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Flavonoid Biosynthesis Genes During Seed Development in *Arabidopsis Thaliana*

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ABSTRACT

Many plants, including agronomically important species, exhibit post-zygotic barriers to hybridisation, in both interploidy crosses within species and interspecific crosses between related species. For instance, crosses between diploid (2x) and tetraploid (4x) plants result in a triploid block where serious endosperm under- or over-proliferation kills the developing triploid embryo. Although most ecotypes of the model species Arabidopsis thaliana tolerate 2x × 4x crosses to create a large viable seed, one ecotype, Columbia (Col-0), exhibits a triploid block when the paternal parent is certainly tetraploid. Recently, lack-of-function mutants in the flavonoid biosynthesis pathway (FBP) that operate in the seed coat have been identified as highly effective maternal suppressors of the Col4x-mediated triploid block. The present hypothesis is that a maternal messenger responsible for regulating the appropriate timing of endosperm cellularisation usually is attenuated or blocked simply by an operating FBP; consequently, mutations in the FBP enhance cellularisation and decrease seed lethality by removing the signalling block. This research attempts to understand the role of the FBP and its products in the seed development regulation following 2x × 4x crosses. To this end, an assortment of some of the FBP mutants was assembled to assess their influence on alleviating the triploid block using confocal and light microscopy. The results revealed that many (although not all) mutations of the FBP alleviated the triploid block and that, specifically, perturbations to FBP that led to a reduced amount of proanthocyanidins was connected with 'rescue' from Col4x-induced seed lethality. These details could have a potential biotechnological utility in overcoming hybridisation barriers when discovering new hybrids to increase endosperm size and, thus, seed yield in crop plants.

1. Introduction

The global population is projected to increase to approximately 10 billion by 2050; therefore, increasing food production is critical [1]. Seeds constitute approximately 68% of the world's food supply and are a vital source of nutrition [2]. Seeds result from sexual reproduction in plants through double fertilisation and are made up of three components: the seed coat (testa), the embryo and the endosperm (Figure 1A) [3]. The development of the seed coat is determined by the maternal genome, whereas the endosperm and the embryo develop under the control of both parental genomes (Figure 1B) [2]. Seed development entails two distinct phases: embryo development and seed maturation [4].

Most flowering plants, including *Arabidopsis thaliana*, have an embryo sac containing seven cells, which consist of four cell types: the egg cell; two synergid cells, which are essential for pollen tube attraction; the central cell; and three cells called antipodal cells, whose function is currently unknown. The embryo sac is surrounded by diploid integument tissues that together constitute the ovule. The egg cell (n) is fertilised by one of the sperm cells (n) delivered through the pollen tube, resulting in a diploid zygote (embryo 2n). The homodiploid central cell (2n) is fertilised by the second sperm cell (n) giving rise to the triploid endosperm (3n), which functions as a nutrient source for the embryo. The triploid endosperm progresses through a free-nuclear multinucleate stage up to the pre-globular embryo stage followed by cellularisation. The ovule integuments differentiate to form the seed coat that protects the seed during embryogenesis, dormancy and germination [5].

The embryo, endosperm and integument cells need to synchronise their growth during seed development. The interaction between the endosperm and integuments during growth may be an important factor in determining the seed size [6]. Thus, the aberrant behaviour of the zygotic (including the endosperm) or maternal tissues can affect the seed size; this can result from unbalancing the programme of endosperm development due to interspecific and interploidy hybridisation [7]. Maternal modifier genes, such as members of the flavonoid biosynthesis pathway (FBP), can re-impose a normal (balanced) programme on the endosperm—in the endothelium—and restore a relatively normal viability [8, 9].

