer	GCGAGTCACAAACTCCTTGC
<i>pROC</i> T-DNA primers	Insertion spanning primer	TGGAGGATCGACATCAA▼GTGTAC
	Forward primer (LBb1)	GCGTGGACCGCTTGTGCAACT
	Reverse primer (RB)	GCGGTTCTGTCAGTTCACAAACG
<i>CTG10/19260</i> shared 5'UTR	(F) 188	GATCGGTGTTCGATTAGAAAAC
<i>CTG10/19260</i> shared coding	(F) 182	GCGAGTCACAAACTCCTTGC
19250 GSP "250"	(F) 186	TATAGTCCTAACCTCTCTAGG
19260 GSP "260"	(R) 187	AGTTAGTGAGATAACACATCC
<i>ctg10</i> complement primers	(F)	<u>atgtcgac</u> TAGTCTCATGCCAAGGCACAGC
	(R)	<u>ataccgt</u> CTGTGAAATGAGATTATACGAGAGC
<i>CTG10</i> Y2H with ASKs	(F)	<u>gacaagggtcgacga</u> ATGGCATACTTAAGCTTCAAGTC
	(R)	<u>cgcgtgtccgcgc</u> TTATTGCAAGAAGTCCATAC
Y2H homologous recombination	(F)	gggactacaaggacgacgacgacaagggtcgacga
	(R)	<u>agtccaaagtcacccgcgtggccgcgc</u>

Primers are presented 5' → 3'. (F): Forward primer; (R): Reverse primer; GSP: Gene Specific Primer. Underlined, lowercase nucleotides are non-binding 5' extensions comprising all or part of various restriction enzyme sites and extraneous nucleotides. Uppercase nucleotides depict that part of a primer binding *Arabidopsis* sequences, T-DNA inserted into the *Arabidopsis* genome, or adaptors ligated onto *Arabidopsis* genomic DNA. The symbol interrupting the 'insertion spanning primer' for the 'ctg10 knockout' (▼) denotes the site of insertion of the pBIN T-DNA in the SALK insertional mutant, 104830.

TEIN (RNP) and a *KELCH REPEAT CONTAINING F-BOX (F-BOX)*. Full length cDNA clones of both were verified to exist in *Arabidopsis* and were acquired using a combination of 5'- and 3'-RACE (Gene Racer®, Invitrogen, Carlsbad, CA, USA) of *Arabidopsis* RNA with a variety of gene-specific and adaptor-specific primers (Table 1).

2.5. Transformation and transgenic analysis

The full length gene (including introns if any) of the *RNP* or the *F-BOX* was cloned into a modified pRTL2 vector (double *CaMV35S* promoter, *TEV* translational enhancer; Table 1 primers) and then into the *NotI* sites of pMLBART. Transgenic plants were generated by *Agrobacterium*-mediated transformation using the floral dip method (Clough and Bent, 1998). Transformed plants were selected using BASTA resistance (*RNP-OE*, *F-BOX-OE*) followed by PCR analysis. *RNP* and *F-BOX* expression was analyzed by RT-PCR using suitable primers.

Once identified as causal for the cold temperature germinating phenotype, the *F-BOX* was assigned the COLD TEMPERATURE GERMINATING 10 (*CTG10*) acronym. An insertional mutant in *CTG10* was identified in the SALK T-DNA lines (Alonso et al., 2003); SALK_104830; SALK, San Diego, CA, USA), and seeds were acquired from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). Homozygotes with an insertion in *CTG10* were identified using PCR with gene-specific primers on either side of the insertion site designed using the Salk Institute Genomic Analysis Laboratory (SIGnAL) iSECT tool and outward facing primers to

the right or left arms of the T-DNA (Table 1). Template DNA was acquired from leaf disks using a kit (Extract-N-Amp, Sigma-Aldrich Inc., St Louis, MO, USA) and amplified using RedTAQ (Sigma-Aldrich Inc.). PCR typing was verified using Southern blot with restriction enzymes chosen to leave the *CTG10* gene and inserted T-DNA intact and permitting discrimination among bands produced by the *CTG10* and the two regions of the genome to which the *CTG10*-specific probe would also bind (at and around At4g19250; Fig. 1A–D; Table 2).

To complement *ctg10*, a 3198 bp fragment consisting of 1097 bp promoter, the 1152 bp *CTG10* gene, and 949 bp 3' of the stop codon were introduced into a modified pRTL2 vector (eliminating the 2 x *CaMV35S* promoter and *TEV* translational enhancer) using *Sall* and *AgeI*. *NotI* sites allowed this *CTG10* gene to be subcloned into the corresponding site of pMLBART and subsequently transformed into *ctg10* (Clough and Bent, 1998). Controls consisted of the empty pRTL2 vector (2 x *CaMV35S* promoter and *TEV* translational enhancer only) introduced into *ctg10* plants and homozygotes were selected using BASTA over several generations.

2.6. DNA isolation and Southern blot analysis

Genomic DNA was isolated from wild type (WT) and *ctg10* *Arabidopsis* leaves according to Murray and Thompson (1980). Genomic DNA (5 µg per lane) was exhaustively digested with restriction endonucleases, electrophoresed through a 0.7% (w/v) agarose gel in 40 mM Tris-acetate, 1 mM EDTA, and denatured using alkali

