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Requirement of aminoacyl-tRNA synthetases for gametogenesis and embryo development in Arabidopsis

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Summary

Aminoacyl-tRNA synthetases (AARSs) are required for translation in three different compartments of the plant cell: chloroplasts, mitochondria and the cytosol. Elimination of this basal function should result in lethality early in development. Phenotypes of individual mutants may vary considerably, depending on patterns of gene expression, functional redundancy, allele strength and protein localization. We describe here a reverse genetic screen of 50 insertion mutants disrupted in 21 of the 45 predicted AARSs in Arabidopsis. Our initial goal was to find additional *EMB* genes with a loss-of-function phenotype in the seed. Several different classes of knockouts were discovered, with defects in both gametogenesis and seed development. Three major trends were observed. Disruption of translation in chloroplasts often results in seed abortion at the transition stage of embryogenesis with minimal effects on gametophytes. Disruption of translation in mitochondria often results in ovule abortion before and immediately after fertilization. This early phenotype was frequently missed in prior screens for embryo-defective mutants. Knockout alleles of non-redundant cytosolic AARSs were in general not identified, consistent with the absolute requirement of cytosolic translation for development of male and female gametophytes. These results provide a framework for evaluating redundant functions of AARSs in Arabidopsis, a valuable data set of phenotypes resulting from multiple disruptions of a single basal process, and insights into which genes are required for both gametogenesis and embryo development and might therefore escape detection in screens for embryo-defective mutants.

Keywords: AARS, chloroplasts, embryo-defective mutant, gametophytic lethal, mitochondria.

Introduction

Aminoacyl-tRNA synthetases (AARSs) are essential components of protein synthesis that catalyze the attachment of amino acids to their cognate tRNAs (O'Donoghue and Luthey-Schulten, 2003) and in some cases perform additional roles in translational regulation, RNA splicing and tRNA proofreading (Szymanski *et al.*, 2000). Because they were probably among the first peptides to evolve (Woese *et al.*, 2000), AARSs have also been examined for clues to the transition from a primitive RNA world to modern life forms. The assumption that all organisms must contain a complete set of 20 aminoacyl-tRNA synthetases, one per amino acid, was challenged in recent years when microbial genome projects confirmed that some AARS genes were dispensable. Two alternative strategies of producing a tRNA charged with the appropriate amino acid have been documented. One involves transamidation of a misacylated tRNA, as seen in the production of tRNAs charged with asparagine and glutamine (Becker and Kern, 1998). This mechanism helps to explain the occasional absence of AsnRS and GlnRS genes. The other strategy involves a single AARS with dual substrate specificity. For example, *Methanococcus* and *Giardia* each contain a ProRS enzyme with additional CysRS activity (Bunjun *et al.*, 2000; Stathopoulos *et al.*, 2001).

In contrast to prokaryotes, most eukaryotes possess more than the basal set of 20 AARS genes. This redundancy arose in part from the acquisition of endosymbionts and the subsequent formation of organelles capable of protein synthesis. Comparison of eukaryotic and prokaryotic proteins suggests that nuclear AARS genes were often acquired from mitochondria (Wolf *et al.*, 1999). *Saccharomyces* appears to contain 35 AARS genes, all located in the nucleus (http://www.yeastgenome.org). In some cases, a single amino acid is associated with two different AARS genes,

one that encodes a mitochondrial protein and the other a cytosolic protein. In other cases, a single gene encodes both the mitochondrial and cytosolic forms (Chatton *et al.*, 1988). When two related AARS genes are present, disruption of the cytosolic enzyme is often more deleterious than loss of the mitochondrial version (Giaever *et al.*, 2002).

Plants received a third compartment capable of protein synthesis with the acquisition of ancestral plastids. The minimal number of AARS genes in plants might therefore be expected to exceed that found in other organisms. Arabidopsis contains 45 putative AARS genes in the nuclear genome. This means that each gene product cannot be uniquely targeted to a different compartment. In some cases, a single gene encodes both the cytosolic and organellar enzymes by alternative transcription and translation. This leads to the production of a short cytosolic protein and a long form with an N-terminal targeting sequence (Mireau *et al.*, 1996; Souciet *et al.*, 1999). More commonly, Arabidopsis contains one gene for the cytosolic AARS and another gene for a related AARS located in both mitochondria and chloroplasts (Akashi *et al.*, 1998; Menand *et al.*, 1998). Several amino acids are associated with multiple AARS genes in Arabidopsis. All four AsnRS genes appear to have evolved by repeated duplication of an ancestral plastid gene and three related CysRS genes appear to have arisen by duplication of a single mitochondrial gene (Peeters *et al.*, 2000). Multiple rounds of gene transfer, duplication, deletion and dual targeting have therefore led to a rather complex picture of AARS gene function in plants.

We have a long-standing interest in the analysis of genes required for embryo development in Arabidopsis (Tzafrir *et al.*, 2004). Many of these *EMB* genes have been identified through forward genetic screens of T-DNA insertion lines (McElver *et al.*, 2001). A complete data set of *EMB* genes identified throughout the community is presented at http://www.seedgenes.org. We originally became interested in AARS genes after noticing that several *EMB* genes in the SeedGenes database (Tzafrir *et al.*, 2003) encoded aminoacyl-tRNA synthetases. These included *EMB1027*, *EMB2247*, *EMB2369*, and *EMB2755*, identified through our collaborative efforts with Syngenta (Tzafrir *et al.*, 2004), and the *TWN2* (Zhang and Somerville, 1997) and *EDD* (Uwer *et al.*, 1998) genes described elsewhere. We reasoned that additional *EMB* genes might be uncovered by screening knockouts of the remaining AARS genes in Arabidopsis. The results of that reverse genetic approach to the identification of *EMB* genes and the analysis of AARS protein function are described here. We document the developmental consequences of disrupting specific AARS genes in Arabidopsis and interpret the mutant phenotypes observed in the context of allele strength, functional redundancy and protein localization. We conclude that elimination of protein synthesis in the cytosol results in male and female gametophytic lethality, disruption of translation in the chloroplast results in embryo lethality, and interference with protein synthesis in mitochondria results in ovule abortion.

Results

General features of Arabidopsis AARS genes

Table 1 presents an overview of the 45 putative AARS genes of Arabidopsis and their loss-of-function mutant phenotypes. The number of AARS genes associated with a single amino acid is variable. Two different AARS genes have been identified for 14 of the 20 amino acids. With another five amino acids, three or four AARS genes seem to be involved. A single gene encoding a cytosolic GlnRS has been found, consistent with the presence of an alternative transamidation pathway in both chloroplasts and mitochondria. Protein localizations shown in Table 1 are based on a combination of computational predictions, proteome analyses and labeling studies. Experimental data confirm that many AARSs are targeted to more than one cell compartment (Duchêne *et al.*, 2001). Phylogenetic studies indicate that most genes encoding cytosolic AARSs in Arabidopsis did not arise from plastids. One exception is AsnRS, where the original cytosolic enzyme appears to have been replaced by a protein that originated from the plastid (Peeters *et al.*, 2000). Some groups of AARS proteins are more similar in sequence than others. Multiple sequence alignments (CLUSTALW at http://www.ebi.ac.uk) revealed five cases of particularly high similarity: two genes each for ArgRS (*At1g66530*, *At4g26300*), AspRS (*At4g26870*, *At4g31180*) and TyrRS (*At2g33840*, *At1g28350*), and all known genes for CysRS and AsnRS (data not shown).