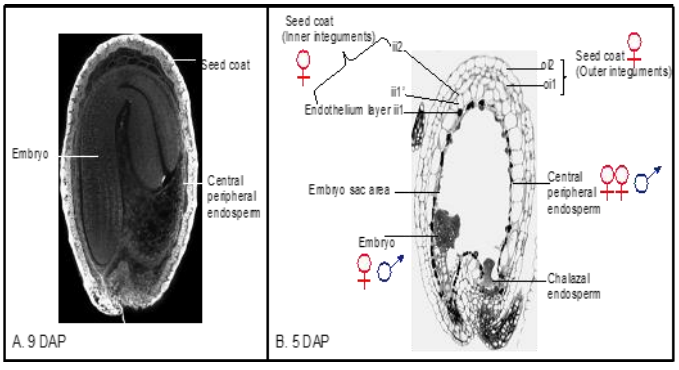

Figure 1. A. *thaliana* seed anatomy at 5 and 9 days after pollination (DAP). A. General seed structure. **B. The seed at the incipient endosperm cellularisation stage.** The outer integument includes the outer integument second layer (oi2) termed 'epidermis' and outer integument first layer (oi1) termed 'palisade'. The inner integument contains ii2, ii1' and ii1 and is called 'endothelium' (adapted from [10]).

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1.1. FBP

Flavonoids, a diverse family of plant secondary metabolites, are synthesised from the phenylpropanoid pathway by converting phenylalanine to 4-malonyl-CoA [11]. In the Brassicaceae, flavonoids have many roles such as attraction of insects to aid with pollen dispersal and regulation of polar auxin transport, as well as a physiological role in seed dormancy or viability [12-16].

1.2. Triploid block in A. thaliana seed development

Generally, the normal development of the endosperm involves a parental genomic ratio of 2m:1p, which is the ideal balance between the paternal proliferation genes (growth promoters) and the maternal growth inhibition genes to form a healthy seed. Any disruption of this balance can cause developmental defects. Any imbalance in this ratio causes negative consequences for the developing seed, which can result in lethality [17].

A triploid block is common in plants, but *A. thaliana* exhibits a tetraploid block [9]. A triploid block in the Col-0 ecotype can be due to mating a diploid parent with a tetraploid parent (2x × 4x or 4x × 2x = 3x), and the result of this mating is abnormal viable progeny [9, 18]. Dilkes *et al.* showed that the outcome for 2x × 4x crosses using the Col-0 ecotype is largely non-viable progeny and a much lower tolerance than the seeds with a parent from the L*er* ecotype in interploidy crosses [8]. Therefore, understanding post-zygotic hybridisation barriers are critical to be able to manipulate and overcome them to potentially create larger seeds for yield increase in agriculture.

1.3. Regulation of endosperm development—a maternal cellularisation signal

As discussed earlier, the behaviour of seeds that result from hybridisation is significantly influenced by endosperm cellularisation. Dilkes *et al.* pointed out that a loss-of-function mutation in the *TRANSPARENT TESTA GLABRA2 (TTG2)* gene in *A. thaliana* – which encodes a transcription factor of the WRKY family [8]; means that the seed parent is almost completely resistant to Col-0 killing, indicating that the maternal integument tissues regulate endosperm cellularisation. Therefore, if *TTG2* plants were to be used as the maternal source, then the absence of *TTG2* proteins act as 'rescuers', modifying the triploid block seen in Col-0 and producing small seeds [5,8]. The FBP is present in an endothelium layer: the ii1 layer of maternal inner integument layers (Figure 1). Hence, by combining our knowledge from the molecular players in the FBP and the effect of the triploid block, we hypothesise that the transport of a cellularising factor between the maternal integuments and the endosperm is inhibited by a functional FBP in *A. thaliana* interploidy cross (Col2x × Col4x). This is known as the maternal cellularisation signal hypothesis (Figure 2). This research investigates the role of the FBP (in a few genes) and its intermediates in regulating paternal Col4x-induced seed lethality ('Col-killer') in *A. thaliana*.