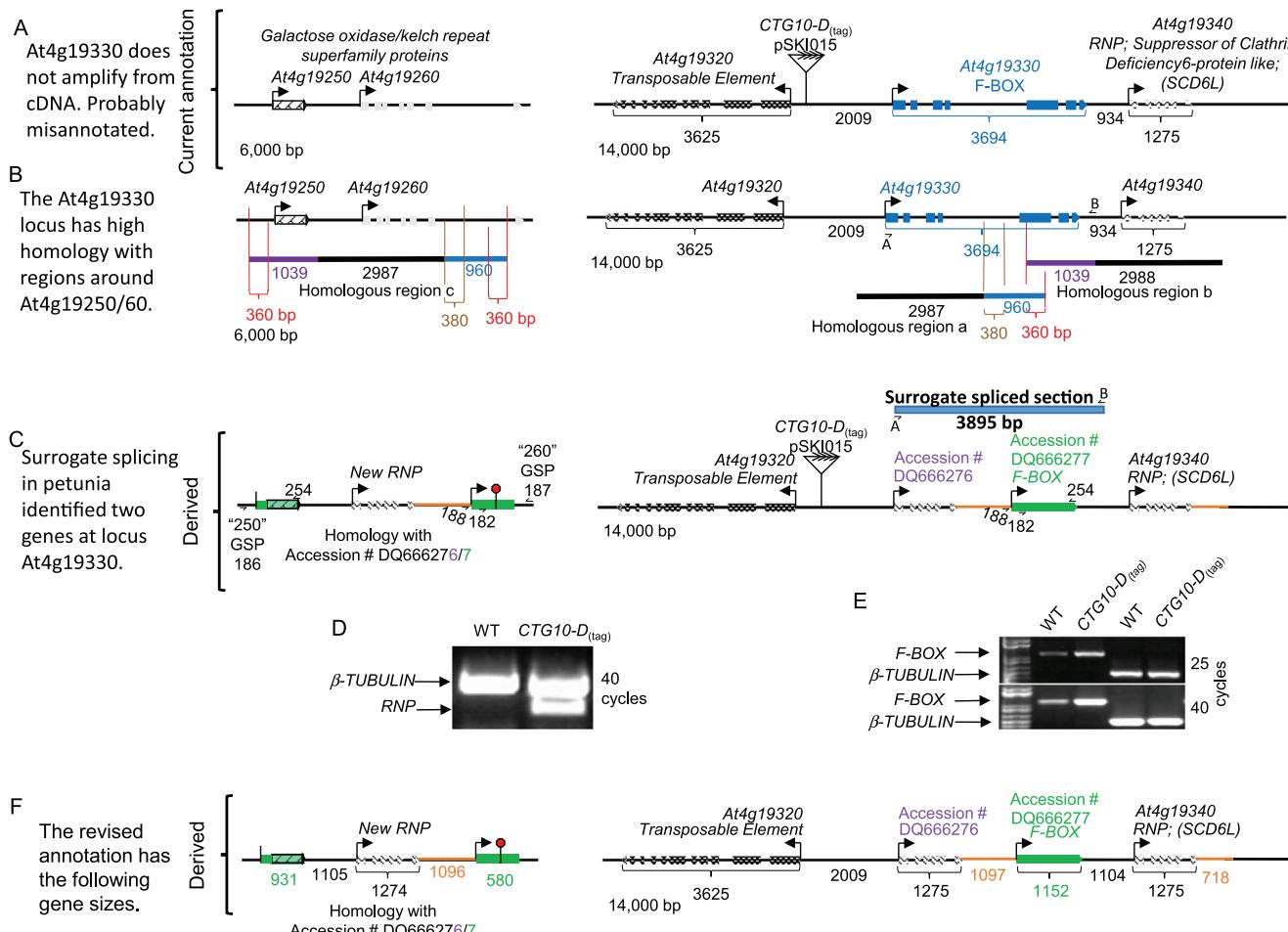


Fig. 1. At4g19330 is misannotated and is actually comprised of two genes. **A)** The site of activation tag insertion (pSKI015, quadruple chevrons) was identified using genome walking on *Sst*I-digested, adaptor-ligated genomic DNA. It inserted between At4g19320, annotated as a suspected transposable element and the 3694 bp At4g19330 locus. It was not possible to amplify the cDNA for At4g19330 in the CTG10_(tag) plants, suggesting it may be misannotated. Transcriptional initiation sites are arrows, thicker bars are exons, lines are introns or intergenic regions. **B)** The region of chromosome 4 into which the pSKI015 T-DNA inserted in the CTG10_(tag) plant contains two 2987/8 m homologous regions (a and b; thick black lines under the chromosomal depiction). A third 2987 nt homologous region (c; thick black line) encompasses At4g19260. Alignments of these three regions (a, b, c) are 99.2% identical over 2987 bp, differing by only 10 nts and 12 insertions/deletions. Regions a and c share an additional 960 bp at their 3' ends (blue lines) that differ by a single base change (C_(region a) → T_(region c)). Regions b and c contain an additional region of 1039 homologous nts at their 5' ends (purple lines), differing in 5 nts and including one insertion/deletion. **C)** Surrogate splicing in petunia (3895 bp surrogate splicing section; large blue bar) identified two genes (Accession #s DQ666276 and DQ666277) comprising the At4g19330 current annotation. One gene, closest to the pSKI015 insertion site in CTG10-D_(tag), is a putative SM-like, RIBONUCLEOPROTEIN (RNPs; identified using InterProScan, (Quevillon et al., 2005)). This RNP gene is nearly identical to the At4g19340 RNP gene produced from homologous region b (1 nt difference in exon 1; Supplemental Fig. 1). Its cDNA is identical to a transcript from homologous region c (New RNP) as these differ only in their introns (2 nt differences in intron 1 and 1 nt difference in intron 3; Supplemental Fig. 1). All three RNPs are represented as hatched exons. Primers are represented in B and C as numbered or capital lettered half-arrows pointing in the direction they elongate. The At4g19330 locus is also comprised of a promoter region (orange line) and an intronless KELCH REPEAT-CONTAINING F-BOX PROTEIN gene (F-BOX; green) terminating earlier than the current annotation. Homologous region c contains two sections homologous to the F-BOX, the 5'-most of which commences + 221 bp 3' to the initiation codon of the F-BOX transcript (green hatched bar overlaying At4g19250). Inclusion of 960 bp of 3' sequence (blue line in B) on homologous region c, recapitulates the F-BOX promoter region (orange lines occurring at three sites in the gene models) and the 5' end of the F-BOX cDNA, with a single C → T base change that results in a premature stop (red octagon at 240 bp). **D)** The like-Sm snRNP gene (RNP) is up-regulated in CTG10-D_(tag) relative to WT as is the F-BOX. Abbreviation is WT for wild type Col-0. **F)** The specific sizes of the genetic elements as delineated in C comprising the re-annotated regions of chromosome 4 are included in this depiction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Sambrook and Russell, 2001). DNA was then transferred to Hybond N⁺ membrane (General Electric Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) using alkaline transfer (Sambrook and Russell, 2001), and the membrane was dried prior to UV cross-linking the DNA fragments at 50 mJ in a GS Gene Linker[®] UV chamber (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After neutralizing, the blot was prehybridized at 65 °C for 12 h in 6 X SSPE (0.9 M NaCl, 60 mM Na₂PO₄, and 6 mM EDTA), 5 X Denhardt's solution ([0.1% (w/v) Ficoll 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA (Fraction V)] (Denhardt, 1966)), 0.5% (w/v) SDS, and 100 µg mL⁻¹ boiled, sheared salmon sperm DNA. Thereafter, hybridization used randomly-labeled (Feinberg and Vogelstein, 1983) radioactive cDNA synthesized in the presence of [α -³²P] dCTP. The probe was first boiled and then snap cooled on ice before introduced into the prehybridization solution.

Membranes were washed twice, 15 min each time, at low stringency (2 X SSC [0.3 M NaCl, 30 mM sodium citrate, pH 7], 0.1% (w/v) SDS at 65 °C) and exposed to a phosphor screen for 2 days. The image was captured using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA, USA).