Identification of T-DNA insertion mutants

We selected Salk (Alonso *et al.*, 2003) and SAIL (Sessions *et al.*, 2002) lines with promising open reading frame (ORF) insertions most likely to generate a null phenotype from the collection of T-DNA insertion mutants presented at http://signal.salk.edu. Emphasis was placed on the least redundant AARSs, where two genes are associated with a single amino acid. New alleles of six genes known to give a seed phenotype (*At1g14610*, *At3g48110*, *At4g04350*, *At4g26300*, *At5g16715*, *At5g56680*) were not requested because we already had information on existing mutants (http://www.seedgenes.org). Putative knockouts of several redundant genes were also not examined. Seeds for 50 knockout lines obtained from the Arabidopsis Biological Resource Center (ABRC) were germinated in soil and the resulting plants initially screened for defects in seed development. Three additional examples of genes with a knockout phenotype in the seed were identified: TyrRS (*At3g02660*), ThrRS (*At2g04842*) and AlaRS (*At5g22800*). A typical heterozygous silique (*emb2761*; ThrRS) is shown in

Table 1. Genes encoding AARSs in Arabidopsis

Amino acid	Total genes	Locus	Protein localization[a]	Localization reference	Mutant phenotype
Gln	1	*At1g25350*	cy		Aborted ovules
Met	2	*At4g13780*	cy	Menand *et al.* (1998)	N.C.[b]
		At3g55400	MT, cp		Aborted ovules
Ile	2	*At4g10320*	cy		N.C.[b]
		At5g49030	CP, mt		Aborted ovules
Trp	2	*At3g04600*	cy		N.R.[b]
		At2g25840	CP, mt	I. Small, pers. comm.	Aborted ovules
Lys	2	*At3g11710*	cy		N.C.[b]
		At3g13490	mt, cp	I. Small, pers. comm.	Aborted ovules
Pro	2	*At3g62120*	cy		N.C.[b]
		At5g52520	mt, cp	I. Small, pers. comm.	Aborted ovules
Glu	2	*At5g26710*	cy		N.A.[b]
		At5g64050	mt, cp	I. Small, pers. comm.	Aborted ovules
Ser	2	*At5g27470*	cy		N.A.[b]
		At1g11870	mt, cp	I. Small, pers. comm.	Aborted ovules
His	2	*At3g02760*	cy		N.C.[b]
		At3g46100	mt, cp	Akashi *et al.* (1998)	N.A.[b]
Leu	2	*At1g09620*	cy, mt		N.C.[b]
		At4g04350	CP, (mt)		Embryo defective
Gly	2	*At1g29880*	cy, mt	Duchêne *et al.* (2001)	N.C.[b]
		At3g48110	CP, mt	Duchêne *et al.* (2001)	Embryo defective
Thr	2	*At5g26830*	cy, mt	Souciet *et al.* (1999)	Uncertain
		At2g04842	mt, cp	I. Small, pers. comm.	Embryo defective
Val	2	*At1g14610*	cy, mt	Souciet *et al.* (1999)	Embryo defective
		At5g16715	CP, mt	I. Small, pers. comm.	Embryo defective
Ala	2	*At1g50200*	cy, mt, (cp)	Mireau *et al.* (1996)	Uncertain
		At5g22800	mt, cp	I. Small, pers. comm.	Embryo defective
Arg	2	*At1g66530*	cy, mt		Viable homozygote
		At4g26300	cy, cp	I. Small, pers. comm.	Embryo defective
Tyr	3	*At2g33840*	cy, (cp)		Viable homozygote
		At1g28350	cy		Viable homozygote
		At3g02660	mt, cp	I. Small, pers. comm.	Embryo defective
Cys	3	*At3g56300*	cy		N.A.[b]
		At5g38830	cy		N.R.[b]
		At2g31170	mt, cp	Peeters *et al.* (2000)	Uncertain
Phe	3	*At1g72550*	cy		N.A.[b]
		At4g39280	cy		N.A.[b]
		At3g58140	mt, cp	I. Small, pers. comm.	N.R.[b]
Asp	3	*At4g26870*	cy		N.E.[b]
		At4g31180	cy		N.E.[b]
		At4g33760	mt, cp	I. Small, pers. comm.	N.E.[b]
Asn	4	*At1g70980*	cy		N.A.[b]
		At3g07420	cy		N.E.[b]
		At5g56680	cy		Embryo defective
		At4g17300	mt, cp	Peeters *et al.* (2000)	Aborted ovules

[a]Based on targeting prediction programs and experimental data: cy, cytosol; mt, mitochondria; cp, chloroplasts; parentheses, ambiguous. Capital letters indicate confirmed presence in known proteomes (http://www.ampdb.bcs.uwa.edu.au). Includes data from Ian Small (Evry, France).
[b]Requested lines were initially limited to Salk and SAIL insertions predicted to result in a null allele. N.A., insertion line not available; N.R., insertion line not requested; N.C., line examined but presence of insertion not confirmed; N.E., not examined because of increased redundancy.

Figure 1(a). Linkage between the insert and mutant phenotype was confirmed by screening 95 Basta-resistant transplants for *At2g04842* and by crossing two mutants with similar phenotypes for *At3g02660* and *At5g22800*.

During the initial screen, a number of insertion lines were found to include plants with an unusually high frequency of unfertilized or aborted ovules. A thorough screen of these lines was then undertaken to identify mutants that consistently exhibited such an ovule abortion (*ova*) phenotype. Nine *OVA* genes with similar knockout phenotypes were uncovered. Immature siliques from one heterozygote (*ova2*; IleRS) are shown in Figure 1. Linkage between the insert and mutant phenotype was confirmed by counting the number of normal seeds and aborted ovules in five siliques from 20 PCR

