RESEARCH ARTICLE

Complex haploinsufficient interaction between *APC11* and *CYCLIN A1;2/TARDY ASYNCHRONOUS MEIOSIS* in embryo development and seed germination in *Arabidopsis* [version 1; peer review: 2 not approved]

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Abstract

Background: Complex haploinsufficiency is characterized by individuals with two heterozygous loci producing a phenotype that is not seen in either of the corresponding single-locus heterozygous individuals. The mutants of the anaphase-promoting complex/cyclosome (APC/C) subunit gene *APC11* and the mitotic cyclin gene *CYCLIN A1;2/TARDY ASYNCHRONOUS MEIOSIS (TAM)* in *Arabidopsis thaliana* are embryo-lethal and defective in meiosis, respectively, but their heterozygous single mutants do not exhibit defective embryo development and seed germination.

Methods: Crosses between two heterozygous *apc11* mutant alleles and two homozygous *tam* mutant alleles, and between two heterozygous *apc11* mutant alleles and a *TAM:TAM-GFP* line were conducted. Phenotypes of the F₁ seeds were analyzed by light microscopy.

Results: We found that F₁ embryos from the crosses between heterozygous *apc11-1* (*APC11/apc11-1*) and homozygous *tam-2* (*tam-2/tam-2*) or between *APC11/apc11-2* and *tam-2/tam-2* were morphologically normal but all the seeds failed to germinate. F₁ embryos from the crosses between *APC11/apc11-2* and *tam-1/tam-1* (weaker allele than *tam-2*) produced morphologically normal seeds that germinated to form mature plants. However, F₁ embryos from the crosses between *APC11/apc11-1* and *tam-1/tam-1* were abnormal and the seeds failed to germinate. Moreover, F₁ embryos from the crosses between *APC11/apc11-1* and a *TAM:TAM-GFP* line were arrested at early developmental stages while F₁ embryos from the crosses between *APC11/apc11-2* and the *TAM:TAM-GFP* line appeared fully developed but the seeds failed to germinate.

Conclusions: Our observations indicate that the *apc11* and *tam*

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mutants have an allele-dependent complex haploinsufficient relationship in embryo development and seed germination.

Keywords

anaphase-promoting complex, mitotic cyclin, complex haploinsufficiency, cell division, dominant negative mutant protein

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Introduction

Haploinsufficiency is a frequently observed phenomenon defined by the inability of a heterozygous mutant locus to produce the same phenotype as the corresponding homozygous wild-type locus in a diploid organism. Since it was first proposed in 1932 by O. L. Mohr (Stern 1943), the word “haploinsufficiency” has long been used to simply describe the dosage effect of one or more heterozygous loci. However, in some cases, two heterozygous loci co-existing in the same organism produce a phenotype not seen in either of the corresponding single heterozygotes, a phenomenon defined by Haarer *et al.* (2007) as complex haploinsufficiency. Establishment of a complex haploinsufficient relationship among genes can help reveal the structure of a genetic network in a biological process especially when lethality prevents the generation and/or analysis of a double mutant of two loci.

Cell cycle regulators form tightly knit networks in eukaryotes (Li *et al.* 2004; Giotti *et al.* 2017; Van Leene *et al.* 2010). However, depending on the circumstances, functional studies of the network of cell cycle regulators in development of complex multicellular organisms are hindered by either lethality of single mutants (Murphy *et al.* 1997; Liu and Finley 2010; Wang *et al.* 2012; Wang *et al.* 2013; Guo *et al.* 2016) or non-existence of a mutant phenotype likely due to redundancy of homologous genes (Jacobs *et al.* 1998; Ye *et al.* 2001; Pérez-Pérez *et al.* 2008). In particular, a mutant phenotype for a mitotic cyclin in embryo development and seed germination has not been observed in plants.

The genome of *Arabidopsis* contains 87 core cell cycle regulators, including 10 A-type and 11 B-type mitotic cyclins (Menges *et al.* 2005). No mutants of these cyclins have been shown to produce an embryo-defective or a seed-germination-defective phenotype, even though gene expression studies have implicated a role in embryo development and/or seed germination for most of them, including *TARDY ASYNCHRONOUS MEIOSIS (TAM)/CYCA1;2* (Masubelele *et al.* 2005; Narsai *et al.* 2011; Hofmann *et al.* 2019). Homozygous *tam-1/tam-1* and *tam-2/tam-2* mutants are defective in meiotic cell cycle progression and in nuclear size regulation in trichomes and guard cells, but no obvious defect was observed in their embryo development and seed germination (Magnard *et al.* 2001; Wang *et al.* 2004; Wang *et al.* 2010; Jha *et al.* 2014; Wang and Yang 2014). The previous studies also did not reveal any defect in heterozygous *TAM/tam-1* and *TAM/tam-2* plants. Thus, it is likely that functional redundancy occurs among TAM and other mitotic cyclins in embryo development and seed germination.

Another major cell cycle regulator, the anaphase-promoting complex/cyclosome (APC/C), is a large E3 ubiquitin ligase complex that mediates the ubiquitination of mitotic cyclins and other cell cycle regulators, which leads to degradation of these regulators by the proteasome (Sudakin *et al.* 1995; Irniger *et al.* 1995; King *et al.* 1995; Yamano 2019). Degradation of cell cycle regulators via the APC/C in a temporal order allows the cell cycle to exit mitosis and prevents the reentry into the next S-phase of the cell cycle (Yamano 2019). On the other hand, cyclin As and/or Bs activate the APC/C via phosphorylation of the APC3 and APC1 subunits or an inhibitor of the APC/C activator CDC20 by cyclin-CDK complexes (Lahav-Baratz *et al.* 1995; Kramer *et al.* 2000; Rudner and Murray 2000; Dienemann and Sprenger 2004; Zhang *et al.* 2016). Cyclin As can also inhibit premature activation of the APC/C by phosphorylating the APC/C activators CDH1 and CDC20 (Sørensen *et al.* 2001; Hein and Nilsson 2016). Although TAM has not been demonstrated to be a substrate of the APC/C, the CDKA1-TAM complex can phosphorylate *in vitro* OSD1, a negative regulator of the APC/C in *Arabidopsis* (Iwata *et al.* 2011; Cromer *et al.* 2012). However, in *Arabidopsis*, unlike single mutants of mitotic cyclins, several single mutants of subunits of APC/C are gametophytic-lethal or embryo-lethal (Capron *et al.* 2003; Kwee and Sundaresan 2003; Wang *et al.* 2012; Wang *et al.* 2013; Guo *et al.* 2016). In particular, embryo development is arrested at the zygote stage in the homozygous *apc11-1/apc11-1* and *apc11-2/apc11-2* mutants of the APC11 subunit of the APC/C although heterozygous *APC11/apc11-1* and *APC11/apc11-2* embryos develop normally (Guo *et al.* 2016). The zygotic lethality of the *apc11* mutants hinders the study of the effect of the mutations on subsequent development in *Arabidopsis*.

In an attempt to investigate how TAM and APC11 genetically interact in *Arabidopsis* development, we conducted genetic crosses between two *tam* mutants and two *apc11* mutants and between a transgenic line containing the *TAM:TAM-GFP* transgene and the *apc11* mutants. Our findings revealed a strong complex haploinsufficient relationship between TAM and APC11 in both embryo development and seed germination, and a dominant-negative effect arising from the coexistence of the *apc11-1* and the *tam-1* alleles or of the *apc11-1* allele and the *TAM:TAM-GFP* transgene.

Methods

Plant materials and growth conditions

The origins and initial characterization of the mutants and the *TAM:TAM-GFP* line used in this investigation were as described in our previous work (Magnard *et al.* 2001; Wang *et al.* 2004; Wang *et al.* 2010; Guo *et al.* 2016). Briefly, *tam-1* and *apc11-1* harbor point mutations that were generated by chemical mutagenesis (Magnard *et al.* 2001; Guo *et al.* 2016), and *tam-2* and *apc11-2* harbor T-DNA insertions (Wang *et al.* 2004; Guo *et al.* 2016), in the *TAM* and *APC11* loci,

respectively. The *TAM:TAM-GFP* line has been characterized with respect to its expression in male meiocytes and vegetative organs and its complementation of the meiotic defect in *tam-1* (Wang *et al.* 2004; Jha *et al.* 2014). All seeds were air dried at least two weeks before sowing. The plants were grown in soil (Sunshine MVP growing Mix, Sungro Horticulture, Agawam, Massachusetts, USA) or on a medium containing 4.3 g/L Murashige and Skoog (MS) salt base (catalogue number 11117-074, Gibco, Waltham, MA, USA), 1% (w/v) sucrose (catalogue number S1695, Spectrum Quality Products, Gardena, CA, USA), and 0.7% (w/v) agar (catalogue number PTC001, Gaisson Laboratories, Smithfield, UT, USA) in a growth chamber at ~22°C. Daily light regime in the growth chamber was 16h fluorescent light supplemented with tungsten light (light intensity = ~50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ near the plants) and 8h darkness.

Genetic crosses

Six types of crosses between a heterozygous *apc11* mutant and a homozygous *tam* mutant or the *TAM:TAM-GFP* line were conducted. Buds of the *tam* mutants and the *TAM:TAM-GFP* line that were about to open were emasculated and their stigmas pollinated with pollen from the *APC11/apc11-1* or *APC11/apc11-2* plants. At least three fertile siliques were obtained from each type of the crosses. Care was taken to remove other buds from the same inflorescences to facilitate harvesting the seeds from the crosses.

Phenotypic characterization

The imbibed seeds were dissected with a pair of 30G½ syringe needles under either a Leica S6D or a Nikon SMZ1000 dissecting microscope with a magnification range of 2x to 4x. Photographs of the seeds were taken using the Leica EC3 imaging system on the Leica S6D microscope. Embryos were observed and photographed on a Nikon Eclipse 80i microscope equipped with the differential interference contrast (DIC) optics and the Nikon DS-Ri1 imaging system. The images shown in the figures were lightly adjusted for contrast and brightness and made black and white on the entire image area in Adobe Photoshop CC 2014.

Results

Complex haploinsufficiency in seed germination observed from crosses between *APC11/apc11-1* and *tam-2/tam-2* and between *APC11/apc11-2* and *tam-2/tam-2*

To investigate how *apc11* mutants genetically interact with *tam* mutants, we crossed *APC11/apc11-1* and *APC11/apc11-2* individuals with *tam-2/tam-2*, respectively. Each of the crosses resulted in more than 50 F₁ seeds. However, these seeds, after fully dried, failed to germinate in more than 14 days on the MS agar medium, in contrast with the corresponding wild-type (Col-0) seeds germinating within 2-3 days (Wang *et al.* 2016). The germination defect observed did not result from abnormally underdeveloped embryos because embryos dissected out from these seeds appeared fully developed (Figure 1). Because the pollen donor plants for these crosses, *APC11/apc11-1* or *APC11/apc11-2*, were heterozygous for the *apc11* mutation as the homozygous mutant plants are not available due to embryo lethality, the genotypes of the F₁ seeds should be either *APC11/APC11;TAM/tam-2*, *APC11/apc11-1;TAM/tam-2*, or *APC11/apc11-2;TAM/tam-2*. Because seeds heterozygous for any of the three mutations alone did not exhibit such a severe germination defect (Wang *et al.* 2010; Guo *et al.* 2016), the germination defect observed indicates complex haploinsufficiency between *apc11-1* and *tam-2*, and *apc11-2* and *tam-2*. Moreover, because all the F₁ seeds failed to germinate and approximately half of their embryos should have the genotype of *APC11/APC11;TAM/tam-2*, our results suggest that *APC11* was also insufficient even in the *APC11/APC11;TAM/tam-2* embryos.

Complex haploinsufficiency in embryo development and seed germination observed from crosses between *APC11/apc11-1* and *tam-1/tam-1*

To provide additional evidence for the complex haploinsufficient relationship between *APC11* and *TAM*, we crossed the *APC11/apc11-1* and *APC11/apc11-2* plants with another mutant, *tam-1/tam-1*, respectively. The *tam-1* mutation is a point mutation that does not result in unreduced gametes as *tam-2* and it produces normal diploid embryos and germinated seeds (Wang *et al.* 2004; Wang *et al.* 2010). Surprisingly, F₁ seeds from the crosses between *APC11/apc11-1* and *tam-1/tam-1* varied in size and showed variable degrees of shrinkage (Figure 2a). These seeds also failed to germinate like the F₁ seeds from the crosses between *APC11/apc11-1* and *tam-2/tam-2*. However, unlike the F₁ seeds from the crosses between *APC11/apc11-1* and *tam-2/tam-2*, small and abnormal embryos were readily found after dissection of these seeds (Figure 2c-f) even though some embryos were morphologically normal (Figure 2f). These observations indicate that *apc11-1* and *tam-1* have a complex haploinsufficient relationship in embryo development and seed germination, given that *APC11/apc11-1* and *TAM/tam-1* individuals do not have such defects (Magnard *et al.* 2001; Wang *et al.* 2004; Guo *et al.* 2016). The level of complex haploinsufficiency between *apc11-1* and *tam-1* is more severe than those between the two *apc11* mutants and *tam-2*. The *apc11-1* and *apc11-2* mutations are also a point mutation and a T-DNA insertion mutation, respectively, with *apc11-2* having a more severe embryo defective phenotype than *apc11-1* (Guo *et al.* 2016). Therefore, the most severe complex haploinsufficiency was found with two relatively weak point-mutation alleles.

Figure 1. F₁ phenotypes. (a-c) From a cross between *APC11/apc11-1* and *tam-2/tam-2*. (a) A representative embryo from an imbibed seed that failed to germinate. (b) and (c) Close-up views of a cotyledon and the root of the embryo in (a), respectively. (d-f) From a cross between *APC11/apc11-2* and *tam-2/tam-2*. (d) A representative embryo from an F₁ seed. (e) and (f) Close-up views of a cotyledon and the root of the embryo in (d), respectively. The embryo in (d) was larger than that in (a) presumably due to an unreduced egg frequently resulting from the *tam-2* mutation (Wang *et al.* 2010; d'Erfurth *et al.* 2010). Scale bar in (a) for (a) and (d) = 200 μm, and scale bar in (b) for (b), (c), (e), and (f) = 50 μm.

The F₁ seeds from the cross between *APC11/apc11-2* and *tam-1/tam-1* appeared normal (Figure 2b) and germinated and developed into mature plants. This result is consistent with *tam-1* being a mild allele that did not result in complex haploinsufficiency in the double heterozygous F₁ individuals during embryo development and seed germination.

Severe defects in embryo development and seed germination in F₁s of crosses between *APC11/apc11-1* and *TAM-GFP/TAM-GFP* and between *APC11/apc11-2* and *TAM-GFP/TAM-GFP*

In an attempt to investigate how the TAM protein is affected in the *apc11* mutants, we crossed *APC11/apc11-1* and *APC11/apc11-2* with a homozygous *TAM:TAM-GFP* line in the Col-0 wild-type background, *TAM-GFP/TAM-GFP*, respectively. Surprisingly, for each type of the crosses, none of the more than 30 F₁ seeds germinated after two weeks on the MS agar medium. Seeds from the crosses between *APC11/apc11-1* and *TAM-GFP/TAM-GFP* appeared small and shrank (Figure 3a). Dissection of their non-germinated seeds revealed that the embryos were arrested at either the apical cell stage (Figure 3b) or the early globular stage (Figure 3c). Most of the F₁ seeds from the crosses between *APC11/apc11-2* and *TAM-GFP/TAM-GFP* were similar in size to normal seeds in two dimensions (length and width) but abnormally thin in the third dimension (depth), i.e., with a flattened appearance (Figure 3d). Some of these seeds were apparently abnormally small (Figure 3d). Dissection of the non-germinated seeds produced embryos at an advanced developmental stage (Figure 3e-g). The *TAM:TAM-GFP* transgene alone does not cause an embryo developmental arrest or a seed non-germination defect (Wang *et al.* 2004). Therefore, the defects observed suggest a dominant negative effect

Figure 2. F₁ phenotypes. (a) and (c-f) From a cross between *APC11/apc11-1* and *tam-1/tam-1*. (a) Seeds of variable sizes and morphologies. (c-f) Embryos from imbibed seeds that failed to germinate, showing variable sizes and morphologies. The variation in seed or embryo size should not have resulted from variable ploidy levels of the egg cells because the *tam-1* mutant, unlike the *tam-2* mutant, produces only haploid gametes (Magnard *et al.* 2001; Ajay *et al.* 2014). (b) Seeds from a cross between *apc11-2* and *tam-1*. Scale bar in (a) for (a) and (b) = 1 mm, and scale bar in (c) for (c-f) = 200 μ m.

of the TAM-GFP protein in the F₁ seeds where the APC11 protein was presumably reduced. In fact, these defects parallel those of the corresponding F₁s from the crosses between *APC11/apc11-1* and *tam-1/tam-1*, and between *APC11/apc11-2* and *tam-2/tam-2*, respectively, although they were more severe with the *TAM:TAM-GFP* transgene than with the *tam* mutations.

Discussion

Using complex haploinsufficiency tests to demonstrate functions of genes in embryo development and/or seed germination

Our findings of complex haploinsufficiency between pairs of *apc11* mutations and *tam* mutations in both embryo development and seed germination are consistent with the knowledge that APC/C and mitotic cyclins reciprocally regulate each other's functions in cell cycle progression in eukaryotes. The role of TAM in embryo development and seed germination and the role of APC11 in seed germination have not been reported before. Therefore, testing if two mutations have a complex haploinsufficient relationship is a useful approach for functional studies of genes acting in embryo development and/or seed germination. This approach can work for a mutant such as *tam-1* or *-2* that by itself does not have an obvious defect in embryo development and seed germination. It can also work for an embryo lethal mutant such as *apc11-1* or *-2* for demonstrating its effect on seed germination.

In Arabidopsis, there are many known recessive embryo lethal mutants (Meinke 2020) and other recessive mutants (e.g. mutants available from the Arabidopsis Biological Resource Center, Columbus, OH) that do not exhibit defects in

Figure 3. F1 phenotypes. (a-c) From a cross between *APC11/apc11-1* and *TAM-GFP/TAM-GFP*. (a) Small and shriveled seeds. (b) A developmentally arrested embryo from an imbibed seed that failed to germinate, showing an enlarged apical cell attached to the suspensor. (c) A developmentally arrested embryo from an imbibed seed that failed to germinate, showing an early globular embryo attached to the suspensor. (d-g) From a cross between *APC11/apc11-2* and *TAM-GFP/TAM-GFP*. (d) Flattened seeds. (e) A representative embryo from an imbibed seed that failed to germinate. (f) and (g) Close-up views of a cotyledon and the root of the embryo in (e), respectively. Scale bar in (a) for (a) and (d) = 1 mm, scale bar in (e) = 200 μm , and scale bar in (b) for (b), (c), (f) and (g) = 50 μm .

embryo development and/or seed germination and yet their wild-type genes are expressed in the embryo (Narsai *et al.* 2011; Hoffmann *et al.* 2019). Any pairs of mutants from these two categories may be tested in the F_1 generation for complex haploinsufficiency if the paired mutants are suspected to affect the same developmental process based on available information. This approach should circumvent the difficulty of identifying the double mutant of such a pair of mutants and be faster than the double mutant analysis because it involves only the F_1 seeds.

Most severe defects were associated with the coexistence of the *apc11-1* and *tam-1* mutant proteins and of the *apc11-1* mutant protein and the TAM-GFP protein

The *tam-2* and *apc11-2* alleles are more severe alleles than the *tam-1* and *apc11-1* alleles, respectively (Magnard *et al.*; zWang *et al.* 2004; Wang *et al.* 2010; Guo *et al.* 2016), but the defects exhibited in the F_1 seeds of this investigation were most severe between *tam-1* and *apc11-1*, comparing to between *tam-2* and *apc11-2*, *tam-1* and *apc11-2*, and *tam-2* and *apc11-1*. The *tam-2* and *apc11-2* alleles are T-DNA insertion mutants that likely do not produce the TAM and APC11 proteins, whereas the *tam-1* and *apc11-1* alleles are expected to produce the mutant proteins because of the nature of their point mutations generated from chemical mutagenesis. Therefore, the presence of both mutant proteins correlates with the most severe defects in comparison with the other combinations without the presence of both mutant proteins. This phenomenon, in principle, may be explained by reciprocal dominant-negative effects of the *apc11-1* and the *tam-1* mutant

proteins. As discussed earlier, both positive and negative interplays exist between the APC/C and mitotic cyclins. This relationship may underpin the phenomenon that the presence of both mutant proteins has a greater functional impact than the presence of one, or absence of both, of the mutant proteins. Interestingly, the *TAM:TAM-GFP* line produced even more severe complex haploinsufficient-like phenotypes than *tam-1* and *tam-2*. This finding further supports the idea that the presence of a malfunctioned protein, which is assumed to be the case for the TAM-GFP protein, is more devastating to the plant than without such a protein, especially when in coexistence with the *apc11-1* mutant protein.

Embryo development and seed germination are sensitive to simultaneous reductions in dosage or otherwise function of the *APC11* and *CYCA1;2/TARDY ASYNCHRONOUS MEIOSIS* genes

Because the crosses in the current investigation were conducted with *APC11/apc11-1* and *APC11/apc11-2*, approximately one half of the F₁ plants should be double heterozygous for the *apc11* and *tam* mutations and the other half homozygous wild type at the *APC11* locus and heterozygous for one of the *tam* mutations. Similar allele frequencies should occur in F₁s of the crosses between the *apc11* mutants and the *TAM:TAM-GFP* line. However, when a defect was present, it was observed in all F₁ seeds of the crosses albeit the severity of embryo developmental defects, if existed, varied. This observation indicates that reconstituting homozygous *APC11* from fertilization could not rescue the F₁s from abnormal embryo development or seed non-germination in the *TAM/tam* background. A plausible explanation for this outcome in the F₁s is that the pollen grains used in the crosses had a reduced level of the APC11 protein or *APC11* transcript due to heterozygosity at the *APC11* locus in the parental tissue, and the APC11 protein or *APC11* transcript could not recover to the normal level in the haploid pollen grains even though they had a wild-type allele of *APC11*. If so, embryo development and seed germination seem to be sensitive to simultaneous reductions in APC11 and TAM levels or to a reduction in the APC11 level along with the presence of a malfunctioned TAM-GFP.

The roles of genes encoding core cell cycle regulators in Arabidopsis seed germination have been demonstrated for two D-type cyclins genes, *CYCD4;1* and *CYCD1;1* (Masubelele *et al.* 2005). Expression of these genes was found to be activated before the onset of cell division in the radicle, and the earliest cell divisions in the radicle preceded root emergence from the seed coat that defines the completion of germination. Cell divisions in the radicles of the *cycd4;1* and *cycd1;1* mutants were reduced, which correlated with the delayed root emergence from their seed coats (Masubelele *et al.* 2005). Interestingly, activation of expression of two A-type cyclin genes, one of which is *TAM* (the other *CYCA3;4*), was also found along with the earliest expressed D-type cyclin genes (Masubelele *et al.* 2005). The findings from this investigation support the roles of these A-type cyclins in seed germination. Furthermore, the non-germination phenotype associated the complex haploinsufficiency lends support to the view that cell divisions are required for seed germination. Detailed genetic analysis with combinations of the D-type and A-type cyclin mutants may shed more light on the essential role of cell division in seed germination in Arabidopsis.

Data availability

Figshare: Complex haploinsufficiency between TAM and APC11.

<https://doi.org/10.6084/m9.figshare.13574891>.

This project contains the following underlying data:

Image files of F₁ dry seeds and imbibed non-germinated seeds, and embryos dissected from imbibed non-germinated seeds.

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Author contributions

MY conceived the idea and designed the experiments. MY and YW performed the experiments. MY analyzed the experiments. LG and C-ML contributed the *apc11* mutants. MY wrote the manuscript with input from YW, LG, and C-ML.

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Takashi Hirayama

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This study described a genetic interaction between the mutations of the genes encoding CYCLIN A1;2/TAM and APC11, of *Arabidopsis*. The author showed that the F1 between APC11+/- and TAM+/- failed to germinate, even though half of the seeds should be APC11+/+ TAM+/- . They used different type of alleles and obtained the similar results. The results described might be interesting, showing the abnormal genetic interaction, haploinsufficiency as the author mentioned. However, the author just described the morphological phenotypes of F1 seeds presenting the unclear photographs of seeds, embryos, and enlarged some tissues. I do not understand what the authors wanted to mention with these photographs. I think these data cannot help readers understand the relation between these two loci. If the author thinks the seed shape is important, they can categorize them and show their frequencies. That might give a clue to understand what has happened.

Haploinsufficiency can be explained by the alteration of the gene or gene-product dosage. As the author described, if the APC and cyclin somehow interact each other, this can be happen due to their importance for cell proliferation. I guess, they can test this idea by introducing the WT transgene. Anyway, I think the author should re-consider the approach to the mechanisms of this genetic interactions.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant molecular genetics, plant hormones, plant stress responses.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 26 Feb 2021

Ming Yang, Oklahoma State University, Stillwater, USA

We understand the reviewer's criticisms and suggestions for further investigation into questions arising from the current results. However, for various reasons, we are not planning to continue this work. We acknowledge that the presentation of results in our paper deviates from the norm that is often found in papers in the related fields. We agree with the reviewer that additional experiments can be done to strengthen the conclusions, but we think the current results can justify for the conclusion that complex haploinsufficiency exists between the *TAM* locus and the *APC11* locus. Our results show 100% seed non-germination in five of the six crosses, and 100% or frequent occurrences of abnormal seeds or embryos in three of the six crosses. Even though the amount of data in our paper is limited, we think that the reported findings are valuable to researchers in the related fields.

Competing Interests: No competing interests were disclosed.

Reviewer Report 22 February 2021

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Xing-You Gu

Agronomy, Horticulture and Plant Science Department, South Dakota State University, Brookings, USA

Bhupinder Singh Batth

South Dakota State University, Brookings, USA

General comments:

The reported research addressed a topic known as "*complex haploinsufficiency*" using a genetic approach in *Arabidopsis thaliana*. Six crosses were made between laboratory lines that were different in the genotype for the Tam and APC11 loci involved in the regulation of cell cycling or embryo development. Female parents for the crosses were genotypes homozygous for the induced mutant tam1 or tam2 at the Tam locus or for the transgene TAM:TAM-GFP; the male parents were genotypes heterozygous for the APC11 locus, which contained an induced mutant apc11-1 or apc11-2. Hybrid F1 seeds, i.e., those from plants of the female parents, were evaluated for germination ability and/or morphologies of the embryos. The F1 seed samples from all these crosses failed to germinate on a medium for >14 days or varied in the embryo size or morphology. These observations were explained by interactions between the two loci on the defect embryo development and the failure of germination, presumably due to the induced mutations at Tam and APC11. Results from this research will be interesting to researchers who are working on gene regulatory networks of embryo development and seed biology. A major weakness of this manuscript is lack of quantitative data for the complex phenotypes.

Specific comments:

1. Based on descriptions in the title, abstract and introduction, "*complex haploinsufficiency*" appears similar to the phenomenon that has been reported for distant crosses between remote ecotypes of a species or between closely related species, in which the F1 hybrids often fail to set seeds or display partial sterility. It is suggested to define the concepts "*haploinsufficiency*", "*complex haploinsufficiency*" and "*complex haploinsufficiency interaction*", explicitly to help more readers or researchers better understand the importance of this work, and to help refine the objective and improve the title, abstract and discussions of the manuscript.
2. This research was conducted using a genetic approach. To help improve the M&M section and the data presentation, it is strongly suggested to develop a table to summarize key information for and from each of the 6 crosses, such as genotypes for the parents and F1s, statistics of phenotypic data (e.g., germination percentage and embryo size) from the F1s, and citations for data from the parental genotypes observed in the previous but not in this research to support the conclusions or hypotheses.
3. The F1 seed samples were segregating for the embryo and endosperm genotypes. More discussions are needed to address the potential influence of the segregation on the data annotation and conclusion.
4. Page 5 "*In an attempt to investigate how the TAM protein is affected in the apc11 mutants, we crossed APC11/apc11-1 and APC11/apc11-2 with a homozygous TAM:TAM-GFP line in the Col-0 wild-type background, TAM-GFP/TAM-GFP, respectively*". It is suggested to indicate the GFP signal in related figures and the difference between the F1 TAM-GFP and APC11 genotypes and the F1 TAM-GFP and APC11-1/2 genotypes.
5. The failure of seed germination can arise from underdeveloped embryos, or other genetic

and environmental reasons, such as dormancy and the requirement of a stratification treatment in *Arabidopsis*. It is suggested to include these factors in experimental design, data analysis and data annotation.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Genetics and seed molecular biology

We confirm that we have read this submission and believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 26 Feb 2021

Ming Yang, Oklahoma State University, Stillwater, USA

We understand the reviewer's criticisms and suggestions for further investigation into questions arising from the current results. However, for various reasons, we are not planning to continue this work. We acknowledge that the presentation of results in our paper deviates from the norm that is often found in papers in the related fields. We agree with the reviewer that additional experiments can be done to strengthen the conclusions, but we think the current results can justify for the conclusion that complex haploinsufficiency exists between the *TAM* locus and the *APC11* locus. Our results show 100% seed non-germination in five of the six crosses, and 100% or frequent occurrences of abnormal seeds or embryos in three of the six crosses. Even though the amount of data in our paper is limited, we think that the reported findings are valuable to researchers in the related fields.

Other points:

1. Our findings are not similar to the sterility phenomenon in F_1 plants from crosses between ecotypes or species. All the plant lines in our paper are in the same ecotype, Columbia (see references in our paper). Furthermore, the defective seeds were the F_1 generation that never had the chance to grow up.
2. We missed the opportunity to examine the GFP signal during embryo development in the seeds from the crosses involving *TAM:TAM-GFP* because we did not suspect these seeds would not germinate later.
3. We don't think that the non-germination phenotype in our experiments resulted from environmental factors, dormancy due to incomplete drying, or the lack of a stratification treatment as all seeds were subjected to the same handling process and seeds from one of the six crosses germinated normally.

Competing Interests: No competing interests were disclosed.

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