2.7. RNA isolation and Northern blot analysis

Total RNA from WT and ctg10 seeds was extracted according to Wan and Wilkins (1994). Total RNA (20 µg per lane) was transferred onto Hybond N⁺ membranes (General Electric Healthcare Bio-Sciences Corp.) in 20 X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7) overnight, the blot dried and UV cross-linked (Bio-Rad) to the membrane. After rinsing the membranes for 5 min in 2 X SSC, blots were placed in pre-

Table 2
Predicted restriction enzyme fragment sizes on Southern blot.

Restriction enzyme	Gene		
	At4g19250	At4g19260 3'-end	At4g19330 (No T-DNA)
BglII	2433	3660	4628
HpaI	2379	3423	2287
NdeI	4257	2602	3761
SpeI	11,746	15,245	4627
XbaI	10,274	14,692	4628

The At4g19330 gene without a T-DNA insertion (At4g19330 (No T-DNA)), the homologous region c encompassing At4g19250 (At4g19250) and the 3' region following At4g19260 (At4g19260 3'-end), when cleaved with five different restriction enzymes, are predicted to provide the band sizes (bp) displayed above. Boxed *Hpa*I restriction fragments for At4g19250 and At4g19330 were difficult to differentiate on Southern blot.

hybridization solution (50% (v/v) formamide, 5 X Denhardt's solution (Denhardt, 1966), 100 µg mL⁻¹ boiled, sheared salmon sperm DNA, 0.2% (w/v) SDS, 6 X SSC [0.9 M NaCl, 90 mM sodium citrate, pH 7.0], (Sambrook and Russell, 2001)) for 4–6 h at 42 °C. Randomly primed, radioactive cDNA probes ([α -³²P] dCTP) were hybridized to the blots for at least 12 h at the same temperature as pre-hybridization. The primary wash was done in 2 X SSC, 0.1% (w/v) SDS, room temperature for 5 min, and repeated at 65 °C for 30 min. The two final high stringency washes were in 0.2 X SSC, 0.1% (w/v) SDS at 65 °C for 30 min each. The hybridized probe was detected using a phosphoimaging screen and a PhosphorImager (Molecular Dynamics).

2.8. Yeast two hybrid assay

For yeast two hybrid analysis of the functionality of the F-BOX moiety of CTG10, ASK cDNAs (ASK1 [At1g75950], ASK2 [At5g42190], ASK4 [At1g20140], ASK5 [At3g60020], ASK9 [At3g21850], ASK11 [At4g34210], ASK12 [At4g34470], ASK13 [At3g60010], ASK16 [At2g03190], ASK18 [At1g10230] (Samach et al., 1999)) were expressed as C-terminal fusions to GAL4-AD in pBI771 and transformed into the haploid strain, LB414α. F-Box cDNAs (CTG10, UFO [At1g30950]) and a 12S CRUCIFERIN STORAGE PROTEIN (CRUCI) negative control were expressed as C-terminal fusions to GAL4BD in plasmid pBI770 and transformed into haploid strain, YPB2a. The CTG10 CDS was inserted into pBI770 using two rounds of PCR performed with primers encoding 5' extensions suitable for homologous recombination into YPB2a (Table 1).

Yeast were grown at 30 °C on YPDA or synthetic complete (SC) media (Becton-Dickinson, San Jose, CA, USA). Yeast strains were mated on YPDA media for 24 h and then grown on SC medium lacking leucine and tryptophan (-leu, -trp). Strains were tested for β -galactosidase activity and for resistance to 10 mM 3 amino-1, 2, 4-triazole added to the selective medium (-leu, -trp, -his). In each case 10 µL of culture with an OD₆₀₀ 1 × 10⁻³ were spotted and grown at 30 °C for 4 days.

2.9. Statistical analysis

Comparisons were performed between WT seeds and those of the insertional mutant *ctg10* (retrieved from the segregating population identified in SIGnAL (SALK) and obtained from ABRC (Ohio State University)) and among the CTG10 over-expressing lines and the vector controls. The percentage germination of independent lines of *ctg10* complemented with an empty vector or with CTG10 were compared on 10 µM paclobutrazol. Differences in percentage germination at representative time points after imbibition assessed at 25 °C were subjected to analysis of variance using the ANOVA procedure of SAS® version 9.3 software. If the ANOVA indicated that there were significant differences among means, Tukey's or Duncan's mean separation test was used to distinguish among them at $\alpha = 0.05$.

3. Results

3.1. The At4g19330 locus is composed of two genes, not one as currently annotated

The CTG10-D_(tag) line was originally identified in an activation tag screen searching for seeds capable of more rapid completion of germination at 10 °C (Salaita et al., 2005). The maintenance of the cold temperature germinating phenotype in heterozygous, F₁ seeds from the original, activation tagged CTG10-D_(tag) line when crossed with WT Col-0, and segregation analysis of BASTA resistance in F₂ seeds from this cross, suggested that the phenotype was caused by the dominant hyper-expression of a gene in the proximity of a single, activating T-DNA (Salaita et al., 2005). The site of T-DNA insertion was identified using genome walking on *Stu*I-digested, adaptor-ligated, genomic DNA (Fig. 1A). A 736-bp amplicon was retrieved from CTG10-D_(tag) plants that consisted of 215 bp of the left T-DNA border and 485 bp of Arabidopsis genomic DNA terminating in half of a *Stu*I site prior to 36 bp from the adaptor. This region resided on chromosome 4 between At4g19320, annotated as a transposable element and, as subsequent investigations determined, not up-regulated in CTG10-D_(tag) plants, and At4g19330, previously annotated as a KELCH REPEAT CONTAINING F-BOX gene (F-BOX, Fig. 1A). The quadruple CaMV 35S promoter battery used to drive activation tag expression was aligned with the F-BOX gene (Fig. 1A) and 5'- and 3'-fragments of the cDNA were demonstrably up-regulated in CTG10-D_(tag) relative to WT. However, repeated attempts to amplify the entire F-BOX cDNA or coding region from a variety of tissues failed using primers that spanned the full length of the predicted transcript. Further investigation led to the conclusion that the F-BOX gene was probably misannotated because no full length EST clones were identified in several database searches and an alternative annotation for At4g19330 existed (Gagne et al., 2002).

Using NCBI-BLAST for short, nearly exact matches it became apparent that any primers designed to scan this region of the Arabidopsis genome for legitimate cDNAs would have difficulty distinguishing among several potential transcripts. These sites clustered on chromosome 4 (on contiguous fragment number 50 [GenBank accession AL161550] and BAC clone T5K18 [GenBank accession AL022580]). This region of chromosome 4 contains three homologous regions (Fig. 1B, regions a–c, and Supplemental Fig. 1). Alignments of these three regions are 99.2% identical over 2987 bp, differing by only 10 nts and 12 insertions/deletions. Regions a and c share an additional 960 bp at their 3' ends that differ by a single base change (C_(region a) → T_(region c); Fig. 1B). Regions b and c contain an additional region of 1039 homologous nts at their 5' ends, differing in 5 nts and including one insertion/deletion (Fig. 1B). Despite this degree of similarity, prediction resulted in three different gene annotations in the three regions, represented by At4g19250, At4g19330, and At4g19340 (current annotation; Fig. 1A). Due to these large regions of homology around At4g19330, any attempt to clarify At4g19330 transcripts using primer scanning and rapid amplification of cDNA ends (RACE) in Arabidopsis was deemed to be difficult (Fig. 1B).