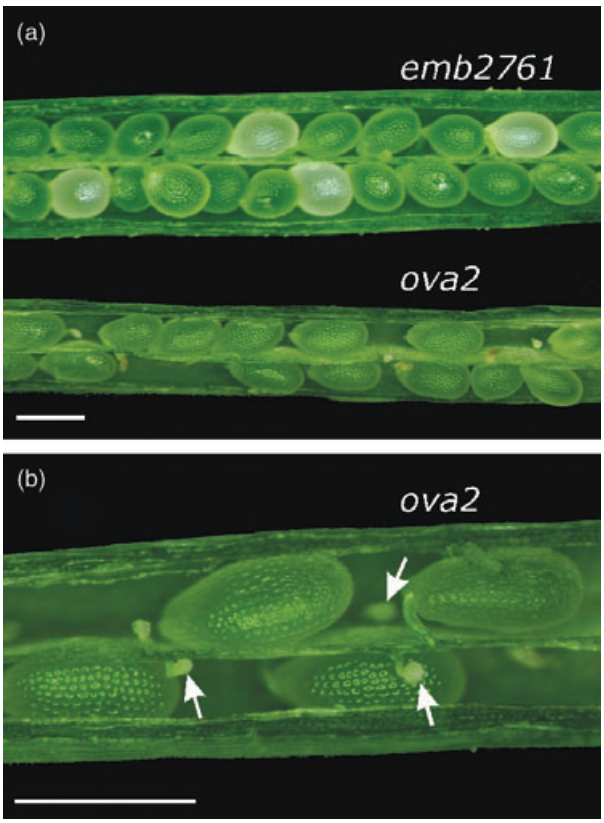

Figure 1. Immature siliques from plants heterozygous for AARS knockouts. (a) Embryo-defective (*emb2761*) phenotype of ThrRS (*At2g04842*) knockout with large white seeds and ovule abortion (*ova2*) phenotype of Ile (*At5g49030*) knockout with small white and tan structures resembling unfertilized ovules. (b) Close-up showing aborted ovules (arrows) in a silique with normal seeds at a cotyledon stage of embryo development. Scale bars, 500 μm.

genotyped plants per line. Two knockout alleles of *At5g64050* (GluRS) were identified with similar *ova* phenotypes. The eight remaining *OVA* genes are each represented by a single mutant allele. Although molecular complementation was not used to confirm gene identities, in part because of the large number of mutants involved, the collective data presented here are consistent with the conclusion that each *ova* phenotype is caused by disruption of an AARS gene.

Knockouts of chloroplast AARSs often exhibit an embryo-defective phenotype

Nine different AARS genes with a loss-of-function phenotype in the seed are listed in Table 2. Seven of these genes encode AARSs predicted or confirmed to be localized in chloroplasts (Table 1). The typical knockout phenotype (Figure 2) is embryo arrest at the globular to transition stage of development. Aborted seeds and arrested embryos are white or pale yellow before the onset of desiccation. Heterozygous siliques generally contain about 25% mutant seeds randomly distributed along the length of the silique, consistent with normal gametophyte development. The ratio of resistant to sensitive plants derived from seeds plated on selection medium is not significantly different from the 2:1 predicted for heterozygotes with a single T-DNA insertion. These combined results indicate that gene disruption results in embryo lethality and that gametophytic transmission of the mutant allele is not affected.

Most AARS knockouts with a seed phenotype appear to be disrupted in protein synthesis in chloroplasts but not in mitochondria. The second gene not affected by the insertion often encodes an AARS that is predicted to function in both the cytosol and the mitochondrion. The characteristic seed phenotype is therefore associated with a loss of chloroplast function. Three potential exceptions to this generalization need to be evaluated. The first is ValRS, where the seed phenotype exhibited by *twn2* (Zhang and Somerville, 1997) is indicative of a weak allele with an insertion in the 5′-untranslated region (UTR) of a gene thought to encode a protein localized to the cytosol and mitochondria. Complete elimination of this gene function should therefore result in a stronger mutant phenotype. Such a null allele remains to be identified. The second exception is AsnRS, where the late seed phenotype exhibited by disruption of a gene predicted to encode a cytosolic AARS may reflect gene redundancy and residual protein function given the location of the T-DNA insertion. The third exception is TyrRS, where the *At3g02660* knockout seed phenotype is atypical (early) and the aberrant segregation ratios and seed distributions may reflect a reduction in male gametophytic transmission. This example is discussed later in relation to viable homozygotes obtained with other TyrRS knockouts. Another informative result is the seed phenotype of a GlyRS (*At3g48110*) knockout, which indicates that in contrast to conclusions of Duchêne *et al.* (2001), cytosolic GlyRS (At1g29880) also appears to function in mitochondria. We conclude from this comprehensive screen that despite the broad requirement of protein synthesis for plant growth and development, only a defined subset of AARS gene knockouts displays an embryo-defective phenotype.

Knockouts of mitochondrial AARSs often exhibit an ovule abortion phenotype

Ten insertion mutants exhibited an ovule abortion phenotype in which heterozygotes produced siliques with a high frequency of unfertilized or aborted ovules. Information on these *ova* mutants is presented in Table 3. Note that insertion sites are consistent with complete elimination of normal gene function. Eight *OVA* genes encode AARSs predicted to be localized to both mitochondria and chloroplasts (Table 1). Linkage between the insert and mutant phenotype was determined by PCR genotyping of individual plants because the kanamycin resistance marker was often

Table 2. Mutants with an embryo-defective phenotype

Amino acid	Locus	Allele symbol	Insertion site[a]	Terminal phenotype[b]	R:S ratio[c]	Mutant seeds (%)[d]	Top half (%)[e]	Original source
Val	*At1g14610*	*twn 2*	5′-UTR	Cotyledon	n.d.	22.5	60.6	Somerville
Val	*At5g16715*	*emb 2247-1*	Exon 19 (27)	Globular	1.4	25.3	48.5	Syngenta/Meinke
		emb 2247-2	Intron 4 (26)	Globular	2.1	27.7	51.7	Syngenta/Meinke
Leu	*At4g04350*	*emb 2369-1*	Exon 10 (20)	Globular	2.1	24.8	45.8	Syngenta/Meinke
		emb 2369-2	Exon 6 (20)	Globular	1.8	25.8	52.2	Genoplante
Arg	*At4g26300*	*emb 1027*	Exon 4 (18)	Globular	2.2	27.0	46.8	Syngenta/Meinke
Asn	*At5g56680*	*emb 2755*	Exon 6 (6)	Cotyledon	1.2	25.8	50.7	Syngenta/Meinke
Gly	*At3g48110*	*edd-1*	Exon 2 (33)	Transition	n.d.	n.d.	n.d.	Altmann
		edd-1S	Exon 8 (33)	Globular	2.0	27.2	52.9	Syngenta/Meinke
Tyr	*At3g02660*	*emb 2768 -1*	Exon 1 (1)	Preglobular	n.d.	19.9*	54.9	Salk/Meinke
		emb 2768 -2	Exon 1 (1)	Preglobular	n.d.	21.5	71.3*	Salk/Meinke
Thr	*At2g04842*	*emb 2761*	Exon 6 (8)	Globular	1.4	23.9	50.3	Salk/Meinke
Ala	*At5g22800*	*emb 1030-2*	Exon 4 (8)	Transition	1.9	25.0	50.0	Salk/Meinke
		emb 1030-1	Uncertain	Transition	1.9	28.5	47.9	Syngenta/Meinke

[a]Based on alignment of flanking sequence; total number of exons or introns noted in parentheses.
[b]See http://www.seedgenes.org for additional details.
[c]Ratio of resistant to sensitive seedlings obtained from selfed heterozygotes; n.d., not determined.
[d]Present in heterozygous siliques; 25% expected; *significant difference, $P = 0.001$.
[e]Mutant seeds in top half of heterozygous siliques; 50% expected. Significant deviation indicates probable effect of mutation on pollen tube growth.