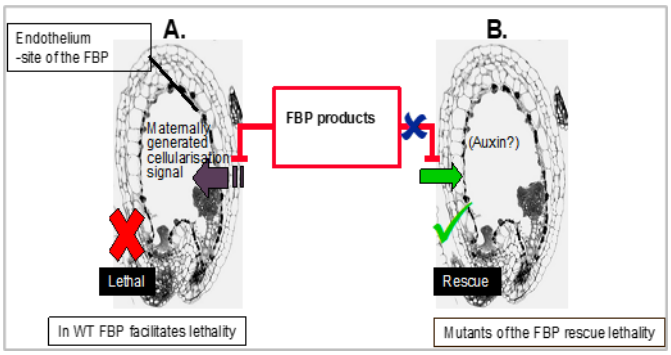

Figure 2. Model displaying the effect of FBP products in wild-type and FBP mutant in *A. thaliana.* **A.** FBP inhibits the action of an endosperm cellularisation factor (2x × 4x). The model predicts that the maternal integument layers create a maternal cellularisation factor that is transported into the endosperm through the endothelium (purple arrow). The FBP (red box) in the endothelium layer inhibits this signal. Consequently, endosperm cellularisation does not occur or is significantly late. Delayed cellularisation allows lethal over-proliferation of the endosperm, as is seen in Col2x × Col4x crosses. **B.** FBP mutants alleviate the inhibition/transport of the cellularisation factor. Presenting a *tt* mutation (blue x) disrupts the FBP (red box) in the endothelium layer. This prevents or causes a decrease in the production of the ultimate products of the pathway (e.g. proanthocyanidins (PAs)). Therefore, the transportation of the cellularisation factor through the maternal integument layers to the endosperm is increased (green arrow). Consequently, endosperm cellularisation happens early, thus rescuing Col4x-mediated seed lethality [19].

2. Results and discussion

Overall, seeds produced from crosses involving various maternal FBP mutants in the Col-0 ecotype and Col4x differed from each other as well as from seeds derived from balanced 2x × 2x in their weight and size (Figures 3, 4 and Table 1). In balanced cross Col2x × Col2x, at 5 DAP, the central peripheral endosperm was cellularised and the embryo already reached the heart stage. By 7 DAP, even though the chalazal endosperm continued to be present, the embryo reached the torpedo stage. By seed maturity, balanced crosses generated viable, plump seeds (Figure 4 and Table 1).

Confocal microscopy analysis revealed well-defined differences in seed development between FBP mutants crossed with Col4x and the control (Col2x × Col4x). The size of seed seemed to increase with seed development generally in most crosses from 5 to 7 DAP. At 5 DAP, the growth of embryos (3n) was inhibited and remained at (a) the early heart stage of development in Col2x and *tt4*, (b) the heart stage in *tt6*, and (c) the globular stage in *tt14/19*, *tt10*, and *anl1*. Notably, by 7 DAP, a significant delay in development was clear for many lines (Col2x, *tt4*, *tt14/19*, *tt10*, and *anl1*) where embryo development remained at a globular-like stage, being overgrown and abnormal in structure. As seen in Figure 4 and Table 1, most crosses between FBP mutants in the Col-0 background and Col4x pollen parents led to dramatic rises in the embryo sac area between 5 and 7 DAP (*tt4, tt6, tt19/14, tt10,* and *anl1).*This contrasts with the control Col2x × Col4x cross, where expansion of the embryo sac between 5 and 7 DAP was small.

The central peripheral endosperm endured accelerated mitosis, resulting in delayed endosperm cellularisation at 5 DAP. The chalazal endosperm size in all crosses was increased, and some were vacuolated between 5 and 7 DAP, as can be clearly observed in *tt6* × Col4x crosses (Figure 4 (C)). At 5 DAP, the chalazal endosperm area in *anl1*× Col4x cross was the smallest, with a mean of 764 µm^2. At 7 DAP, the chalazal endosperm area in *anl1* × Col4x cross was the largest, with a mean area of 52,555 µm^2. