Natural Variation in Sensitivity to a Loss of Chloroplast Translation in Arabidopsis[1][W][OPEN]

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Mutations that eliminate chloroplast translation in Arabidopsis (*Arabidopsis thaliana*) result in embryo lethality. The stage of embryo arrest, however, can be influenced by genetic background. To identify genes responsible for improved growth in the absence of chloroplast translation, we examined seedling responses of different Arabidopsis accessions on spectinomycin, an inhibitor of chloroplast translation, and crossed the most tolerant accessions with embryo-defective mutants disrupted in chloroplast ribosomal proteins generated in a sensitive background. The results indicate that tolerance is mediated by *ACC2*, a duplicated nuclear gene that targets homomeric acetyl-coenzyme A carboxylase to plastids, where the multidomain protein can participate in fatty acid biosynthesis. In the presence of functional *ACC2*, tolerance is enhanced by a second locus that maps to chromosome 5 and heightened by additional genetic modifiers present in the most tolerant accessions. Notably, some of the most sensitive accessions contain nonsense mutations in *ACC2*, including the "Nossen" line used to generate several of the mutants studied here. Functional ACC2 protein is therefore not required for survival in natural environments, where heteromeric acetyl-coenzyme A carboxylase encoded in part by the chloroplast genome can function instead. This work highlights an interesting example of a tandem gene duplication in Arabidopsis, helps to explain the range of embryo phenotypes found in Arabidopsis mutants disrupted in essential chloroplast functions, addresses the nature of essential proteins encoded by the chloroplast genome, and underscores the value of using natural variation to study the relationship between chloroplast translation, plant metabolism, protein import, and plant development.

Embryo development in Arabidopsis (*Arabidopsis thaliana*) requires the coordinated expression of a large number of essential genes (Muralla et al., 2011). Recessive mutations that disrupt these nuclear genes result in an embryo-defective (*emb*) mutant phenotype (Meinke, 2013). Many *EMB* genes of Arabidopsis encode chloroplast-localized proteins involved in basic metabolism, protein import, and chloroplast gene expression (Hsu et al., 2010; Bryant et al., 2011; Savage et al., 2013). Functional plastids are therefore required for embryo development in Arabidopsis. Mutations that disrupt photosynthesis alone interfere with embryo and seedling pigmentation, not embryo development. Multiple examples of *EMB* genes that encode chloroplast-localized aminoacyl-tRNA synthetases, RNA-binding proteins, translation factors, and ribosomal proteins have been described in the literature (Berg et al., 2005; Bryant et al., 2011; Muralla et al., 2011; Romani et al., 2012; Tiller and Bock, 2014). Translation of some chloroplast-encoded mRNAs is therefore essential for seed development. This raises a basic question: which chloroplast genes are required? In this report, we used natural variation and genetic analysis to evaluate the model (Bryant et al., 2011) that a single chloroplast gene, *acetyl-coenzyme A carboxylase D* (*accD*), needed for the initial stages of fatty acid biosynthesis, underlies the requirement for chloroplast translation during heterotrophic growth and embryo development in Arabidopsis.

Targeted gene disruptions in tobacco (*Nicotiana tabacum*) have identified four chloroplast genes with essential functions that extend beyond photosynthesis: *accD*, *caseinolytic protease P1* (*clpP1*), hypothetical chloroplast open reading frame1 (*ycf1*), and *ycf2* (Drescher et al., 2000; Kuroda and Maliga, 2003; Kode et al., 2005). Comparative genomics have shown that all four genes are retained in the plastid genomes of most angiosperms, including chlorophyll-deficient, parasitic species (dePamphilis and Palmer, 1990; Funk et al., 2007; Jansen et al., 2007). Several examples of essential chloroplast genes that relocated to the nucleus have also been described (Magee et al., 2010; Rousseau-Gueutin et al., 2013). The absence of *ycf1* and *ycf2* in grasses (Jansen et al., 2007) and the replacement of *accD* with a nuclear gene that targets functional protein back to the chloroplast (Konishi and Sasaki, 1994; Chalupska et al., 2008) remain to be explained.

The *accD* gene in Arabidopsis (AtCg00500) encodes one subunit of the chloroplast-localized heteromeric acetyl-coenzyme A carboxylase (ACCase), an essential enzyme in fatty acid biosynthesis that converts acetyl-CoA to malonyl-CoA. Three other subunits are encoded by nuclear genes, one of which is also known to be required for embryo development (Li et al., 2011). Disruptions of three additional genes (At3g25860, At1g34430, and At2g30200) associated with the reactions that precede and follow the step catalyzed by heteromeric ACCase also result

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in embryo lethality (Lin et al., 2003; Bryant et al., 2011; Muralla et al., 2011). Embryo lethality is also encountered in auxotrophic mutants unable to produce biotin, an essential vitamin required for ACCase function (Schneider et al., 1989; Patton et al., 1998; Muralla et al., 2008). The conversion of acetyl-CoA to malonyl-CoA during fatty acid biosynthesis within the plastid is therefore required for embryo development in Arabidopsis.

In addition to the chloroplast-localized, heteromeric ACCase found in most angiosperms, there is also a cytosolic, homomeric ACCase involved in later stages of fatty acid biosynthesis. In both Arabidopsis and *Brassica napus*, the gene that encodes this homomeric enzyme is duplicated (Yanai et al., 1995; Schulte et al., 1997). One copy (*ACC1*; At1g36160) encodes an essential protein localized to the cytosol. Disruption of this gene in Arabidopsis (*EMB22*, *GURKE*, and *PASTICCINO3* [*PAS3*]) results in an embryo-defective phenotype distinct from that seen following a loss of chloroplast translation (Meinke, 1985; Baud et al., 2004). Weak alleles exhibit cold sensitivity and glossy inflorescence stems resulting from changes in cuticular wax composition (Lü et al., 2011; Amid et al., 2012). The adjacent copy (*ACC2*; At1g36180) is expressed at low levels and is predicted to encode a chloroplast-localized protein (Yanai et al., 1995; Baud et al., 2003; Babiychuk et al., 2011). Knockouts of this gene exhibit no obvious phenotype under normal growth conditions (Babiychuk et al., 2011).

In *Brassica* spp., plants with albino leaves devoid of chloroplast ribosomes have been produced by germinating seeds on spectinomycin, an inhibitor of chloroplast translation, and then transplanting the young seedlings to basal medium (Zubko and Day, 1998). This experimental approach was initially described as a promising system for generating stable albinism without mutagenesis. However, different results were obtained with tobacco and Arabidopsis seedlings, which were much more sensitive to spectinomycin. In light of this reported variation in seedling responses to spectinomycin and the known duplication of *ACC1* in the Brassicaceae, we decided to explore whether natural accessions of Arabidopsis differed in their ability to tolerate a loss of chloroplast translation and whether genetic analysis in Arabidopsis could uncover some of the genes involved. The results described here confirm the value of this approach, provide insights into the phenotypes of mutants defective in essential chloroplast functions, and help to explain the requirement of chloroplast translation for plant growth and development.

RESULTS

Arabidopsis Accessions Differ in Seedling Sensitivity to Spectinomycin

Several factors were considered before deciding which accessions to evaluate on spectinomycin: geographical location, genetic diversity (McKhann et al., 2004; Nordborg et al., 2005; Clark et al., 2007), inclusion among mutants defective in chloroplast translation (Bryant et al., 2011), availability of genomic sequence data (Weigel and Mott, 2009), and flowering time. Emphasis was placed on early-flowering accessions to facilitate genetic analysis. A list of accessions tested is presented in Supplemental Table S1. Spectinomycin was chosen to inhibit chloroplast translation based on past work (Zubko and Day, 1998; Dudas et al., 2012), known mode of action (Wirmer and Westhof, 2006), and limited impact on mitochondrial translation. The extent of seedling development was evaluated based on the size and number of leaves produced.

Seedling responses of 52 accessions after 5 weeks on medium containing spectinomycin and Glc are presented in Supplemental Figure S1. Because only 20 seedlings were initially tested for each accession, minor differences in growth responses are not significant. We focused instead on striking differences observed between the most and least tolerant accessions identified. Three tolerant accessions (Jl-3, Bensheim-1 [Be-1], and Tsu-0), three sensitive accessions (Oystese-0 [Oy-0], Nie1-2, and "Nossen"), and one intermediate accession (Columbia) were chosen for thorough analysis, including crosses with embryo-defective mutants disrupted in chloroplast translation. Seedlings from tolerant accessions developed albino rosettes with multiple pairs of leaves after 5 weeks on spectinomycin. By contrast, seedlings from sensitive accessions developed at most rudimentary leaf initials along with expanded cotyledons. Examples of seedling phenotypes are shown in Figure 1. The consistency of growth responses is evident in Figure 2A. Spectinomycin tolerance did not correlate with seedling growth rates on basal medium or with overall plant vigor in soil. Greening was restricted to seedlings with poor root contact with the growth medium. To rule out the possibility that accession-specific differences were unique to spectinomycin, we evaluated the effect of another inhibitor of chloroplast translation with a different mode of action (lincomycin) on tolerant and sensitive accessions. Similar results were found with four accessions examined in detail (Supplemental Fig. S2). Spontaneous resistance, reduced uptake, increased detoxification, and enhanced compartmentalization of antibiotics are unlikely explanations for these differences in seedling responses because, as noted later, the most tolerant accessions also supported the most advanced growth of mutant embryos defective in chloroplast translation, where antibiotics were not involved. Based on these results, we conclude that natural accessions of Arabidopsis differ in their ability to tolerate a loss of chloroplast translation.

Crossing Tolerant and Sensitive Accessions Results in Phenotypic Segregation in the F2 Generation

To explore the genetic basis of differences in spectinomycin tolerance, wild-type plants from three tolerant accessions (Jl-3, Be-1, and Tsu-0) were crossed with a sensitive accession ("Nossen") derived from segregating populations of RIKEN insertion mutants (Bryant et al., 2011). We designated this sensitive accession "Nossen" because it differs from the sequenced No-0 accession (Kuromori et al., 2004). In all crosses examined, sensitive and tolerant seedlings segregated in the F2 generation (Supplemental Table S2). Many F2 seedlings also

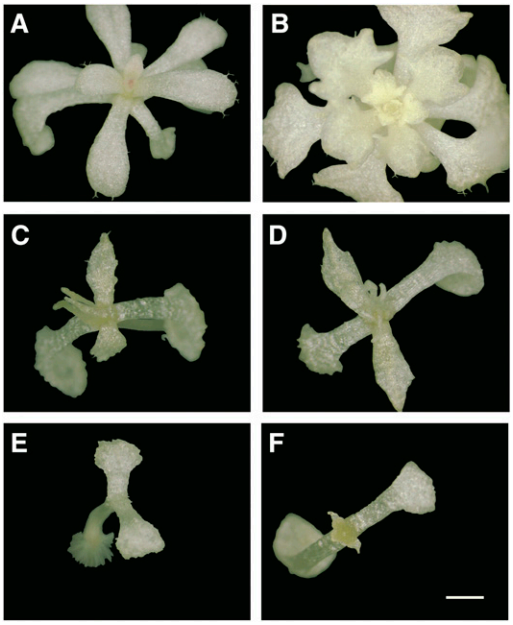

Figure 1. Differential seedling responses of Arabidopsis accessions on spectinomycin. A and B, Tolerant accessions (Jl-3 and Be-1). C and D, Intermediate accession (Columbia). E and F, Sensitive accessions ("Nossen" and Oy-0). Bar = 1 mm.

exhibited intermediate phenotypes. We focused on the Tsu-0 accession for subsequent analyses because its F2 response on spectinomycin was most consistent with a single, semidominant locus conferring tolerance (Fig. 2B). However, F1 seedling phenotypes seemed to indicate that tolerance was determined by multiple genes instead. Overall, these studies revealed a genetic component to accession-specific differences in seedling growth in the absence of chloroplast translation. But the overlapping phenotypes observed suggested that identifying the genes involved would be difficult. We therefore pursued a second, complementary approach that involved crosses between tolerant accessions and embryo-defective mutants disrupted in chloroplast ribosomal proteins in a sensitive background. This approach led to the identification of distinct genetic loci that impacted the extent of embryo development in the absence of chloroplast translation and later to the demonstration that variation in these same genes contributed to the tolerance of seedlings from different accessions on spectinomycin.

Spectinomycin-Tolerant Accessions Partially Rescue Mutant Embryos Defective in Chloroplast Translation

Most studies on embryo-defective mutants of Arabidopsis have overlooked the impact of genetic background (accession) on mutant phenotype. We took the opposite approach here once we realized that embryo phenotypes of mutants defective in chloroplast translation are sensitive to genetic background. Information on 33 mutant alleles disrupted in chloroplast translation is presented in Supplemental Table S3. Embryos from 19 Salk and Syngenta mutants (Columbia accession) reach a late globular stage. Embryos from six RIKEN mutants ("Nossen" accession) arrest at a preglobular stage. Embryos from two other mutants (Landsberg *erecta* [L*er*] accession) reach an intermediate (early globular) stage. Most importantly, after discounting four weak alleles and mutations in less essential genes, where screening for embryo rescue is ineffective because the original mutant allele supports continued growth, differences in the extent of embryo development in the absence of chloroplast translation correlate with differences in seedling growth on spectinomycin. In other words, the smallest mutant embryos are found in accessions that are most sensitive to spectinomycin. We therefore tested whether the tolerant Tsu-0 accession could rescue mutants in the sensitive "Nossen" accession. Two nuclear genes (*EMB3126* and *EMB3137*) encoding chloroplast ribosomal proteins (L1 and S13) with mutant alleles in different genetic backgrounds ("Nossen", Columbia, and L*er*) and different embryo phenotypes were chosen for analysis (Table I). Work on the L*er* mutant (*emb3126-3*) was later discontinued because of variable seed size in that accession.

To screen for dominant suppressors of early embryo arrest in RIKEN mutants (*emb3126-1* and *emb3137-1*),

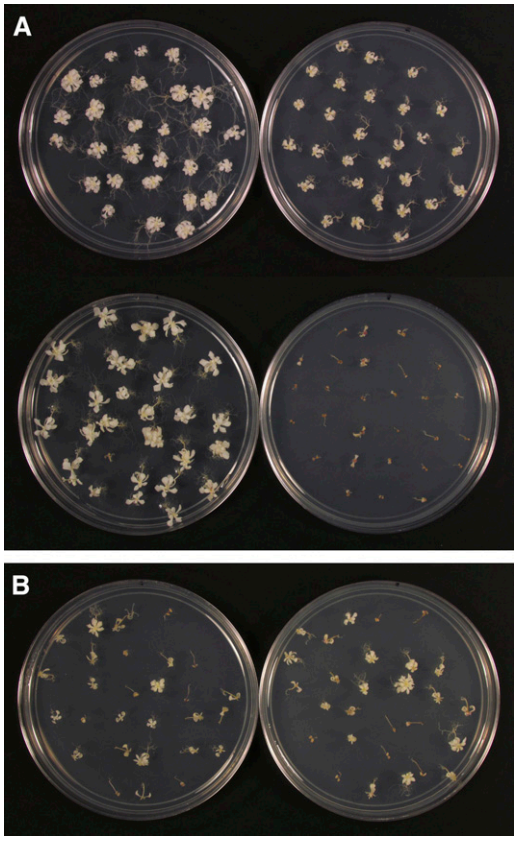

Figure 2. Consistent seedling responses of parental accessions on spectinomycin compared with segregating phenotypes in the F2 generation. A, Parental accessions (clockwise from lower left): tolerant, Jl-3, Be-1, and Tsu-0; and sensitive, "Nossen". B, Segregating F2 seedlings from a Tsu-0 cross with "Nossen." Plate diameter = 9 cm.

we crossed Tsu-0 plants with *emb/EMB* heterozygotes. A dominant Tsu-0 suppressor unlinked to the *EMB* locus should enable 75% of the mutant seeds in siliques of selfed F1 heterozygotes to reach a later stage of development. In addition, three classes of F2 heterozygotes are expected in the next generation: those with a late seed phenotype, those with an early seed phenotype, and those with a mixture of both (Supplemental Fig. S3). Plants with more advanced embryos should be included among the late class if additional modifiers are present. Using this approach, we identified a single dominant suppressor in the Tsu-0 accession that significantly increases the size of mutant seeds and supports embryo development to a globular stage. As expected, 75% of the mutant seeds in F1 siliques reach a later (globular) stage of development (Table II, rows 1 and 2). Increased seed size is consistent with continued endosperm development. The remaining 25% of mutant seeds resemble those found in the parental RIKEN mutant. Three classes of F2 plants were found in the expected 1:2:1 ratio: *TT* plants with all rescued (globular and beyond) mutant seeds; *SS* plants with all parental (preglobular) mutant seeds; and *ST* plants with a 3:1 ratio of rescued to parental mutant seeds (Table III). Because e*mb3126-1* and e*mb3137-1* are both rescued to some extent by the dominant suppressor, the effect is not limited to a specific ribosomal protein. Partial rescue was also observed, although not examined in detail, when *emb3126-1* and *emb3137-1* were crossed with the tolerant Jl-3 and Be-1 accessions. The ability to rescue mutant seeds defective in chloroplast translation is therefore not unique to the Tsu-0 accession. As expected, rescue was less striking when *emb3126-1* and *emb3137-1* were crossed with two sensitive accessions (Oy-0 and Nie1-2); mutant seeds in F1 siliques failed to progress beyond an early globular stage and were reduced in size when compared with those from crosses involving the tolerant Tsu-0 accession (Table II, rows 3–6). Surprisingly, some F2 mutant seeds reached a much later (transition or cotyledon) stage of development when Tsu-0 was crossed with *emb3126-1* but not with *emb3137-1*. These results are explained later, in the context of mapping a second locus known as the enhancer of the suppressor.