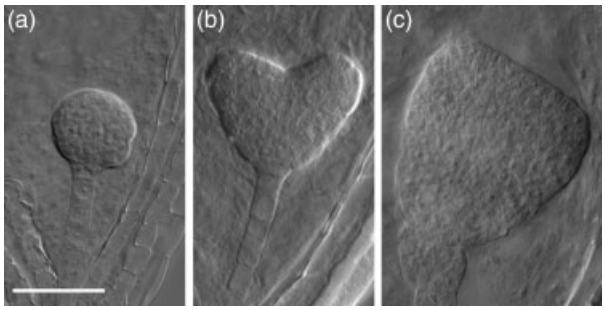

Figure 2. Nomarski images showing terminal embryo phenotypes for knockouts of AARSs predicted to function in chloroplasts. Mutant seeds were removed from heterozygous siliques containing normal seeds at a transition to early cotyledon stage of embryo development.
(a) ArgRS (*At4g26300*) knockout (*emb1027*).
(b) AlaRS (*At5g22800*) knockout (*emb1030-1*).
(c) AsnRS (*At5g56680*) knockout (*emb2755*). Scale bar, 50 μm.

suppressed. Heterozygotes consistently had more aborted ovules than wild-type controls (Figure 3) but often fewer than the 50% expected for female gametophytic lethals. Two lines of evidence suggested that some of these aborted ovules might correspond to mutant seeds arrested after fertilization: (i) their combined frequency often approached 25% of total ovules, as expected for zygotic lethals, and (ii) they occasionally appeared somewhat larger and more pigmented than unfertilized ovules. The low ratio of heterozygous to wild-type plants among selfed progeny of heterozygotes, however, was more consistent with reduced gametophytic transmission.

Two different approaches were taken to search for evidence of zygotic and gametophytic lethality. One involved reciprocal crosses between heterozygous and wild-type plants to determine rates of gametophytic transmission. A second compared the frequency of normal seeds and aborted ovules in heterozygous siliques produced after either self-pollination or crosses with wild-type pollen. Results shown in Table 4 demonstrate that with the exception of *ova9*, which involves an atypical cytosolic GlnRS, all *ova* mutant alleles can be transmitted through both male and female gametes. In many cases, male and female transmission efficiencies for a given mutant allele were reduced to similar extents. Based on these observed rates of transmission, most *ova* mutants should produce a mixture of defective ovules and aborted seeds following self-pollination. Because we failed to detect an appreciable number of mutant seeds with an advanced phenotype in these siliques, we conclude that some of the structures that resemble aborted ovules must represent mutant seeds arrested immediately after fertilization. Similar types of seeds have been noted in screens for female gametophytic mutants (Pagnussat *et al.*, 2005) but escaped detection in screens for embryo-defective mutants (McElver *et al.*, 2001) because they resembled unfertilized ovules. Consistent with this view, the percentage of aborted ovules in F_1 siliques decreased somewhat when *ova1*, *ova2* and *ova3-1* heterozygotes were crossed with wild-type pollen (Table 4), although the extent of reduction did not always match that predicted from the observed transmission rates. In contrast, the frequency of aborted ovules in *ova9* heterozygotes did not change after pollination with wild-type pollen, in agreement with the observed absence of female transmission of that mutant allele.

Based on these observations, we expected to find the following structures in siliques of selfed heterozygotes: (i)

Table 3. Mutants with an ovule abortion phenotype

Amino acid	Locus	Allele symbol	Insertion line[a]	Insertion site[b]	Percent aborted ovules[c]	OVA/ova : OVA/OVA (total plants genotyped)[d]	
						PCR	Plating
Met	*At3g55400*	*ova 1*	SK_029422	Exon 4 (11)	33 (10)	1.2 (115)	n.d.
Ile	*At5g49030*	*ova 2*	SK_088424	Exon 16 (22)	42 (11)	1.1 (115)	n.d.
Glu	*At5g64050*	*ova 3-1*	SK_118914	Intron 6 (12)	32 (9)	1.1 (76)	n.d.
		ova 3-2	SK_064910	Exon 6 (13)	26 (9)	0.8 (39)	1.4 (297)
Trp	*At2g25840*	*ova 4*	SK_042930	Exon 1 (13)	26 (7)	1.2 (40)	1.3 (96)
Lys	*At3g13490*	*ova 5*	SK_112490	Exon 7 (14)	37 (4)	0.6 (37)	0.5 (349)
Pro	*At5g52520*	*ova 6*	SK_045080	Exon 9 (10)	56 (5)	0.7 (40)	n.d.
Ser	*At1g11870*	*ova 7*	SL_1303_G01	Exon 4 (10)	29 (6)	0.4 (40)	0.7 (250)
Asn	*At4g17300*	*ova 8*	SK_011751	Intron 2 (13)	26 (6)	0.4 (40)	n.d.
Gln	*At1g25350*	*ova 9*	SK_116777	Intron 5 (21)	39 (6)	0.6 (40)	0.9 (50)

[a]SK, Salk; SL, SAIL (see http://signal.salk.edu).
[b]Based on alignment of flanking sequence; total number of exons or introns noted in parentheses.
[c]Average calculated for 50 heterozygous siliques (10 per plant); average for wild-type plants from the same insertion line noted in parentheses.
[d]Ratio of progeny plants (heterozygotes:wild type) obtained from selfed heterozygotes and genotyped either by PCR or by plating on selection medium; total number of plants genotyped noted in parentheses. Ratios could not be determined (n.d.) by plating for lines with a suppressed resistance marker.

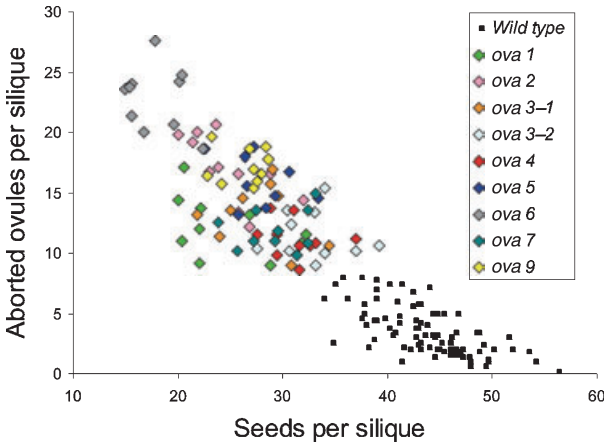

Figure 3. Frequency of aborted ovules and normal seeds in siliques from insertion lines with an ovule abortion phenotype. Each data point represents the average of five siliques from a single plant. Immature siliques from 20 plants of known genotype (10 *ova*/*OVA;* 10 *OVA*/*OVA*) determined by PCR were examined from each mutant. Note that heterozygotes consistently produce on average fewer normal seeds and more aborted ovules than sibling wild-type plants.

normal seeds; (ii) unfertilized ovules; (iii) ovules aborted prior to fertilization; (iv) mutant ovules fertilized with wild-type pollen and (v) mutant ovules fertilized with mutant pollen. Distinguishing between these classes was difficult because unfertilized ovules often resembled ovules that aborted before fertilization and some fertilized mutant ovules appeared to develop slowly at first and then recover. Unfertilized ovules at the preglobular stage of normal development [Figure 4(c)] are nevertheless smaller than developing seeds [Figure 4(a)] and slightly smaller than other structures found in some *ova* heterozygotes [Figure 4(b)], which we interpret as fertilized mutant ovules that degenerate by the globular stage of normal development [Figure 4(d)]. These results are consistent with the conclusion that disruption of mitochondrial function in Arabidopsis results in reduced gametophytic transmission and embryo lethality shortly after fertilization.

Most knockouts of cytosolic AARSs have not been confirmed

Disruption of non-redundant cytosolic AARSs appears to have a more serious effect on reproductive development in Arabidopsis. As shown in Table 1, most of these insertions were either not represented in the Salk database, not requested because they were located in genomic regions unlikely to generate a null phenotype or not confirmed through PCR reactions involving progeny plants. Although negative results can be difficult to interpret, the recurring pattern observed here for multiple AARSs with similar predicted localizations is consistent with the conclusion that significant disruption of cytosolic translation results in both female and male gametophytic lethality. Because we limited our initial genetic analysis to insertion lines generated at Salk and Syngenta, several GABI lines (Rosso *et al.*, 2003) predicted to contain insertions within coding regions of AARS genes were not examined in detail. Consistent with the gametophytic lethality model, PCR confirmation of T-DNA inserts later failed in five GABI lines disrupted in genes encoding cytosolic AARSs for Met, Ile, Lys, Pro and Ala (Y. Li, M.G. Rosso and B. Weisshaar, MPIZ, Cologne, Germany, personal communication). We suspect that fol-

Table 4. Gametophytic transmission of mutant alleles and phenotype analysis of progeny plants and seeds

Allele symbol	Female transmission[a] (total plants)	Male transmission[a] (total plants)	% Aborted ovules after crossing[b] (total siliques)	% Aborted ovules after selfing[b] (total siliques)
ova 1	0.6 (36)	0.8 (36)	6 (10)	23 (20)
ova 2	0.7 (36)	0.6 (36)	32 (25)	40 (16)
ova 3-1	0.7 (36)	1.0 (36)	17 (10)	27 (20)
ova 3-2	0.8 (159)[c]	0.8 (235)[c]	n.d.	n.d.
ova 4	0.8 (323)[c]	1.0 (313)[c]	n.d.	n.d.
ova 5	0.8 (146)[c]	0.7 (218)[c]	n.d.	n.d.
ova 9	0.0 (159)[c]	0.8 (143)[c]	42 (15)	44 (22)

[a]Number of heterozygous plants found/number expected with full transmission after reciprocal crosses to wild type involving heterozygotes as either female or male parents.
[b]Percent aborted ovules found in immature siliques produced after heterozygotes were crossed with wild-type pollen or selfed.
[c]Results obtained from plating on selection medium.

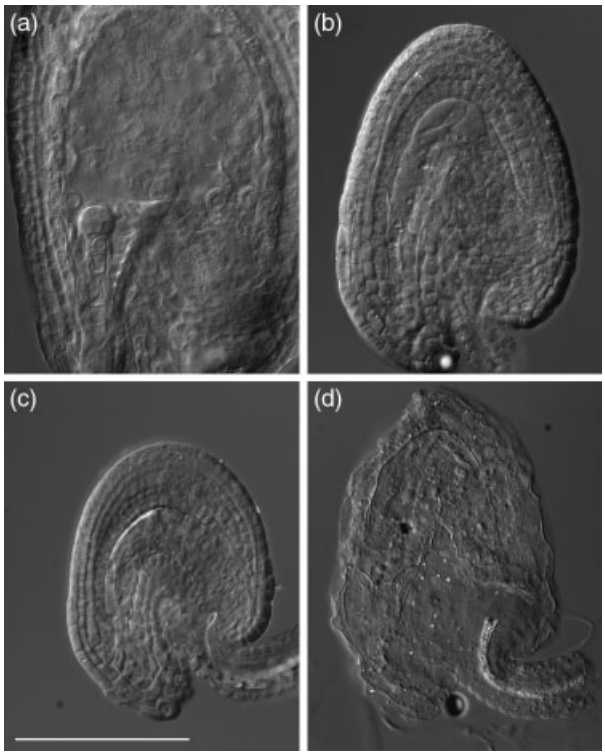

Figure 4. Nomarski images of seeds and ovules from heterozygous siliques of an IleRS knockout (*ova2*) with an aborted ovule phenotype.
(a) Normal seed at the preglobular stage of embryo development.
(b) Aborted ovule lacking a developed embryo from a silique at the preglobular stage.
(c) Unfertilized or aborted ovule from a silique at the preglobular stage. Scale bar, 100 μm.
(d) Degenerated mutant ovule from a silique at the globular stage.

lowing initial detection of these insertions in parental (T_1) leaf tissue, transmission of the mutant allele to progeny (T_2) seeds was blocked. We encountered a similar situation with SAIL lines where the expected insertion could not be confirmed in progeny plants using the same PCR primers that generated the original flanking sequence. The obvious limitation when dealing with individual lines is that one cannot prove that seeds obtained from the stock center were derived from the same plant that generated the flanking sequence. The difference here is that the same negative result was observed repeatedly for different genes predicted to encode non-redundant, cytosolic AARSs. Because the Salk collection of insertion lines was generated in a different manner, with DNA extracted from progeny (T_2 or T_3) seedlings rather than parental (T_1) leaves, the scarcity of Salk insertions disrupting cytosolic AARSs was not surprising. This highlights an important distinction between the Salk collection and the Syngenta and GABI insertion lines with respect to gametophytic lethals deficient in both male and female transmission. With Salk lines, these insertions are often eliminated before sequencing, whereas in GABI and Syngenta lines they can be sequenced once but then are usually not confirmed in subsequent generations.