A surrogate splicing procedure (Wu et al., 2005) in a species removed from Arabidopsis was employed to clone the At4g19330 cDNA(s). A 3895 bp region amplified from genomic DNA, encompassing At4g19330 (Fig. 1B), was cloned into an over-expression vector and introduced into petunia (*Petunia X hybrida*) leaves using *Agrobacterium* infiltration (Wu et al., 2005). Following RNA extraction, DNase I treatment, and reverse transcription, a battery of primers was used to define two separate mRNAs produced from the At4g19330 locus (Fig. 1C). The transcriptional start sites and poly-adenylation sites were identified by 5'- and 3'-RACE. The mRNA produced from the gene lying closest to the promoters in CTG10-D_(tag) has highest homology to a LIKE-SM SMALL RIBONUCLEAR PROTEIN (RNP; (Quevillon et al., 2005); GenBank accession number DQ666276) (Fig. 1C). The more 3' gene, further from the promoters in CTG10-D_(tag), is intronless and

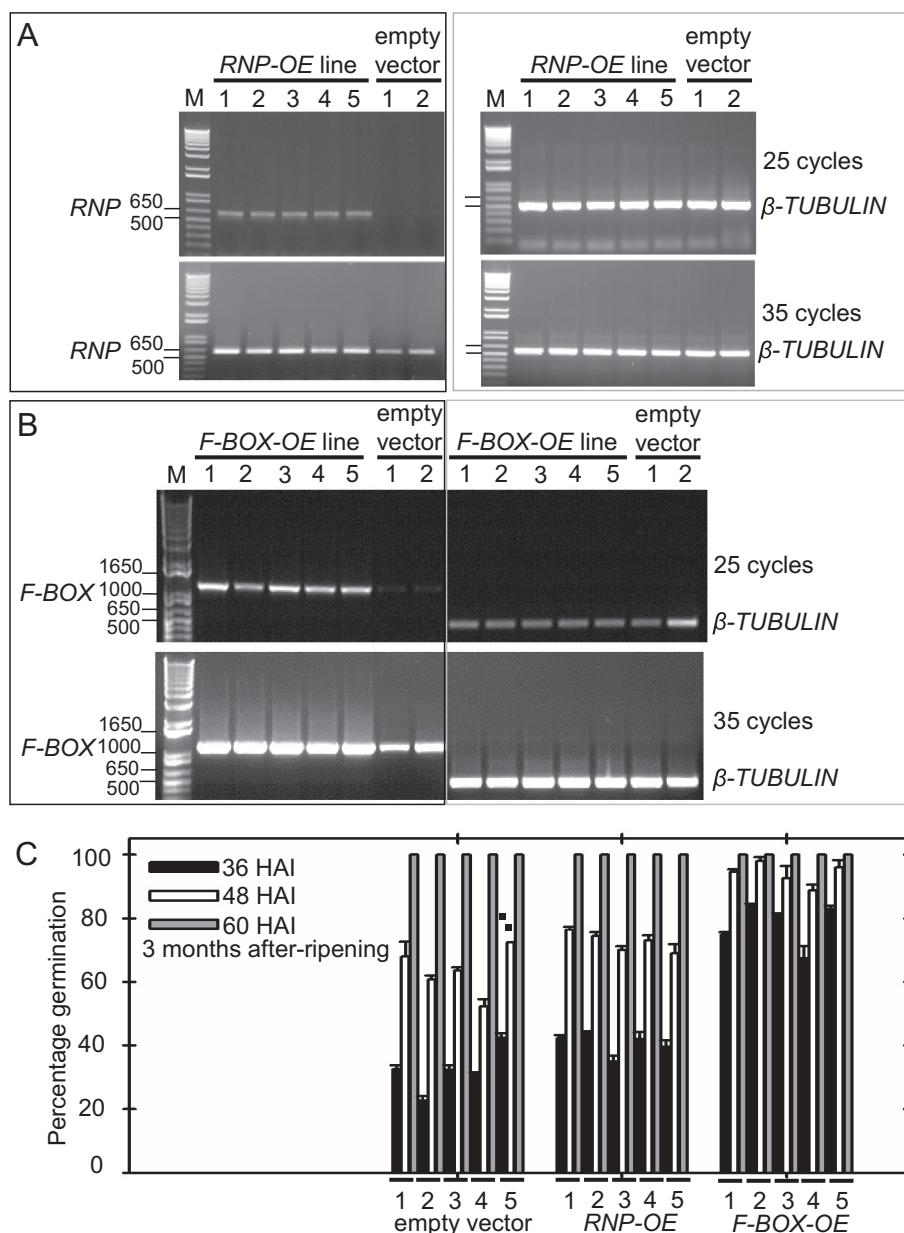


Fig. 2. The KELCH-REPEAT CONTAINING F-BOX, not the RIBONUCLEOPROTEIN is responsible for the *CTG10-D_(tag)* germination phenotype. Independent lines over-expressing either A) *RNP* (*RNP-OE*) or B) the *F-BOX* (*F-BOX-OE*) produced more of each respective transcript (as determined by RT-PCR) than empty vector control lines whereas β -TUBULIN transcript amounts remained uninfluenced. C) The similarity of average percentage germination values among genotypes within each designated time point (HAI represents hours after imbibition) was assessed using ANOVA and significantly deviating means were identified using Duncan's multiple pairwise comparison with an experiment-wide error rate of $\alpha = 0.05$. The standard error of the mean ($n = 3$) is depicted by a capped line emanating from each bar. All *F-BOX-OE* but no *RNP-OE* lines completed germination significantly more rapidly than empty vector control lines following 3 months after-ripening. Average percentage germination for all *F-BOX-OE* seeds, but not those of any of the *RNP-OE* lines, was statistically significantly greater at both 36- and 48-HAI relative to the best empty vector control (line 5, offset dots over bars).

produces a transcript encoding a KELCH REPEAT CONTAINING F-BOX protein (GenBank accession number DQ666277) (*F-BOX*, Fig. 1C).

Once specific transcript sequences had been identified, the production of cDNA from these two transcripts from the At4g19330 locus could be attempted in *Arabidopsis* using RT-PCR. It was impossible to distinguish between the *RNP* transcripts produced from homologous regions a or c (Fig. 1B; Supplemental Fig. 1). The *RNP* encoded by homologous region b produces a cDNA that differs from those of regions a and c by a single base in the first exon ($A_{\text{regions a,c}} \rightarrow T_{\text{region b}}$) (Supplemental Fig. 1). At4g19260 (in homologous region c; Fig. 1B) can be spliced to generate the same *RNP* transcript produced by homologous region a (Fig. 1B, C). The nucleotides 3' of the new At4g19260 *RNP* stop codon and the 960 bp 3'-extended area of homology (blue in Fig. 1B) reiterates the 1096 bp promoter of the *F-BOX* (with 1 insertion/

deletion; orange lines in Fig. 1C, F). It is also homologous with the first 580 bp of the *F-BOX* coding region (with a single C \rightarrow T change at nt 240 resulting in a premature stop; Fig. 1C, F). Similarly, the 1039 bp 5'-extended region of homology including At4g19250 on homologous region c, reconstitutes the 3' end of the *F-BOX* on homologous region b, commencing 221 nts into the open reading frame and proceeding to the stop codon with a single deletion (at nt 232, changing the reading frame) and one base change (Fig. 1B, C, F).

Based on the difference in *RNP* amplicon abundance following PCR of reverse transcribed RNA from WT and *CTG10-D_(tag)* plants, either the specific *RNP* gene which is 5' to the *F-BOX* gene (Fig. 1D), or both *RNP* genes on homologous regions a and b, is(are) up-regulated in *CTG10-D_(tag)* plants (Fig. 1D). Primers capable of selectively amplifying the *F-BOX* cDNA encoded by the 3' end of At4g19330 determined that it was

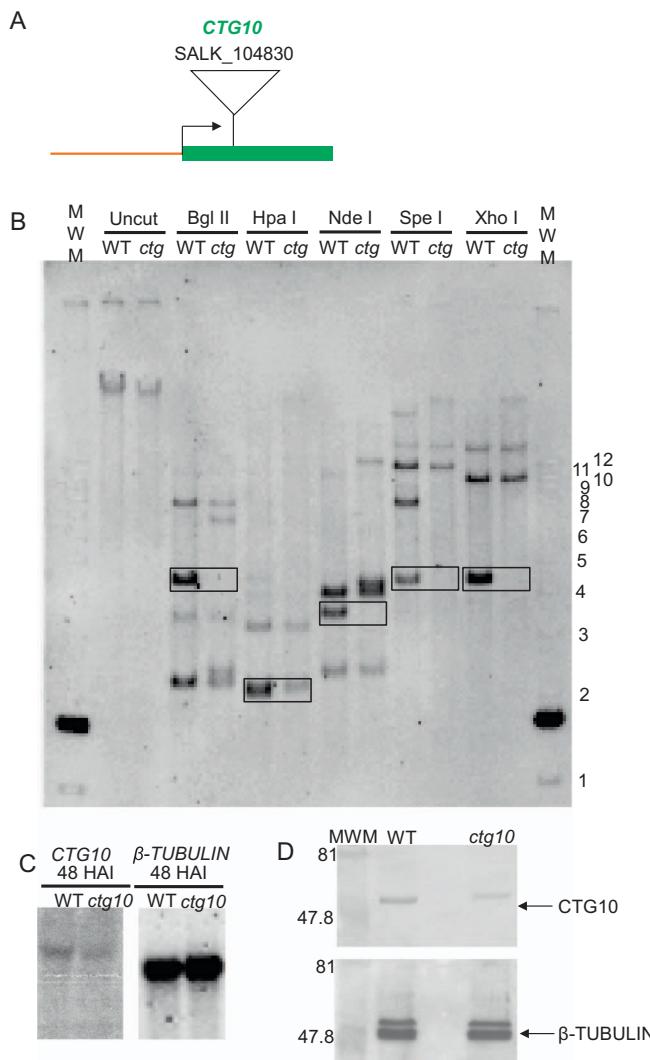


Fig. 3. The CTG10 mRNA and protein persist in the *ctg10* mutant. **A**) A cartoon of the *CTG10* locus showing the promoter (orange line), intronless gene (green bar), and location of the T-DNA insertion (SALK_104830) situated 359 nts 3' of the commencement of the coding sequence, 793 nts from the stop codon. **B**) Southern blot with restriction enzymes capable of distinguishing between *CTG10* and the homologous regions encoded in the genome at and around At4g19250 (Fig. 1, Table 2) probed with a *CTG10* cDNA probe, indicated that the SALK line was homozygous for the T-DNA insertion (*CTG10*-specific regions boxed); however, **C**) Northern blot of 48 HAI, dark-imbibed seeds, probed with the same probe as in **A**, indicated that full length *CTG10* mRNA was still formed in the T-DNA insertional mutant acquired from SALK, albeit at slightly lower amounts than WT. The blot was also probed with β -TUBULIN to demonstrate equal loading. **D**) Western blots of proteins from dry seed of WT and *ctg10* probed with *CTG10* antibody indicated that the *ctg10* plants still had *CTG10* present, although at lower amounts, relative to WT. Blots were also probed with TUBULIN antibody to determine the equality of the protein amounts loaded between the two genotypes. Both were developed colorometrically. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

also up-regulated in *CTG10-D_(tag)* plants (primers 188 \times 254 or 182 \times 254; Fig. 1C, E; Table 1). The reannotation of At4g19330 into two genes was partially supported by Illumina polyadenylation tags (PATs; Wu et al., 2011) from transcripts that were located in the region of the current annotation designated as the sixth (last) At4g19330 intron (Supplemental Fig. 2). Although tags 3' to the last exon of the current At4g19330 annotation are also present, they may also belong to DQ666277 as polyadenylation clusters (PACs) are not uncommon in *Arabidopsis* (Wu et al., 2011). No PATs specific for transcripts from the *RNP* gene at the 5' end of the current annotation of At4g19330 were observed in seed mRNA (Supplemental Fig. 2). However, evidence

supporting the designation of At4g19260 as another *RNP* homolog was forthcoming from the identification of PATs from seed mRNA located in the sixth (last) intron of At4g19260 in the current annotation. No PATs were identified (at least in seed mRNA) for transcript that would be produced from the current annotation (i.e. 3' to the seventh exon, Supplemental Fig. 3).

The three RNP proteins have been studied to some extent and form a clade of paralogs among the 42 members of the Sm RNP family in *Arabidopsis* (Cao et al., 2011). That they are all present in this single clade underlines their considerable homology; even among their genomic sequences, both exons and introns are nearly identical (Supplemental Fig. 1).

Examining species' genomes using Phytozome v11.0, there are intronless, open reading frames of putative *CTG10* orthologs present in both *Arabidopsis lyrata* (v1.0: scaffold_7:10266181..10271377 reverse) and *Capsella rubella* (v1.0: Carubv10006603m.g scaffold_7:8277913..8281167 reverse); although, in these two species, the annotations follow the current annotation existing in *Arabidopsis thaliana* for At4g19330, probably propagating the error. Using BLINK, a query identified a putative *CTG10* ortholog in *Camelina sativa* (NC_025695.1, LOC104727919; (Kagale et al., 2014)). The *C. sativa* locus was annotated in a manner consistent with our reannotation of *CTG10* in *A. thaliana* presented here.

3.2. The F-BOX encoded by the At4g19330 locus is responsible for the seed germination phenotype

Upon the resolution of the two genes in this region of chromosome 4, the primary objective remained to ascertain if up-regulation of the *RNP* or *F-BOX* was responsible for the germination phenotype distinguishing *CTG10-D_(tag)* from WT (Salaita et al., 2005). Multiple independent, over-expression lines were constructed for both genes and tested against empty vector control lines. Five independently transformed lines were chosen for *RNP*, the *F-BOX*, and two for the empty vector; and the *RNP* and *F-BOX* transgenes were verified to result in greater than usual transcript accumulation while the empty vector did not enhance the abundance of either transcript relative to WT (Fig. 2A, B, and data not shown). Plants over-expressing the *RNP* did not vary from the vector control in any germination attribute (Fig. 2C). Following 3 months dry storage at room temperature (after-ripening), there was no difference in the final percentage germination between seeds from plants over-expressing the *F-BOX* and vector control plants; however, the speed with which the seeds from *F-BOX-OE* plants completed germination was superior to that of the vector control lines, commencing before 36 HAI and being virtually complete by 48 HAI (Fig. 2C). Furthermore, following 2 weeks after-ripening, seeds harvested from plants over-expressing the *F-BOX* gene completed germination to no less than 75% within 5 days of being placed on water while seeds from vector control lines completed germination in this time to only 20% or less (data not shown). Based on these results, the gene encoding the FBP has been named COLD TEMPERATURE GERMINATING10 (*CTG10*; GenBank accession DQ666276), and will be referred to as *CTG10* in the following text (Fig. 1F).

If an over-abundance of *CTG10* results in faster than usual completion of germination, reduction of *CTG10* amounts should detrimentally influence seed germination percentage and or speed. A T-DNA insertional mutant in the *CTG10* coding region was identified in the SALK SIGNAL database (Alonso et al., 2003) and acquired from The *Arabidopsis* Biological Resource Center (ABRC, The Ohio State University, Columbus, OH, USA; Fig. 1F). Plants homozygous for the insertion (*ctg10*, Fig. 3A) were identified using PCR of genomic DNA (data not shown) and verified using Southern blot (Fig. 3B). Despite the insertion in the single exon of *ctg10*, it only resulted in reduction (not elimination) of *CTG10* transcript and protein (Fig. 3C, D).

Neither the percentage nor speed of seed germination was influ-

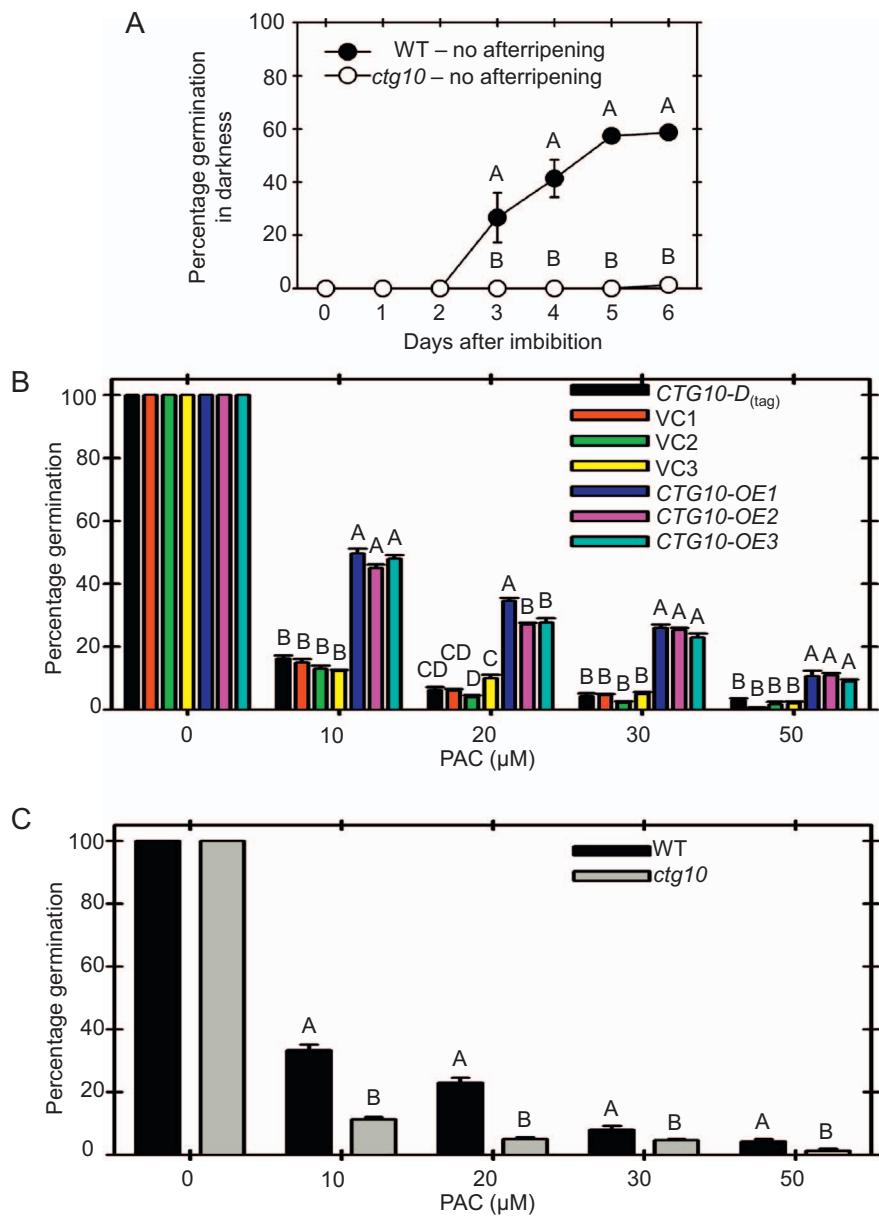


Fig. 4. The *ctg10* seeds complete germination to a lesser percentage than WT in darkness and are hypersensitive to the GA biosynthetic inhibitor, paclobutrazol. A) Freshly harvested seeds from siliques (approximately 18 days after anthesis) of a *ctg10* knockdown imbibed on water are delayed in completing germination in constant darkness. Three month old, afterripened seeds of; B) the original *CTG10-D_(tag)* line, independent *CTG10-OE* lines, and empty vector controls (VC) or; C) the *ctg10* and WT, were sown on various concentrations of paclobutrazol (without prior moist chilling) and incubated in constant light at 25 °C. The germination percentage was calculated 7 days after the start of imbibition. B) Direct overexpression of *CTG10* (without the intervening intergenic regions and D0666276 RNP; see Fig. 1A and B) results in paclobutrazol hypersensitivity while; C) a decrease in *CTG10* amounts has the opposite effect. The average percentage germination values and standard error bars (n = 3) are depicted. The values either among (B) or between (C) genotypes within a designated paclobutrazol concentration were assessed using ANOVA and significantly deviating means (distinguished with different uppercase letters) identified using Tukey's multiple pairwise comparison with an experiment-wide error rate of $\alpha = 0.05$.

enced by the T-DNA insertion when afterripened seeds were germinated along with WT controls at 25 °C under constant light or under alternating 12-h cycles of light and dark (data not shown). However, freshly harvested *ctg10* seeds that were not moist chilled were significantly more delayed completing germination in darkness than were WT control seeds harvested and treated in the same manner (Fig. 4A).

A role for *CTG10* in GA-stimulated seed germination was initially discounted based on the seemingly normal GA sensitivity of the original activation tagged line, *CTG10-D_(tag)* (Salaita et al., 2005). However, germination of seeds from independent *CTG10-OE* lines is less, and that of *ctg10* seeds more, sensitive to repression by the GA biosynthetic inhibitor, paclobutrazol than are empty vector control or WT seeds, respectively (Fig. 4B and C). This is not the case for seeds from the

CTG10-D_(tag) which are sensitive to paclobutrazol to the same degree as the vector control lines (Fig. 4B). The original activation tagged mutant (*CTG10-D_(tag)*) was reported to be similar to WT in its sensitivity to paclobutrazol (Salaita et al., 2005) and this result was confirmed in this study (Fig. 4B).

A complementation approach was pursued, where the sensitivity of *ctg10* seed germination percentage to 10 μM paclobutrazol could be rescued by introduction of the *CTG10* promoter, gene, and 3' end fragment but not by an empty vector control (Fig. 5A).

3.3. The product of the F-BOX gene has the capacity to interact with *Arabidopsis* SKiP-like proteins

A variety of prediction analyses and published literature (Gagne

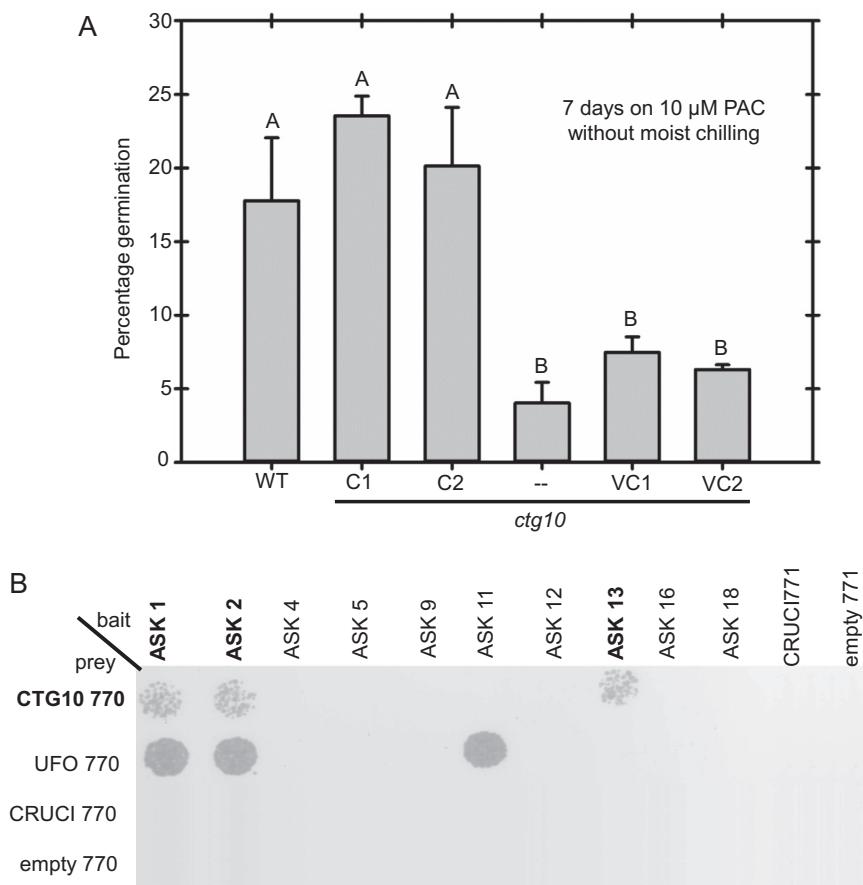


Fig. 5. Completion of germination on 10 μ M paclobutrazol of the *ctg10* mutant is rescued by complementation with *CTG10* and *CTG10* possesses a functional F-BOX region. A) WT Col-0 seeds, incubated on 10 μ M paclobutrazol for 7 days in constant light completed germination to the same percentage as two independent lines of the *ctg10* mutant complemented with *CTG10* (C1, C2: *CTG10*-complemented lines). The uncomplemented *ctg10* mutant completed germination statistically significantly less than WT, C1, or C2, as did two independent lines complemented with an empty vector (VC1, VC2: vector control-complemented *ctg10*). The final percentage germination on 10 μ M paclobutrazol of the VC lines were not statistically different from the uncomplemented *ctg10* mutant seeds (—). Averages are from 3 replications and error bars depict the standard error. Significantly deviating average percentage germination was identified using ANOVA and Duncan's multiple pairwise comparison (experiment-wise error rate of $\alpha = 0.05$) and are represented by different uppercase letters. B) Using a yeast two hybrid approach, the predicted F-Box domain at the N-terminus of *CTG10* interacts with three of 10 *Arabidopsis* SKP1-like protein (ASK) tested, namely ASK1, 2, and 13. Abbreviations used are: UFO, UNUSUAL FLORAL ORGANS; CRUCI, 12S CRUCIFERIN storage protein; empty 770, empty vector pBI770; 771, pBI771.

et al., 2002) suggested that *CTG10* encoded an FBP. An assessment of this prediction using yeast two-hybrid analysis with 10 different *Arabidopsis* SKP1-like proteins and appropriate controls (ASK1 and ASK2 with UFO (Samach et al., 1999)), provided evidence supporting this contention with three of the 10 ASKs tested resulting in a positive interaction with *CTG10* (Fig. 5B).

4. Discussion

While the archived genome sequence of any organism remains static once completed, the annotation of that genome is a perpetual work-in-progress (Bradbury et al., 2013; Seaver et al., 2014). A wealth of new biological information (splicing variants, alternative transcriptional start- or polyadenylation-sites, RNA edits, pseudogenes, etc.) is continuously being discovered and added to the existing annotations. This higher level information, superimposed on the raw sequence of the genome, is of fundamental importance to endeavors to understand the function of gene products encoded by an organism (Ashurst and Collins, 2003). For more complex information to accrue for any locus, the existence and transcriptional boundaries of that locus must be verified from experimental data. However, in those genomes that are completely sequenced, there currently exist numerous computer-based annotations that are without experimental support. A variety of potential errors in genome-wide annotations, and in annotations of well-studied genes, have been described (Goesmann et al., 2002; Linial, 2003; Khatri

et al., 2005; Pfeiffer and Oesterhelt, 2015). Given sufficient homology, instances of misidentification of which gene is transcribed to produce a specific mRNA exist (Laird et al., 2004). Misannotations in one genome which have been propagated to others are so-called “transitive annotation errors” (Schinoes et al., 2009).

Surrogate splicing has been used previously to acquire cDNAs from genes belonging to highly homologous gene families containing multiple introns for functional analysis of the predicted proteins (Wu et al., 2005). These authors were cognizant of the potential of surrogate splicing to assist in delimiting genes, identifying transcriptional boundaries, and defining correct splice sites in regions of genomes they described as ‘problematic’. The current study benefited from the ability of surrogate splicing to correctly identify two transcriptional units (genes) from genomic DNA misannotated to represent one gene (At4g19330). While in most circumstances such a discovery would be most easily accomplished using primer scanning and 3' and 5'-RACE on RT-RNA from the organism of interest, highly homologous (identical) regions in the *A. thaliana* genome frustrated these endeavors. In addition to the surrogate splicing data, excellent support of the revised annotations proposed here was acquired from libraries of RNA-Seq tags focused on the polyadenylation sites of transcripts from *Arabidopsis* (Wu et al., 2011).

Using the information acquired by surrogate splicing, it became apparent that the RNP annotated on homologous region b (At4g19340; *SUPPRESSOR OF CLATHRIN DEFICIENCY6-PROTEIN LIKE PROTEIN*;

(*SCD6L*) could be recapitulated from regions a and c. Indeed, petunia cells produced just such a transcript from a genomic DNA fragment encompassing homologous region a. If homologous region c is spliced in the same manner, and the 1039 bp 5'- and 960 bp 3'-extended regions of homology are included, it is evident that fragments of the *CTG10* gene (DQ666277), including its promoter, were present on either side of the RNP on homologous region c. It would seem that this area of chromosome 4 has undergone substantial alterations leading to partial length copies of the *CTG10* gene residing around At4g19260.

The discovery that there were two genes present in the At4g19330 locus and that both were up-regulated in the *CTG10-D*_(tag) line led to uncertainty with regards to which gene product was responsible for faster completion of germination, the phenotype of interest. Over-expression of each gene separately demonstrated that the *F-BOX* gene product was capable of affecting rapid seed germination when over-produced and hence was named *CTG10*. The consequences on transcript and protein abundance of T-DNA insertion into the coding sequence of *CTG10* were slight. Nevertheless, a negative seed germination phenotype for *ctg10* seeds in darkness or on paclobutrazol further convinced us that *CTG10* is involved in stimulating seed germination.

The difference in paclobutrazol sensitivity between the *CTG10-D*_(tag) line from the screen (Salaita et al., 2005) and the *CTG10-OE* lines subsequently produced (Fig. 4B) is possibly attributable to the difference in the position of the CaMV35S promoters in these two genotypes. It was previously noted that the *CTG10-D*_(tag) seeds exhibited a normal sensitivity to paclobutrazol (Salaita et al., 2005). While this has been verified for the original line in this report, over-expression of *CTG10* by placing the CaMV35S promoters immediately upstream of the gene (independent *CTG10-OE* lines) rather than over 4000 bp distant (*CTG10-D*_(tag)) results in paclobutrazol hyposensitivity (Fig. 4B). The *CTG10-OE* lines, previously thought to be acting on seed germination in a manner divorced from alteration in the abundance or sensitivity to plant hormones (Salaita et al., 2005), may in fact be predisposing the seeds to respond to GA. The FBPs SLEEPY (McGinnis et al., 2003) and SNEEZY (Strader et al., 2004) act in just such a manner, enhancing GA sensitivity during germination by targeting the DELLA proteins for poly-ubiquitination and degradation by the 26S proteasome.

5. Conclusions

The ubiquitin/26S proteasomal proteolytic pathway is one of the most elaborate regulatory mechanisms in plants, components of which are estimated to be encoded by greater than 1400 genes in the model plant *Arabidopsis* (Smalle and Vierstra, 2004). One means by which proteins are recognized for (poly)ubiquitination involves SCF E3 ligases with FBPs imparting substrate specificity to the process. The diversity of FBPs is considerable in plants (Gagne et al., 2002) in which the pathway plays a key role in many cellular processes (Xie et al., 1998; Samach et al., 1999; Zhao et al., 1999; Callis and Vierstra, 2000; Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

It is reasonable to expect protein degradation to play a very important role in orthodox seed germination. The latter phase of seed germination is a period when proteins associated with seed maturation and longevity in the dehydrated state (oleosins, late embryogenesis abundant proteins, etc.) cease to have a meaningful role and/or to be required in the abundance needed during seed maturation. Literature documenting transcriptional reprogramming (Zlatanova and Ivanov, 1988) and associated alterations in protein populations in the transition from seed development to maturation desiccation (Blackman et al., 1991; Finnie et al., 2002), and from quiescence and/or dormancy to germination leading to radicle protrusion (Gallardo et al., 2001; Gallardo et al., 2002; Maltman et al., 2002) abound. Although exceptions have been documented (Kamura et al., 1998), presumably the *CTG10* protein identified here derives at least part of its biological function from its activity during the seed germination event through integration with a(several) SCF complex(es). Based on the accumulated

evidence, *CTG10* probably functions to remove a(several) protein(s) negatively influencing seed germination.

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Appendix A. Supplementary data

Supplemental Fig. 1: Clustal alignment of the RIBONUCLEOPROTEIN genes present in each homologous region a–c.

Supplemental Fig. 2: Illumina poly(A) tags identify a series of polyadenylation sites downstream of the experimentally derived annotation for the *CTG10 F-BOX*.

Supplemental Fig. 3: Illumina poly(A) tags identify a polyadenylation cluster downstream of the experimentally derived annotation for At4G19260. Supplementary data associated with this article can be found in the online version, at doi:<http://dx.doi.org/10.1016/j.plgene.2017.05.012>.

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