The Dominant Tsu-0 Suppressor of Embryo Arrest Maps to the *ACC2* Region of Chromosome 1

Because a variety of changes in *ACC2* itself might lead to the phenotypes observed, we pursued a candidate gene approach to determine whether *ACC2* corresponds to the suppressor. Three classes of F2 plants from Tsu-0 × *emb3126-1* and Tsu-0 × *emb3137-1* populations were analyzed (Table III): *SS* plants with two sensitive ("Nossen") alleles of the suppressor; *TT* plants with two tolerant (Tsu-0) alleles; and *ST* plants with one copy of each. PCR primers were designed to amplify DNA adjacent to *ACC2* in one accession but not the other (Fig. 3). PCR genotyping of 80 F2 plants (49 *TT*, 29 *SS*, and 2 wild type) revealed perfect linkage between *ACC2* and the suppressor. This corresponds to a genetic distance of less than 1 centimorgan (cM). To provide further evidence that *ACC2* is the suppressor, we analyzed seedling growth responses on spectinomycin of two insertion mutants disrupted in *ACC2* (Salk lines, Columbia accession). Both of these mutants exhibited hypersensitivity to spectinomycin. None of the wild-type accessions examined had a more severe phenotype. We then crossed *acc2* knockout homozygotes, which exhibit no visible phenotype on soil, with a Salk mutant (*emb3137-2*) in the Columbia background, which exhibits embryo arrest at the late globular stage, to determine whether some mutant embryos in F1 siliques arrested at an earlier stage of development. As expected, 25% of the mutant seeds in these F1 siliques arrested at the preglobular stage instead of the globular stage (Supplemental Table S4). Collectively, these results support the conclusion that the Tsu-0 suppressor represents an allele of *ACC2*.

The Tsu-0 Suppressor Improves Seedling Growth in the Absence of Chloroplast Translation

To determine whether the suppressor impacts both embryo development in the absence of chloroplast translation and seedling response to spectinomycin, we PCR genotyped sensitive and tolerant F2 seedlings from Tsu-0 × "Nossen" populations using the *ACC2*-linked markers noted above. The results (Table IV) indicated that seedlings with a sensitive phenotype similar to the "Nossen" parent were typically homozygous for the "Nossen" allele of the suppressor and that both highly tolerant and somewhat tolerant seedlings were either heterozygous or homozygous Tsu-0 for the suppressor, consistent with a dominant pattern of inheritance. To determine whether the suppressor found in the Tsu-0 accession is also present in other tolerant accessions, we genotyped F2 seedlings on spectinomycin derived from a "Nossen" cross with the tolerant Be-1 accession (Table

Table I. *Mutant alleles chosen for initial crosses with spectinomycin-tolerant accessions*

Allele Symbol	Ribosomal Protein	Insertion Line[a]	Background Accession	Embryo Phenotype	Embryo Size[b]
					μm
emb3126-1	L1	RIKEN	"Nossen"	Preglobular	25
emb3126-3	L1	JIC[c]	L*er*	Small globular	60
emb3137-1	S13	RIKEN	"Nossen"	Preglobular	25
emb3137-2	S13	Salk	Columbia	Large globular	90

[a]Additional details are presented in Supplemental Table S3. [b]Values are rounded here to highlight major differences and to simplify comparisons with values reported elsewhere. [c]JIC, John Innes Centre.

Table II. *Partial embryo rescue in F1 siliques from crosses between natural accessions and embryo-defective mutants*

Mutant Allele[a]	Wild-Type Accession	Siliques Screened	Seeds Screened	Percentage Mutant Seeds	Percentage Mutant Seeds Exhibiting Embryo Rescue	Phenotype of Rescued Embryo[b]	Average Size of Rescued Embryo
							μm
emb3126-1	Tsu-0	40	1,842	24.1	71.4	Most large globular[c]	84
emb3137-1	Tsu-0	40	1,939	24.3	75.4	Large globular	78
emb3126-1	Oy-0	11	474	24.5	72.4	Small globular	55
emb3137-1	Oy-0	20	965	26.5	75.6	Small globular	55
emb3126-1	Nie1-2	11	550	24.2	Not determined[d]	Tiny globular	49
emb3137-1	Nie1-2	10	491	27.1	Not determined[d]	Tiny globular	50

[a]Embryo arrest in parental ("Nossen") lines occurs at the preglobular stage. [b]Embryo rescue was more pronounced in crosses with a spectinomycin-tolerant accession (Tsu-0) than in crosses with spectinomycin-sensitive accessions (Oy-0 and Nie1-2). [c]Some embryos reached a later stage. [d]Rescued mutant seeds did not differ sufficiently in size from parental mutant seeds.

IV). The same dominant suppressor appears to mediate spectinomycin tolerance. We conclude that the suppressor likely functions across multiple accessions to improve growth in the absence of chloroplast translation.

ACC2 Expression Levels in Seedlings Are Not Correlated with Spectinomycin Tolerance

Arabidopsis microarray data indicate that *ACC2* is expressed at low levels throughout growth and development. Thus, one mechanism for improving spectinomycin tolerance might be to increase the amount of *ACC2* transcript produced. This model was not supported by repeated reverse transcription (RT)-PCR and RT-quantitative PCR (qPCR) experiments. *ACC2* transcripts in wild-type seedlings were consistently less abundant than *ACC1*, and tolerant accessions did not produce more *ACC2* transcript than sensitive or intermediate accessions (Fig. 4). This does not eliminate the possibility that some tolerant accessions overexpress *ACC2* at the seedling stage. But increased *ACC2* transcription does not underlie the suppressor effects analyzed here. We then searched for variations in ACC2 protein sequences that might explain the differences observed between tolerant and sensitive accessions and between late and early stages of embryo arrest in the absence of chloroplast translation. This ultimately led to the discovery that some of the most sensitive alleles of the suppressor found in natural accessions are associated with a complete loss of ACC2 protein function.

ACC2 Nonsense Mutations Are Present in Several Sensitive Accessions

Initially, we assumed that ACC2 was functional even in the most sensitive accessions and that the Tsu-0 suppressor allele of *ACC2* improved its enzymatic activity, stability, or transport into chloroplasts. After sequencing the *ACC2* genomic region from the sensitive "Nossen" accession used in crosses, we realized that this assumption was incorrect. The "Nossen" allele of *ACC2* is interrupted by a nonsense mutation that removes two essential transcarboxylase domains located in the C-terminal half of the protein. Additional examples of *ACC2* nonsense mutations (Supplemental Table S5) were then found among sequenced accessions at the Salk 1001 Genomes Web site (http://signal.salk.edu/atg1001). Two accessions with deletions or rearrangements that eliminate essential domains were also uncovered. Similar mutations are not found in the adjacent *ACC1* locus, which is comparable in length but is essential for growth and development. Seedlings from accessions with major *ACC2* disruptions are hypersensitive to spectinomycin (Supplemental Table S5), consistent with the knockout mutant phenotype. These results demonstrate that loss of *ACC2* function is associated with heightened sensitivity to a loss of chloroplast translation in Arabidopsis and that functional ACC2 protein is not always required in natural environments, where heteromeric ACCase encoded in part by the chloroplast genome can function instead.

Table III. *Classes of F2 plants identified from Tsu-0 crosses with mutants in a sensitive ("Nossen") background*

Parental Mutant	F2 Class Symbol	Description of F2 Plant Phenotype	Total Plants Identified	Total Seeds Screened	Percentage Mutant Seeds	Percentage Embryo Visible
emb3126-1	*SS*	No embryo rescue	21	3,199	27.0	0.8
	ST	Partial rescue segregating	49	6,103	26.3	76.7
	TT	Partial rescue consistent	31	7,862	25.3	99.4
	Wild type	Wild-type plants	45	1,549	0.2	–
emb3137-1	*SS*	No embryo rescue	9	1,491	24.5	0.5
	ST	Partial rescue segregating	30	5,259	24.7	72.8
	TT	Partial rescue consistent	19	3,144	26.6	99.4
	Wild type	Wild-type plants	37	3,993	0.9	–

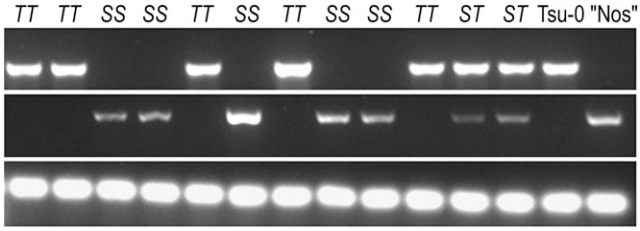

Figure 3. PCR genotyping of a segregating population of F2 plants from a Tsu-0 cross with *emb3126-1*. Primers were designed to amplify sequences adjacent to the *ACC2* coding region in either Tsu-0 (top row) or "Nossen" (middle row). The bottom row shows the genomic region encoding 18S ribosomal RNA. *SS*, Plants homozygous for the "Nossen" allele of the suppressor; *ST*, heterozygotes; *TT*, plants homozygous for the Tsu-0 suppressor.

ACC2 Sequences Are More Variable Than ACC1 Sequences

Protein sequences from 855 accessions with sequenced genomes were then searched for evidence of reduced ACC2 function. First, we examined amino acid residues altered in lethal *acc1* missense mutants from Arabidopsis and elsewhere. One accession with a substitution (E1689G) at the same location as the *pas3-1* mutant was identified. However, seedling responses of this accession on spectinomycin were inconclusive. We then analyzed 416 amino acid residues (out of 2,355 total) perfectly conserved in a multikingdom alignment of 20 different ACC1/ACC2 sequences (Supplemental Fig. S4). We reasoned that substitutions at these locations were most likely to reduce protein function. More variation was found in ACC2 (8.2% of the conserved residues differed in at least one accession) than in ACC1 (2.2% differed; z test $P < 0.001$). Similar results were obtained when six members of the Brassicaceae with sequenced genomes were compared: 17 residues differed in ACC2 compared with just two for ACC1. When the frequencies of synonymous (K_s) versus nonsynonymous (K_a) substitutions were evaluated, the K_a/K_s ratios obtained (*ACC1*, 0.08; *ACC2*, 0.20) suggested a relaxation of purifying selection for *ACC2*. These analyses support the conclusion that *ACC2* exhibits more variation in sequence and function than *ACC1*. We then examined the distribution of *ACC1* and *ACC2* in the Brassicaceae. Tandem gene duplications are present in the sequenced genomes of Arabidopsis, *Arabidopsis lyrata*, *Capsella rubella*, and *Eutrema parvulum*. Unlinked copies of *ACC1* and *ACC2* are found in *Brassica rapa*. A nonsense mutation is located in the third exon of *ACC2* in *Leavenworthia alabamica*. No evidence of *ACC2* was found in *Aethionema arabicum* or *Boechera stricta*. Overall, these results confirm that *ACC2* structure, function, and retention differ widely throughout the Brassicaceae.

A Semidominant Enhancer of ACC2 Function Is Present in the Tsu-0 Accession

While screening F2 plants from crosses between Tsu-0 and *emb3126-1* heterozygotes, we noticed that plants homozygous for the Tsu-0 suppressor differed in the extent of embryo rescue beyond the globular stage. One group (early *TT*) produced mutant embryos that consistently arrested at the globular stage and rarely exceeded 100 μm in diameter. A second group (late *TT*) produced mutant embryos that were typically 100 μm or larger and often progressed to a later (elongated or cotyledon) stage of development. A third group (intermediate *TT*) produced a mixture of both types of mutant embryos. Information on these phenotype classes is summarized in Table V. The number of progeny plants assigned to each class (26:46:26) is consistent with the 1:2:1 ratio expected for a second locus impacting the extent of embryo development. We named this locus the enhancer of the suppressor because its effect required the presence of the Tsu-0 suppressor and enhanced the ability of that suppressor to promote continued embryo development. Progeny testing of selected *TT* plants in the F3 generation confirmed that late plants were homozygous for the Tsu-0 allele of the enhancer, early plants were homozygous for the "Nossen" allele, and intermediate plants were heterozygous (Table VI). Furthermore, when F3 progeny of late *TT* plants were examined, it appeared that at least two additional modifiers increased the extent of embryo rescue. Once again, three phenotypic categories (late advanced, late moderate, and late reduced) were recognized (Supplemental Table S6). Plants in the first category produced mutant embryos that averaged 255 μm in length, a dramatic increase beyond the preglobular (25 μm) embryo observed in the parental mutant. Embryo rescue was even more extensive in F5 mutant seeds found in siliques of F4 plants derived from the most advanced F3 parental plants. All of these mutant embryos were elongated and

Table IV. *Suppressor genotypes of F2 seedlings from "Nossen" crosses with tolerant accessions*

Tolerant Accession	F2 Seedling on Spectinomycin	*ACC2* Genotype[a]	Seedlings	Seedlings Genotyped
			%	
Tsu-0	Tolerant	T, H	100	9
	Intermediate	T, H	100	13
	Sensitive	N	94[b]	17
Be-1	Tolerant	T, H	100	7
	Intermediate	T, H	95[b]	19
	Sensitive	N	100	10

[a]H, Heterozygous; N, homozygous "Nossen" allele; T, homozygous tolerant allele. [b]Rare exceptions had marginal seedling phenotypes that were likely misclassified.

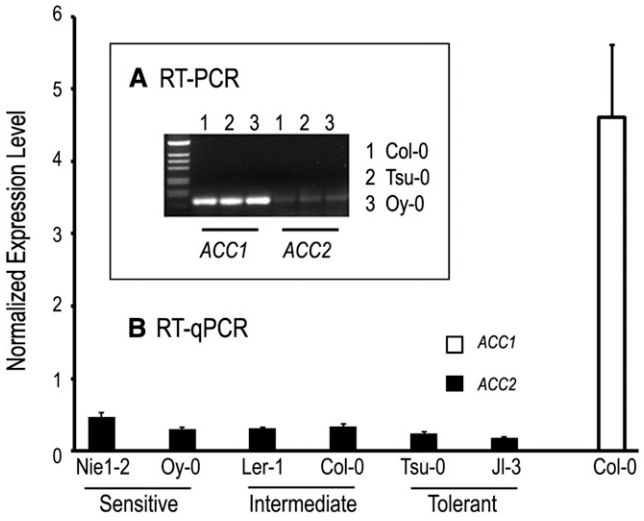

Figure 4. Homomeric ACCase transcript levels in seedlings from Arabidopsis accessions that differ in spectinomycin tolerance. Note that *ACC2* transcripts are less abundant than *ACC1* and that their level does not increase with tolerance.

more than 70% reached a cotyledon stage of development, frequently surpassing 300 μm in length. Cumulative effects of the Tsu-0 suppressor, enhancer, and modifier alleles on the development of *emb3126-1* mutant embryos are presented in Figure 5. Examples of arrested embryo phenotypes are shown in Figure 6. Collectively, these results demonstrate that multiple genes from the spectinomycin-tolerant Tsu-0 accession impact the extent of embryo development in the absence of chloroplast translation.

The Tsu-0 Enhancer Maps Near the Top of Chromosome 5

One feature of Tsu-0 crosses with *emb3137-1* was at first difficult to explain: F2 plants homozygous (*TT*) for the Tsu-0 allele of the enhancer lacked the late-embryo (elongated and cotyledon) phenotypes characteristic of *emb3126-1* crosses (Supplemental Table S7). It seemed unlikely that this reflected differences in protein function because the Salk *emb3137-2* allele had the same globular embryo phenotype found in other Columbia mutants defective in chloroplast translation. Instead, we thought the difference might result from linkage between the enhancer locus and *EMB3137*. In that case, most homozygous mutant embryos should also be homozygous for the "Nossen" allele of the enhancer. To explore this possibility, we assembled information on the map locations of known genes associated with chloroplast protein import, which represented logical candidates for an enhancer that improved the ability of *ACC2* to compensate for a loss of heteromeric ACCase (Supplemental Fig. S5). Several candidate genes linked to *EMB3137* were identified. When late *TT* and early *TT* plants from Tsu-0 crosses with *emb3126-1* were genotyped (Supplemental Fig. S6) using PCR primers that revealed accession-specific DNA sequence polymorphisms, linkage between the enhancer and *EMB3137* was confirmed. We then progeny tested plants with questionable phenotype assignments, adjusted their classifications as needed, and genotyped 110 plants of interest using PCR primers for *EMB3137*, *OUTER ENVELOPE PROTEIN80* (*OEP80*), located 10 cM below *EMB3137*, and *TRANSLOCON OUTER CHLOROPLAST34* (*TOC34*), located 10 cM above *EMB3137*. The results demonstrated that the enhancer is tightly linked to *EMB3137*, in a region devoid of genes that represent logical candidates based on known protein function.

To confirm that linkage between *EMB3137* and the enhancer was responsible for the absence of late-embryo phenotypes in crosses between Tsu-0 and *emb3137-1*, we analyzed F2 seeds from crosses between Tsu-0 and two additional mutants, one disrupted in a closely linked gene (*EMB3136*) and the other (*EMB1473*) unlinked. Both genes encode chloroplast ribosomal proteins. As expected, late embryos were found with *emb1473*, which is unlinked to the enhancer, but not with *emb3136*, which is linked (Supplemental Tables S8 and S9).

PCR genotyping of sensitive and tolerant F2 seedlings from Tsu-0 × "Nossen" populations using linked markers revealed that the Tsu-0 enhancer is semidominant. The most tolerant seedlings were homozygous Tsu-0 for the enhancer, somewhat less tolerant seedlings were typically heterozygous, and the least tolerant (but not sensitive) seedlings tended to be homozygous "Nossen". Sensitive seedlings, which are homozygous "Nossen" for the suppressor, had variable enhancer genotypes, as predicted. This pattern of inheritance was more difficult to document at the embryo stage because mutant embryos were not genotyped. When combined with corresponding data for *ACC2*, these results demonstrate that both the suppressor and enhancer function at two different stages of development (embryo and seedling) in the Tsu-0 accession, validating our decision to use seedling responses to spectinomycin as a rapid assay for differences between accessions that might impact seed

Table V. *Enhancer phenotype classes of TT plants from a Tsu-0 cross with emb3126-1*

Plants Analyzed[a]		Mutant Embryos Analyzed		Embryo Lengths		Embryo Phenotypes			
Enhancer Class	No. Screened	No. Measured	Average Length	Less Than 100 μm	Greater Than 200 μm	Globular	Triangular	Linear	Cotyledon
			μm			%			
Late	26	1,220	154	3.6	11.3	9.5	47.8	33.9	8.8
Intermediate	46	1,928	92	62.7	3.5	74.6	14.9	8.7	1.8
Early	26	965	66	94.8	0.1	99.4	0.5	0.1	0.0

[a]Limited to F2 plants and F3 plants derived from F2 plants assigned to the intermediate enhancer class.

Table VI. *Progeny testing of TT plants from different enhancer classes from a Tsu-0 cross with emb3126-1*

Parental F2 Plants Enhancer Class	*EMB* Genotypes of F3 Plants		Seeds in Siliques of F3 Heterozygotes		Enhancer Classes of F3 Plants		
	Heterozygote	Wild Type	Total Screened	Percentage Mutant	Late	Intermediate	Early
Late	24	11	6,359	26.2	24	0	0
Intermediate	19	13	2,716	24.7	6	8	5
Early	35	12	2,004	23.7	0	0	35

development. Analysis of arrested embryos, however, was a more sensitive method for identifying the genes involved, as both genotype and phenotype information could be confirmed in subsequent generations.

Knockout Mutants of a Chloroplast Protein Import Gene Exhibit Hypersensitivity to Spectinomycin

To explore the role of chloroplast import proteins in modulating the ability of *ACC2* to compensate for a loss of chloroplast translation, two knockout mutants disrupted in the *TRANSLOCON INNER CHLOROPLAST20-IV* (*TIC20-IV*) gene required for the import of housekeeping proteins through the inner chloroplast membrane were tested on spectinomycin. As shown in Figure 7, mutant seedlings were much more sensitive to spectinomycin than seedlings from the parental Columbia accession, resembling the response of *acc2* knockouts. Because *tic20-IV* null mutants lack an obvious phenotype when grown on soil, the paralogous *TIC20-1* gene, which targets photosynthetic proteins, must be less effective at importing *ACC2* in the absence of chloroplast translation than other housekeeping proteins essential for growth and development in the presence of chloroplast translation. A different result was obtained when a *toc34* knockout was tested on spectinomycin. Mutant seedlings were not more sensitive than the parental Columbia accession. In this case, the *TOC33* paralog appears to compensate for the loss of *TOC34* in both the presence and absence of chloroplast translation.

DISCUSSION

Natural variation among wild-type accessions of Arabidopsis has been widely used to address fundamental questions in plant biology, from physiology and development to molecular ecology and plant-pathogen interactions (Alonso-Blanco et al., 2009; Weigel, 2012). We took a different approach here by analyzing natural variation in the developmental consequences of disrupting a basic cellular function: chloroplast translation. Our interest was driven by reports of differential responses of Arabidopsis, *Brassica* spp., and maize (*Zea mays*) to a loss of chloroplast translation (Zubko and Day, 1998; Asakura and Barkan, 2006) and by intriguing correlations noted between the extent of embryo development in Arabidopsis mutants defective in chloroplast translation (Bryant et al., 2011; Muralla et al., 2011) and the genetic background (accession) in which those mutations were generated. We demonstrate here that heterotrophic growth in the absence of chloroplast translation in Arabidopsis is mediated by *ACC2*, a duplicated nuclear gene that targets homomeric ACCase to plastids, and enhanced by genetic modifiers present in the most tolerant accessions. Further analysis of this experimental system could lead to a better understanding of essential chloroplast gene functions in flowering plants and to improved delivery of homomeric ACCase to plastids during seed development.

Comparative Genomics and the Loss of Essential Chloroplast Genes

Two approaches have been used to identify essential chloroplast genes in plants: targeted gene disruption in tobacco and comparison of sequenced chloroplast genomes, especially from parasitic, nonphotosynthetic plants. The first approach identified four candidate genes that seemed to explain the importance of chloroplast translation: *accD* (Kode et al., 2005); *ycf1* (Drescher et al., 2000), which functions in protein import (Kikuchi et al., 2013); *ycf2* (Drescher et al., 2000), whose function remains unknown; and *clpP1* (Kuroda and Maliga, 2003), which encodes part of the ClpP protease that regulates protein degradation (Kim et al., 2013). All four genes are retained in the plastids of most flowering plants, consistent with their proposed essential functions. Nevertheless, repeated examples of selective gene loss or transfer to the nucleus have been documented in the literature.

In *Trachelium caeruleum* and *Trifolium subterraneum*, a truncated *accD* variant is found in the nucleus, where it likely targets functional protein back to the chloroplast (Haberle et al., 2008; Magee et al., 2010; Rousseau-Gueutin et al., 2013). In other species, *accD* appears to be

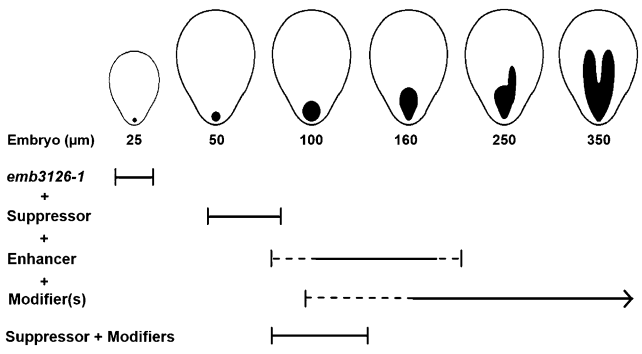

Figure 5. Combined effects of the Tsu-0 suppressor, enhancer, and modifier on seed and embryo rescue in *emb3126-1* ("Nossen" background). Ellipses represent mutant seeds, filled images depict mutant embryos, and bars define the stage of arrest.

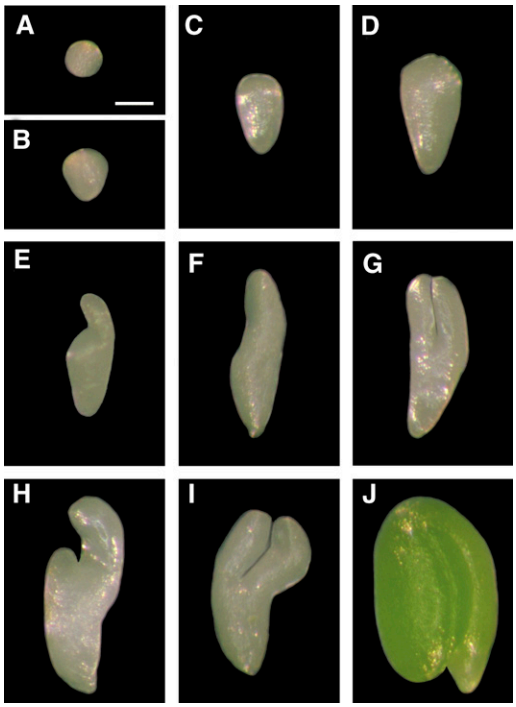

Figure 6. Examples of embryos in siliques of plants homozygous for the Tsu-0 suppressor. A, Late globular embryo. B, Triangular embryo. C and D, Elongated linear embryos with an expanded basal region and a small apical dome. E to I, Cotyledon stage embryos with one or two cotyledons. J, Sibling wild-type embryo. Bar = 100 μm.

nonfunctional or missing altogether (Chumley et al., 2006; Guisinger et al., 2011; Straub et al., 2011; Li et al., 2013). This implies that a redundant nuclear gene remains to be identified because fatty acid biosynthesis is essential. In the Poaceae, *accD* is replaced by a nuclear gene that encodes a chloroplast-localized homomeric ACCase (Goremykin et al., 2005; Jansen et al., 2007; Guisinger et al., 2010). Several common herbicides target this novel enzyme (Kaundun, 2014). The chloroplast *ycf1* gene is also absent from the Poaceae and is missing or disrupted in selected members of other families (Chumley et al., 2006; Magee et al., 2010; Guisinger et al., 2011; Straub et al., 2011; Li et al., 2013). The *ycf*2 gene is lost in many of these lineages as well. Both of these genes are large, and when curated genomic sequences are available, related genes are not found in the nucleus. Examples of *clpP1* loss or disruption have been noted in several angiosperm families (Haberle et al., 2008; Guisinger et al., 2011; Straub et al., 2011) and in Japanese cedar (*Cryptomeria japonica*), a gymnosperm (Hirao et al., 2008).

These examples seem to indicate that *ycf1*, *ycf2*, and *clpP1* are dispensable. However, their retention in the reduced plastid genomes of parasitic plants (dePamphilis and Palmer, 1990; Funk et al., 2007; McNeal et al., 2007; Delannoy et al., 2011; Logacheva et al., 2011) argues that they are indeed essential, with functions not limited to photosynthesis. This appears to conflict with the model (Bryant et al., 2011) that *accD* alone is required for embryo development in Arabidopsis. How can ACC2 uptake by plastids defective in translation extend growth and development when Ycf1, Ycf2, and ClpP1 are not produced? One possibility is that these proteins are needed later in development than AccD. This might explain the failure of the Tsu-0 enhancer and modifier alleles described here to bring about complete embryo rescue. Alternatively, the consequences of *clpP1* single gene disruption in transgenic tobacco, where other chloroplast proteins are still being made, might be more severe than when all chloroplast-encoded proteins are missing. Kikuchi et al. (2013) recently demonstrated that *ycf1* is part of an essential 1-MD complex that facilitates chloroplast protein import. Understanding how mutant embryos defective in chloroplast translation continue development in the absence of *ycf1* function, therefore, requires a consideration of how chloroplast proteins are imported from the cytosol.

Essential Components of Chloroplast Protein Import

Multiple components of the chloroplast protein import system have been identified in Arabidopsis (Jarvis, 2008; Kessler and Schnell, 2009; Shi and Theg, 2013): (1) cytosolic chaperones that participate in protein folding and targeting (Flores-Pérez and Jarvis, 2013); (2) Toc receptor

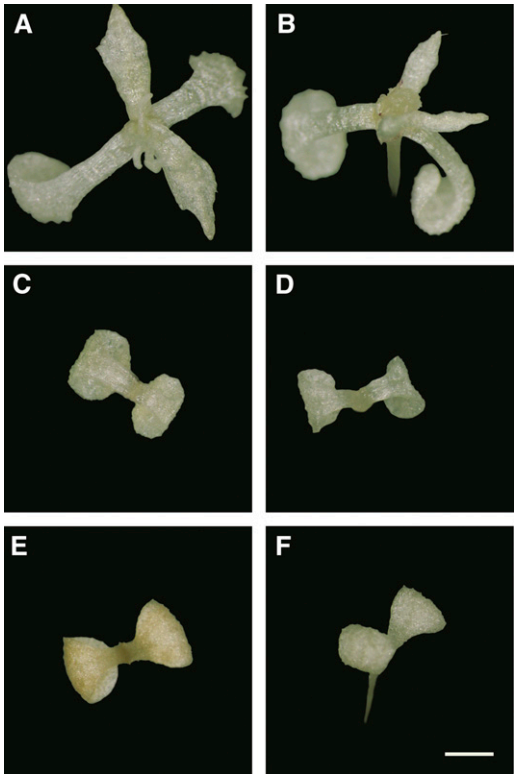

Figure 7. Responses of selected mutant and wild-type seedlings on spectinomycin. A, Parental Columbia accession. B, *toc34-1* (*ppi3-2*). C, *tic20-IV-1* (SAIL_97_F10). D, *tic20-IV-2* (Koncz 11324). E, *acc2*-1 (Salk_148966c). F, Most sensitive accession (Sav-0) identified in the initial screen. Bar = 1 mm.

GTPases in the outer chloroplast membrane that interact with these chaperones and the transit peptide of incoming proteins (Kubis et al., 2004; Inoue et al., 2010); (3) Toc75 outer membrane channel proteins (Baldwin et al., 2005); (4) inner membrane Tic20 channel proteins and associated Tic complex members (Hirabayashi et al., 2011; Kasmati et al., 2011; Kikuchi et al., 2013); (5) stromal chaperones, motor proteins, and heat-shock proteins such as Hsp93 (Kovacheva et al., 2007; Chu and Li, 2012), Hsp90C (Inoue et al., 2013), and chloroplast Hsc70 (Su and Li, 2010); and (6) a stromal processing peptidase (SPP) that cleaves the transit peptide (Trösch and Jarvis, 2011). Features of the N-terminal transit peptide that target proteins to plastids have also been analyzed (Chotewutmontri et al., 2012; Li and Teng, 2013).

Housekeeping and photosynthetic proteins are reportedly directed to different import complexes (Inoue et al., 2010; Hirabayashi et al., 2011). Complexes with Toc132/120, Toc34, and Tic20-IV are thought to associate with housekeeping proteins, whereas those with Toc159, Toc33, and Tic20-1 appear to import photosynthetic proteins. These differences are relevant here because disruption of complexes that import photosynthetic proteins should have less impact on embryo development in the absence of chloroplast translation than those involved with housekeeping proteins. Some overlap must still exist between these complexes, as both *toc34* and *tic20-IV* insertion mutants produce viable plants in soil (Constan et al., 2004; Kasmati et al., 2011). The increased sensitivity of *tic20-IV* mutant seedlings to spectinomycin described here is consistent with a nonredundant, essential role for *TIC20-IV* in plastid import of ACC2 protein.

Many genes required for chloroplast protein import in Arabidopsis exhibit an embryo-defective mutant phenotype: *TOC75-III* (Baldwin et al., 2005), *OEP80/EMB213* (Patel et al., 2008; Meinke et al., 2009), *Hsp90C/EMB1956* (Tzafrir et al., 2004; Inoue et al., 2013), *TIC100/EMB1211* (Tzafrir et al., 2004; Liang et al., 2010), *TIC56* (Kikuchi et al., 2013), *TIC32* (Hörmann et al., 2004), and *SPP* (Trösch and Jarvis, 2011). Several double knockouts of genes with overlapping functions also exhibit embryo or gametophyte lethality: *toc159 toc132* (Kubis et al., 2004), *toc33 toc34* (Constan et al., 2004), *tic20-I tic20-IV* (Hirabayashi et al., 2011), *hsp93-III hsp93-V* (Kovacheva et al., 2007), and *cphsc70-1 cphsc70-2* (Su and Li, 2008). Based on the work presented here, the most severe embryo phenotypes should be found among knockouts of genes most essential for ACC2 import. The early embryo arrest observed in *toc75-III* single mutants (Baldwin et al., 2005) and in the double mutants *toc33 toc34* (Constan et al., 2004), *hsp93-V hsp93-III* (Kovacheva et al., 2007), and possibly *tic20-I tic20-IV* (Hirabayashi et al., 2011) indicate that these proteins are essential for chloroplast protein import in general and ACC2 import in particular. The *toc159 toc120 toc132* triple mutant would likely exhibit a similar phenotype if construction were not limited by lethality. The late phenotypes of *tic100* and *tic56* mutants defective in the 1-MD complex are consistent with a limited role for *ycf1*, a component of the same complex, in the uptake of housekeeping proteins late in development.

Potential Functions of the Tsu-0 Enhancer and Modifier Alleles

We propose two contrasting models for how the Tsu-0 enhancer improves growth in the absence of chloroplast translation. Both models are consistent with the requirement that a tolerant (functional) allele of the suppressor (*ACC2*) be present. According to the first model, the Tsu-0 enhancer facilitates the import of ACC2 protein into plastids. Candidate genes include some of the protein import components detailed above. Enhanced uptake of inactive ACC2 protein should have no effect, consistent with observations. One problem with this model is the absence of promising candidate genes in the enhancer map location. With the second model, the Tsu-0 enhancer increases the amount or stability of functional ACC2 protein made, for example by improving translational efficiency or decreasing protein degradation. Other modifiers in tolerant accessions could have similar, complementary functions. Another possibility for modifier action involves partial compensation for the absence of Ycf1, Ycf2, or ClpP1. This explanation is less compatible with enhancer function because it is more independent of suppressor activity. Two different types of gene disruption are consistent with our genetic analysis. In one case, the Tsu-0 enhancer acts as a gain-of-function mutant allele when combined with the "Nossen" allele. Alternatively, the Tsu-0 enhancer may be a loss-of-function mutant allele of a locus that exhibits haploinsufficiency. These alternative mechanisms predict different types of enhancer functions, with the Tsu-0 allele promoting ACC2 function in the first case, as detailed above, and the "Nossen" allele decreasing ACC2 function in the second. One practical application of identifying the enhancer and modifiers described here concerns improved targeting of overexpressed homomeric ACCase to plastids in transgenic plants. The modest increase in fatty acid biosynthesis observed with this approach in the past (Roesler et al., 1997; Klaus et al., 2004) may reflect inefficient stabilization or uptake of functional ACCase into plastids.

Chloroplast Translation and Embryo Development in Maize and Arabidopsis

The ability of some angiosperms to survive a localized loss of chloroplast translation was first revealed through studies with the maize *iojap* and barley (*Hordeum vulgare*) *albostrians* mutants deficient in chloroplast ribosomes (Walbot and Coe, 1979; Siemenroth et al., 1981). Subsequent work on chloroplast splicing mutants provided further evidence that grasses tolerate disruptions in chloroplast translation that cause embryo lethality in Arabidopsis (Asakura and Barkan, 2006). Extensive research on embryo-defective mutants of Arabidopsis was consistent with this conclusion (Bryant et al., 2011). At first, it appeared that differences in fatty acid biosynthesis alone could explain the phenotypes observed. However, at least five mutants with severe defects in maize embryogenesis are disrupted in chloroplast translation: *lem1* (Ma and Dooner, 2004), *prpl35-1* (Magnard et al., 2004),

ppr8522 (Sosso et al., 2012), *emb16/why1* (Zhang et al., 2013), and *emb12* (Shen et al., 2013). Thus, a loss of chloroplast translation in maize is sometimes associated with embryo lethality. How can this observation be reconciled with the survival of maize *iojap* leaves devoid of chloroplast ribosomes? Interestingly, all of the embryo mutants noted above belong to the embryo-specific class in which development of the mutant endosperm tissue is normal. Embryo lethality is therefore not the result of a metabolic defect that limits growth. Furthermore, some of the phenotypes are sensitive to genetic background, with the same mutation resulting in embryo or seedling lethality, depending on the inbred line (Zhang et al., 2013).

This background effect in maize is different from the natural variation described here for Arabidopsis because it does not involve fatty acid biosynthesis. Instead, the model proposed for maize (Shen et al., 2013; Zhang et al., 2013) invokes the loss of a retrograde signal produced in functional plastids (Woodson et al., 2011; Terry and Smith, 2013) that activates the expression of nuclear genes, including some required for embryogenesis but not for endosperm or leaf development. The embryo-specific phenotype in maize may therefore reflect the loss of an essential chloroplast function that extends beyond photosynthesis. This raises the possibility that disruption of a retrograde signal during embryo development in Arabidopsis is also associated with a loss of chloroplast translation and that this defect contributes to the inability of the Tsu-0 enhancer and modifier alleles to bring about complete rescue of mutants defective in chloroplast translation.

Significance of the *ACC1-ACC2* Tandem Gene Duplication in Arabidopsis

One question that remains to be addressed concerns the function of *ACC2* in natural populations of Arabidopsis, where chloroplast translation is not blocked. Clearly, *ACC2* function is not required in all environments or genetic backgrounds, because multiple accessions contain nonsense mutations or deletions that remove essential protein domains. Furthermore, most plant species lack *ACC2* altogether. Whether *ACC2* contributes to fatty acid biosynthesis in chloroplasts under selected conditions and what impact such contributions have on plant growth and development in natural environments remain to be evaluated. One possibility is that ACC2 might lack the feedback regulation system described for heteromeric ACCase in the Brassicaceae (Andre et al., 2012; Bates et al., 2014). In such a case, ACC2 might contribute to fatty acid biosynthesis when the heteromeric enzyme is down-regulated. Alternatively, ACC2 may contribute to one or more metabolic pathways in the cytosol, especially in genotypes or environments where such a function is advantageous and where plastid import of ACC2 protein is restricted.

A different pattern of genetic redundancy is encountered in mammals, where a duplicated ACCase (*ACC2*) performs an essential function in fatty acid oxidation in mitochondria (Abu-Elheiga et al., 2005). Yeast (*Saccharomyces cerevisiae*) also contains a duplicated ACCase that functions in mitochondria (Hoja et al., 2004). Notably, that duplication is not widespread among yeast genomes examined to date. *Drosophila melanogaster* and zebrafish also contain ACCase proteins with N-terminal extensions consistent with mitochondrial localization. Duplication and diversification of ACCase genes has therefore occurred multiple times during the evolution of eukaryotes. In Arabidopsis, many duplicated genes appear to lack a knockout phenotype. One common explanation is that appropriate growth conditions were not evaluated. The work presented here documents an intriguing and informative phenotype that appears only under specialized laboratory conditions. Why functional ACC2 protein has been retained in selected members of the Brassicaceae, and in some Arabidopsis accessions but not others, remains to be explained.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The embryo-defective mutants of Arabidopsis (*Arabidopsis thaliana*) used for crosses with natural accessions have been described elsewhere (Bryant et al., 2011; Muralla et al., 2011) and are included in the SeedGenes database of essential genes (www.seedgenes.org). Seeds for *emb3126-1* (RATM-53-3245-1), *emb3126-3* (GT-5-101962), *emb3137-1* (RATM-15-0663-1), and *emb3136* (RATM-51-2522-3) were obtained from Kazuo Shinozaki at the RIKEN Plant Science Center. Seeds for *emb3137-2* (Salk-133412), *acc2-1* (Salk-148966c), *acc2-2* (Salk-110264), and wild-type accessions were obtained from the Arabidopsis Biological Resource Center. Stock numbers for each accession are listed in Supplemental Tables S1 and S5. Paul Jarvis provided seeds for *tic20-IV-1* (SAIL_97_F10), *tic20-IV-2* (Koncz 11324), and *toc34* (*ppi3-2*; Salk-059206), all in the Columbia background. Seeds for the "Nossen" accession were obtained from wild-type plants segregating in populations of mutants (*emb3126-1* and *emb3137-1*) grown in our laboratory. These plants were designated "Nossen" because they differ from the sequenced Nossen accession (Kuromori et al., 2004). Internal seed stocks were used for *emb1473* (Syngenta 24154) in the Columbia background, and duplicates are available through the Arabidopsis Biological Resource Center. Mature seeds were germinated on agar plates (Meinke et al., 2009), and seedlings were transplanted to pots containing a mixture of soil, sand, and vermiculite. Plants were grown under fluorescent lights (16-h-light/8-h-dark cycle) in a growth room maintained at room temperature (23°C ± 1°C) and watered daily with a nutrient solution as described by Berg et al. (2005).

Seedling Responses on Spectinomycin

Antibiotics known to inhibit chloroplast translation were added to a basal germination medium containing Murashige and Skoog salts, 3% (w/v) Glc, and 0.8% (w/v) agar. Spectinomycin (50 mg L^{-1}) was used for most experiments; lincomycin (200 mg L^{-1}) was tested with several accessions to confirm the spectinomycin results. Seedling responses were characterized 5 weeks after plating. The following scale was used to classify F1 and F2 seedlings from crosses between accessions, with specified lengths corresponding to the maximal distance from leaf tip to leaf tip: A, cotyledons only; B, first pair of leaves only (less than 1.5 mm); C, multiple leaves (less than 1.5 mm); D, multiple leaves (1.5 to less than 2.5 mm); E, first pair of leaves only (more than 1.5 mm); F to J, multiple leaves: F, 2.5 to less than 4 mm; G, 4 to less than 6 mm; H, 6 to less than 9 mm; I, 9 to less than 12 mm; and J, 12 mm or more. For the initial screen of 52 accessions, a numerical score with fewer categories was used to facilitate the averaging of results: 1, A; 2, B; 3, C + D; 4, E; 5, F + G; and 6, H and above. Seedlings with evidence of greening due to limited root contact with the medium were excluded.

Crosses and Embryo Phenotyping

Crosses between wild-type accessions and plants heterozygous for an embryo-defective mutation were performed in both directions. When a heterozygous (*emb/EMB*) plant was the female parent, the harvested silique was expected to lack aborted seeds, unlike adjacent siliques produced from selfing.

When the accession was the female parent, successful crosses were confirmed by segregation of mutant seeds in F1 plants. Crosses between two different accessions were confirmed by PCR genotyping. Methods used to phenotype mutant seeds and embryos are described at the tutorial section of the SeedGenes Web site. Heterozygous plants were identified by screening siliques for the presence of 25% aborted seeds. Seed and embryo measurements were performed with a stage micrometer using a dissecting microscope. The estimated accuracy of each measurement was 10 μm for embryos and deflated seeds and 20 μm for inflated seeds. Average lengths were calculated without SD; attention was focused instead on the frequency and distribution of size classes observed. Mutant embryos were removed from aborted seeds prior to desiccation using fine-tipped (Dumont no. 4) forceps. The smallest embryos measured were globular and 50 μm in diameter. Overall, embryos were less susceptible than seeds to a reduction in length resulting from seed desiccation.

Four categories of embryo phenotypes were recognized: globular (round), triangular (pointed basal region of the embryo visible), linear (further elongation of the basal region; sometimes accompanied by extension of the apical region without cotyledon formation), and cotyledon (presence of one or two cotyledons). Triangular embryos were typically more than 100 μm in diameter, and linear embryos were 150 μm or more in length. The extent of embryo development did not appear to be influenced by silique position along the stem. Most siliques analyzed contained aborted seeds that were slightly deflated and had begun to turn brown, which ensured that mutant embryos had reached a terminal stage of development. When siliques contained a mixture of aborted seeds at the globular and preglobular stages, the preglobular seeds were often collapsed and more highly desiccated than those with globular embryos. Even at maturity, globular embryos could often be detected as a small bump that distorted the shape of the seed.

PCR Genotyping of Plants

ACC2, *TOC34*, *EMB3137*, and *OEP80* primers were designed by aligning sequences of relevant accessions from the 1001 Genomes Project (http://signal.salk.edu/atg1001). Additional sequencing and PCR were required for the "Nossen" accession examined here. A complete listing of all primers is presented in Supplemental Table S10. Primers were purchased from Integrated DNA Technologies. Accession-specific primers used for mapping the suppressor (*ACC2*) were designed to amplify a product from either the Tsu-0 or "Nossen" accession. Other primers used for mapping the enhancer locus were designed to amplify products from both accessions, followed by DNA sequencing of PCR products to distinguish between heterozygotes and homozygotes (Supplemental Fig. S5). Genomic DNA was isolated from seedlings and inflorescences using a modified cetyl trimethyl ammonium bromide protocol (Lukowitz et al., 2000). Salk insertion mutants (*toc34-1*, *acc2-1*, and *acc2-2*) were genotyped to confirm that the appropriate seed stock had been received. A standard transfer DNA left border primer (LB1a) was used in combination with gene-specific primers for *TOC34* and *ACC2*. PCR was performed using the Qiagen PCR Master Mix and a Biometra Uno II thermocycler. PCR products were separated on 1% agarose gels containing GelRed Nucleic Acid Stain (Phenix). The AlphaImager HP system (Proteinsimple) was used to visualize the bands. For DNA sequencing, PCR products were purified with the Qiagen MinElute PCR Purification Kit and sequenced at the Oklahoma State University Recombinant DNA/Protein Resource Facility.

RT-qPCR and Subsequent Analysis

Total RNA was isolated from seedlings grown on a basal germination medium (described above) using an RNeasy plant mini kit (Qiagen) and reverse transcribed with a QuantiTect reverse transcription kit (Qiagen) that included elimination of genomic DNA for RT-qPCR. Reactions were performed using a SYBR Green PCR kit (Roche) with a Roche480 Light Cycler (Roche). PCR amplifications were completed with a melt curve analysis to confirm the specificity of amplification and the lack of primer dimers. PCR efficiencies were calculated based on serial dilution. The *ACTIN2* and 18S ribosomal RNA genes were used as references. LightCycler 480 software version 1.5 was used for analysis. Three RNA samples, independently isolated from each accession, were used for RT-qPCR analysis. Reactions were performed four times for each sample. Primer sequences are shown in Supplemental Table S10.

Homomeric ACCase Sequence Analyses

To identify conserved amino acids important for ACCase function, 20 ACC1 and ACC2 protein sequences were analyzed from representative plant, animal, and fungal species: Arabidopsis (two), *Brassica rapa* (two), *Medicago truncatula* (one), wheat (*Triticum aestivum*; two), maize (*Zea mays*; two), *Homo sapiens* (two), *Mus musculus* (two), *Danio rerio* (two), *Drosophila melanogaster* (one), yeast (*Saccharomyces cerevisiae*; two), *Schizosaccharomyces pombe* (one), and *Neurospora crassa* (one). Details are presented in Supplemental Table S11. Sequences were aligned using ClustalW2 (Larkin et al., 2007). In several cases, small gaps were identified that spanned conserved residues. These were assumed to be annotation errors and were corrected based on genomic data. Homomeric ACCase sequences from 855 Arabidopsis accessions were obtained from the Salk Web site for the Arabidopsis 1001 Genomes project (http://signal.salk.edu/atg1001). For determination of Ka/Ks ratios, *ACC1* and *ACC2* coding sequences were obtained for six members of the Brassicaceae: Arabidopsis, *Arabidopsis lyrata* (Hu et al., 2011), *B. rapa* (Cheng et al., 2011), *Capsella rubella* (Slotte et al., 2013), *Leavenworthia alabamica*, and *Sisymbrium irio*, along with *Theobroma cacao* (Motamayor et al., 2013). Sequences were downloaded from the Phytozome (www.phytozome.net) and CoGe (www.genomevolution.org/CoGe/) Web sites (Lyons et al., 2008; Goodstein et al., 2012). Ka/Ks ratios were calculated using MEGA version 6 (Tamura et al., 2013).

Genomic DNA spanning the *ACC2* locus from "Nossen" plants grown in our laboratory was sequenced at the Oklahoma State University Recombinant DNA/Protein Resource Facility. PCR primers were designed to avoid amplification of the adjacent *ACC1* locus (Supplemental Table S10). Takara (Clontech) PrimeSTAR GXL DNA polymerase was used to amplify large fragments of *ACC2*. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced with primers within the *ACC2* gene. A similar approach was used to confirm *ACC2* nonsense mutations and deletions present in other accessions.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Seedling responses of 52 Arabidopsis accessions on spectinomycin.

Supplemental Figure S2. Seedling responses of selected accessions on lincomycin.

Supplemental Figure S3. Expected F2 progeny from a selfed F1 plant heterozygous for an embryo-defective mutation and a second locus conferring sensitivity or tolerance to a loss of chloroplast translation.

Supplemental Figure S4. Conserved amino acid residues in eukaryotic ACCases and mutations analyzed in model genetic organisms.

Supplemental Figure S5. Chromosome locations of Arabidopsis genes encoding known components of the chloroplast protein import system and *EMB* genes encoding chloroplast ribosomal proteins.

Supplemental Figure S6. DNA sequence polymorphisms used to genotype plants for *ACC2*, *TOC34*, *EMB3137*, and *OEP80* in Tsu-0 crosses with *emb3126-1*.

Supplemental Table S1. Natural accessions of Arabidopsis examined on spectinomycin.

Supplemental Table S2. Seedling responses on spectinomycin of parental accessions and F2 progeny from crosses between accessions.

Supplemental Table S3. Phenotypes and genetic backgrounds (accessions) of embryo-defective mutants of Arabidopsis disrupted in chloroplast translation.

Supplemental Table S4. Reduced embryo development in F1 siliques from *acc2* (Columbia) crosses with *emb3137-2* (Columbia).

Supplemental Table S5. *ACC2* gene disruptions identified in natural accessions of Arabidopsis.

Supplemental Table S6. Modifier phenotype classes of late *TT* plants from a Tsu-0 cross with *emb3126-1*.

Supplemental Table S7. Differences in the extent of embryo rescue in *TT* plants from Tsu-0 crosses with *emb3137-1* and *emb3126-1*.

Supplemental Table S8. Partial embryo rescue in F1 siliques from a Tsu-0 cross with *emb1473* (Columbia)

Supplemental Table S9. Limited embryo rescue in F1 siliques from a Tsu-0 cross with *emb3136* ("Nossen").

Supplemental Table S10. PCR primer sequences used for RT-qPCR and plant genotyping.

Supplemental Table S11. Sequences used for ACC1/ACC2 alignments and determination of Ka/Ks ratios.

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