Several apparent exceptions to the proposed generalization concerning cytosolic AARSs merit consideration. One is the ovule abortion phenotype observed in a knockout of the cytosolic GlnRS (*At1g25350*). This suggests that either the mutant examined is not a null allele, which is unlikely but possible because the insertion site is located within an intron, or the proposed alternative pathway of producing Gln-tRNA in chloroplasts and mitochondria also contributes somewhat to translation in the cytosol during male gametophyte development. Partial complementation of a defect in cytosolic translation by an enzyme primarily targeted to organelles may also explain why a knockout allele (*GABI_728E05*) of cytosolic ThrRS (*At5g26830*) was recently confirmed (Y. Li, M.G. Rosso, B. Weisshaar, MPIZ, Cologne, Germany, personal communication). A second apparent contradiction, the embryo defects found in the *twn2* mutant disrupted in the cytosolic ValRS, has already been explained as an atypical phenotype associated with a weak allele. No

confirmed null alleles of this locus have been described. Another problematic gene is *At1g50200* (AlaRS), which was reported to have a seed phenotype associated with a 3′-UTR T-DNA insertion (Ge *et al.*, 1998) that we could not confirm using seeds obtained from the Nottingham stock center. We have therefore listed the status of this mutant phenotype as uncertain in Table 1. The failure to confirm the insert in a putative knockout allele of this gene (*GABI_439B04*) is consistent with results obtained for other cytosolic AARS mutants.

Several AARS knockouts produce viable homozygotes

Viable homozygotes were identified for knockouts of three different AARS genes (Table 1). Additional examples of knockout homozygotes may be found among insertion lines disrupted in redundant AARSs (Asn, Asp, Phe, Cys) not examined in detail. Two independent knockout alleles of *At1g66530* (ArgRS; *SK_007461* and *SL_100D11*) with insertion sites confirmed in our laboratory were found to produce viable homozygotes with no obvious phenotype. This observation is significant because only two ArgRSs have been identified in Arabidopsis. The other gene (*At4g26300*) must therefore supply functional protein to all three compartments of the cell, provided a third ArgRS has not escaped detection. Knockouts of *At4g26300* are embryo defectives (Table 1), which suggests that the first gene product (At1g66530) cannot support translation in chloroplasts. These two proteins are the most similar of all known AARSs in Arabidopsis, consistent with a recent duplication event. Viable homozygotes with normal phenotypes and confirmed insertion sites were also identified for two TyrRSs: *At2g33840* (*SK_089092*) and *At1g28350* (*SK_027145*, *SK_125634* and *SK_004125*). Disruption of the third gene encoding TyrRS (*At3g02660*) results in an atypical (early) embryo phenotype and reduced male transmission of the mutant allele. This pattern of arrested development, which resembles a weak *ova* phenotype, may reveal the consequences of interfering somewhat with mitochondrial but not chloroplast functions.

Discussion

Forward genetics has long been used to identify genes with critical functions during embryo development in Arabidopsis (Meinke and Sussex, 1979). Large numbers of embryo-defective (*emb*) mutants have been recovered following chemical (Franzmann *et al.*, 1995) and insertional mutagenesis (McElver *et al.*, 2001) and examined for defects in cellular processes required for plant growth and development (e.g. Bellec *et al.*, 2002; Geldner *et al.*, 2003; Ishikawa *et al.*, 2003; Lukowitz *et al.*, 2004; Stacey *et al.*, 2002). The frequency of *emb* mutants obtained to date is consistent with the presence of 500–1000 total *EMB* genes in Arabidopsis (McElver *et al.*, 2001). Information on 250 genes isolated and characterized throughout the community is presented at the SeedGenes website (Tzafrir *et al.*, 2003; http://www.seedgenes.org).

A major challenge for the future is to identify those remaining *EMB* genes that have so far escaped detection. Forward genetics becomes less efficient with increasing saturation because a greater proportion of mutants identified represent duplicate alleles of known *EMB* genes. Although these new alleles may help to confirm gene identities and provide clues to gene function, alternative strategies involving reverse genetics are needed to identify additional genes. The issue then becomes which candidate genes represent the most promising targets for reverse genetics. We have addressed this question in some detail (Tzafrir *et al.*, 2004) and identified several classes of genes that merit further analysis, including those that resemble essential genes in other model organisms, those for which a knockout homozygote cannot be identified and those that share an interacting protein partner, metabolic pathway or cellular process with a known *EMB* gene. In this report, we pursued the shared process strategy by examining knockouts of genes encoding aminoacyl-tRNA synthetases, which were known to be included among existing *EMB* genes. The surprising paucity of *EMB* genes with expected phenotypes among these knockouts and the presence of multiple lines with an ovule abortion phenotype serve as a reminder that mutants disrupted in basal cellular functions will exhibit an embryo-defective phenotype only if they survive gametogenesis. Future attempts to saturate for *EMB* genes in Arabidopsis will therefore need to address the issue of gametophytic lethality in more detail and work towards the establishment of a comprehensive data set of genes required for multiple stages of reproductive development.

Although considerable progress has been made in the identification of genes required for male and female gametophyte development in Arabidopsis (McCormick, 2004; Yadegari and Drews, 2004), the total number of genes identified to date remains uncertain, in part because there is no central database that summarizes information obtained from different laboratories. Defining gametophytic genes is also difficult because many loss-of-function mutations appear to reduce but not eliminate gametophytic transmission. The largest collection of published gametophytic mutants is from a screen of transposon-tagged lines that revealed the identities of more than 100 genes required for female gametophyte development (Pagnussat *et al.*, 2005). Potential roles of these genes in male gametophyte development remain to be documented. Forward genetic screens for gametophytic mutants have often focused on reduced transmission of an associated selectable marker (Feldmann *et al.*, 1997; Lalanne *et al.*, 2004; Procissi *et al.*, 2001). Plants heterozygous for a tagged mutant allele that interferes with gametophyte development typically exhibit a ratio of resistant to sensitive progeny that is below the 2:1 ratio expected

for a recessive embryo lethal. An alternative strategy for identifying gametophytic mutants that utilizes the *quartet* mutation has also been described (Johnson *et al.*, 2004). Mutations that are never passed through either male or female gametes will not be maintained and cannot be studied. The total number of such genes remains unknown. Some mutations disrupt female gametophyte development but not pollen development, germination or tube growth. These result in heterozygotes with 50% aborted ovules (Pagnussat *et al.*, 2005). Other mutations interfere with male transmission but not with female transmission. Some of these exhibit visible pollen defects; others are disrupted only in pollen tube growth or fertilization (Johnson *et al.*, 2004; Lalanne *et al.*, 2004). Another class of mutants is defined by heterozygotes that produce a mixture of defective seeds and aborted ovules that can vary between 25% and 50% of total ovules produced. The distinction between genes required for gametogenesis and those required for embryogenesis is therefore not absolute. Some genes with basal cellular functions may nevertheless be eliminated from data sets of potential *EMB* genes because knockouts are defective in gametogenesis and homozygous mutant embryos are not formed.

Several different factors can influence the knockout phenotype of a gene with a basal cellular function: (i) functional redundancy of the gene, protein product, biochemical pathway and cellular process; (ii) expression patterns of redundant genes and localization of their protein products; (iii) amount of functional gene product contributed from surrounding diploid cells to haploid gametophytes or from heterozygous sporocytes to descendant haploid spores; and (iv) strength of the mutant allele examined. The relative contributions of these factors have been difficult to evaluate in the past because data sets of known gene disruptions are incomplete. It has therefore been hard to predict which knockouts should be embryo lethals and which might be gametophytic lethals.

The approach described here makes it possible to examine the developmental consequences of knocking out multiple genes with related but distinct roles in protein synthesis. The results obtained are consistent with the conclusion that disruption of protein synthesis in chloroplasts results in embryo lethality and interference with protein synthesis in mitochondria results in ovule abortion. Because complete elimination of protein synthesis in the cytosol appears to cause both male and female gametophytic lethality, we conclude that stored AARS gene products from diploid sporocytes, contributions to gametophytes from surrounding heterozygous cells and overlapping functions of redundant AARSs targeted to organelles are insufficient to rescue the mutant phenotype and must therefore serve a minor role in supporting cytosolic translation during normal reproductive development. Furthermore, knockouts of other factors required for cytosolic translation should survive gametogenesis only if some level of redundancy is involved, contributions from heterozygous cells are more significant or the gene product is not essential.

Genes encoding components of the cytosolic (80S) ribosome provide an informative example. Eight of these genes (*AML1*, *EMB3010*, *EMB1080*, *EMB1129*, *EMB2207*, *EMB2296*, *EMB2386*, *EMB2171*) have a loss-of-function phenotype in the seed and are included in the SeedGenes database. Four other genes, *PFL1* (Van Lijsebettens *et al.*, 1994), *PFL2* (Ito *et al.*, 2000), *ARS27A* (Revenkova *et al.*, 1999) and *STV* (Nishimura *et al.*, 2004), have later mutant phenotypes. All of these genes are redundant at the DNA sequence level, with up to four genes encoding the same ribosomal protein (Barakat *et al.*, 2001). Ribonucleic acid interference (RNAi) experiments with *Caenorhabditis elegans* (Sonnichsen *et al.*, 2005) have confirmed that most of these proteins are essential, consistent with their critical role in translation. It therefore appears that genomic redundancy, perhaps with some contribution of gene product from heterozygous cells, enables Arabidopsis knockouts of genes encoding cytosolic ribosomal proteins to survive gametogenesis. However, these contributions eventually become insufficient to support normal development of the embryo and mature sporophyte. Knockouts of the remaining 80S ribosomal genes, all of which are redundant to some extent, should therefore result in gametophytic lethality only if these genes have non-overlapping patterns of expression.

The conclusion here that disruption of an essential chloroplast function often results in embryo lethality is consistent with results obtained from other studies dealing with knockouts of genes encoding chloroplast-localized proteins, including those required for replication (Wall *et al.*, 2004), metabolism (Lin *et al.*, 2003), protein import (Hormann *et al.*, 2004; Kovacheva *et al.*, 2005), protein assembly (Apuya *et al.*, 2001) and chloroplast development (Despres *et al.*, 2001). The mixture of embryo and female gametophytic lethality described for two *SIG5* knockout alleles may reflect separate roles for this RNA polymerase sigma factor in both chloroplasts and mitochondria (Yao *et al.*, 2003). Many other genes that encode ribosomal proteins and pentatricopeptide repeat (PPR) proteins localized to chloroplasts are also included in the SeedGenes database. The requirement of chloroplast functions for embryo development has therefore been clearly established.

Predicting the developmental consequences of a complete loss of mitochondrial function in Arabidopsis is more difficult because mutants devoid of mitochondrial activity have not been described. In light of the metabolic activity required to support pollen tube growth, we were surprised to find that disruption of aminoacyl-tRNA synthetases predicted to function in mitochondria did not interfere more completely with male gametophytes. We had assumed that male transmission of the mutant allele would be more

severely disrupted than female transmission because mutant eggs seemed more likely to be partially rescued by parental gene products. Instead, it appears that storage of paternal gene products may be more widespread than expected and capable of rescuing at least some deleterious mutations. Several lines of evidence are consistent with this hypothesis: (i) the scarcity of Arabidopsis genes with knockout phenotypes limited to male gametophytes, especially when compared with the wealth of *EMB* genes; (ii) the diversity of transcripts identified in microspores and male gametophytes (Honys and Twell, 2004) and (iii) recent evidence for male-biased transmission of deleterious mutations in Arabidopsis (Whittle and Johnston, 2003).

Because transcriptome analyses of male gametophytes have been unable to distinguish between RNAs produced before and after meiosis, the possibility remains that some RNAs present in mutant microspores are products of the wild-type allele in heterozygous parental microsporocytes. We propose that knockouts of many non-redundant genes with basal cellular functions in Arabidopsis are capable of surviving male gametophyte development in part because transcription in microsporocytes results in the production of sufficient functional protein later in development to meet the needs of mutant gametophytes. Knockouts of cytosolic AARSs represent a potential exception that may reflect the inability of stored components of the protein synthesis machinery to meet high translational demands during pollen tube growth. Mitochondrial-localized AARSs may be somewhat more stable and less in demand than their cytosolic counterparts, which could explain the transmissibility of their knockout alleles. The ovule abortion phenotype of these mutants is nevertheless consistent with an absolute requirement of mitochondrial functions for initial divisions of the zygote and endosperm. Mutant eggs and central cells that survive gametogenesis become arrested immediately after fertilization with mutant sperm, consistent with the phenotype observed. Stress-induced activation of ovule abortion (Sun *et al.*, 2004) represents another possible mechanism for reducing female transmission of these deleterious mutant alleles by arresting development of mutant ovules either before or immediately after fertilization.

Despite rapid growth in knockout collections and transcriptome data, we remain unable to predict on a global scale which disruptions of non-redundant genes should be gametophytic lethals, which should have reduced gametophytic transmission and which should be embryo defectives. Our inability to assess the stability of stored gene products in male and female gametophytes continues to be a limiting factor. We can nevertheless begin to ask whether significant overlap exists between existing collections of embryo-defective and gametophytic mutants. The surprising answer is that the amount of overlap identified to date is minimal. We expected to find a significant number of loci where null alleles were gametophytic lethals and weak alleles were embryo defectives. Very few of these genes have been identified. More common are genes where putative null alleles are embryo defectives and weak alleles have a vegetative or floral phenotype. Examples include *CYT1*/*VTC1* (Lukowitz *et al.*, 2001), *SUS1*/*CAF*/*SIN1* (Schauer *et al.*, 2002), *DGL1* (Lerouxel *et al.*, 2005) and *APG10* (Noutoshi *et al.*, 2005). Established collections of female gametophytic (Pagnussat *et al.*, 2005) and embryo-defective (Tzafrir *et al.*, 2004) mutants exhibit <3% overlap among genes identified. One confirmed example is *At3g34780*, which encodes a protein of unknown function. Different mutant alleles of this gene have been identified in screens for female gametophytic mutants (*mee22*), embryo-defective mutants (*emb1611*) and mutants with vegetative abnormalities (J. Fletcher, University of California, Berkeley, personal communication). More common are individual mutant alleles with a combination of gametophytic and embryonic defects. One example is the *hap15* (*emb2719*) mutant identified in dual screens for gametophytic (Johnson *et al.*, 2004) and embryo-defective (McElver *et al.*, 2001) mutants. The reduced segregation ratio and abnormal distribution of aborted seeds found in heterozygous siliques from this mutant are consistent with observed defects in pollen tube growth. Several additional *emb* mutants with aberrant ratios and seed distributions are included in the SeedGenes database, consistent with the conclusion that some mutations disrupt gene products required for both embryo development and pollen tube growth (Meinke, 1982).

The extent of overlap between existing collections of male and female gametophytic mutants also remains to be determined. Apparent contradictions in phenotypes and transmission rates need to be resolved as well. For example, the Arabidopsis ortholog (*At1g02140*) of the regulatory gene *mago nashi*, which is required for proper localization of mRNA at the posterior end of the *Drosophila* oocyte (Mohr *et al.*, 2001), has been identified as the disrupted locus in a female gametophytic mutant (*mee63*) with a slight reduction in female transmission (Pagnussat *et al.*, 2005) and in a male gametophytic mutant (*hap1*) with normal female transmission and drastically reduced male transmission (Johnson *et al.*, 2004). Because of the considerable effort required to establish definitive transmission data and characterize subtle defects in male and female gametogenesis it may take some time before overlaps between expanding collections of gametophytic and embryonic mutants are resolved. A long-term objective of the Arabidopsis community should therefore be the establishment of a comprehensive data set of genes with a loss-of-function phenotype at any stage of reproductive development from meiosis to seed maturation. Such a data set could eventually help to reveal the importance of a wide range of gene products and cellular processes throughout plant growth and development.

Experimental procedures

Plant materials and growth conditions

Seed stocks for Salk (Alonso *et al.*, 2003) and SAIL (Sessions *et al.*, 2002) insertion lines (Columbia ecotype) were obtained from the ABRC at The Ohio State University. Plants were grown in 7.5 cm pots containing a mixture of vermiculite (Strong-Lite coarse; Sun-Gro Horticulture, Pine Bluff, AR, USA), potting soil (Scotts Redi-Earth, Plug and Seedling Mix, Scotts-Sierra Horticultural Products, Marysville, OH, USA) and sterilized sand (12:3:1 v/v/v), placed in a growth room at 24 $\pm$ 2°C under fluorescent lights on 16-h light/8-h dark cycles, and watered daily from below with a nutrient solution (0.35 g l^{-1}) containing Excel 15-5-15 fertilizer (Scotts Miracle-Gro, Port Washington, NY, USA). Detailed information on plant growth conditions, screening immature siliques for seed phenotypes and preparation of Nomarski images of cleared seeds is presented in the tutorial section at http://www.seedgenes.org.

Reverse genetics of insertion lines

Putative insertion lines for the selected AARS genes were identified using the database at http://signal.salk.edu. Seeds were requested for Salk and SAIL lines with open reading frame insertions likely to generate a null phenotype. Nineteen insertion lines chosen for reverse genetic analysis yielded informative phenotypic data: SALK_116777 (*At1g25350*), SALK_029422 (*At3g55400*), SALK_088424 (*At5g49030*), SALK_042930 (*At2g25840*), SALK_112490 (*At3g13490*), SALK_045080 (*At5g52520*), SALK_118914 and SALK_064910 (*At5g64050*), SAIL_1303_G01 (*At1g11870*), SALK_003349 (*At2g04842*), SALK_100540 (*At5g22800*), SAIL_100_D11 and SALK_007461 (*At1g66530*), SALK_027145, SALK_125634 and SALK_004125 (*At1g28350*), SALK_016722 and SALK_045570 (*At3g02660*) and SALK_011751 (*At4g17300*). T-DNA inserts from five additional lines representing cytosolic AARSs failed to confirm in our laboratory, consistent with the conclusion that gametophytic transmission was blocked: SAIL_310_D04 (*At3g62120*), SAIL_837_G08 and SAIL_655_D04 (*At3g02760*), SAIL_1250_D03 (*At1g09620*) and SAIL_108_C12 (*At1g29880*). Confirmation by PCR of five GABI lines for cytosolic AARSs also failed before shipment to our laboratory: GABI_338B08 (*At4g13780*), GABI_310G02 (*At4g10320*), GABI_258B07 (*At3g11710*), GABI_192A04 (*At3g62120*), and GABI_439B04 (At1g50200). Another 26 lines requested from the stock center were assigned a low priority because they belonged to the redundant class of AARSs, represented weak alleles, or appeared from initial characterization to be problematic. Information on 10 additional lines with seed phenotypes identified through forward genetics is presented at http://www.seedgenes.org.

We initially planted six pots containing nine seeds each per mutant. Immature siliques were then screened from resulting plants and leaf tissue harvested for PCR analysis. Genomic DNA was extracted using a modified CTAB protocol (Lukowitz *et al.*, 2000). Tissue from nine plants in a single pot was pooled for DNA extraction. Single plants from pots with confirmed insertions were tested further. Dry seeds were harvested for detailed analysis from single plants with confirmed insertions. Plating of seeds on selection media was performed as described by McElver *et al.* (2001).

Primer design and PCR analysis

Gene-specific primers for each mutant line were designed using the SIGnAL iSect Primer Design program at http://signal.salk.edu and purchased from IDT (Coralville, IA, USA). Primers for the left border of T-DNA in Salk LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) and SAIL lines LBsail (5′-TTCATAACCAATCTCGATACAC-3′) were used in combination with the appropriate gene-specific primers to detect and confirm insertions. The PCR parameters used were: 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 56°C for 40 sec, 72°C for 80 sec and a final elongation step of 72°C for 5 min. Reactions were performed with a Biometra (Göttingen, Germany) Uno II thermocyler. Polymerase chain reaction products were separated in agarose gels, stained with ethidium bromide and visualized with a Kodak (New Haven, CT, USA) EDAS 290 camera. Amplified products were gel purified (Qiagen, Valencia, CA, USA) and sequenced at the Oklahoma State University Recombinant DNA/Protein Resource Facility as needed to confirm T-DNA insert locations. Confirmed insertion sites for ten different Salk and SAIL lines verified through additional sequencing were located within 160 bp of the locations presented at http://signal.salk.edu.

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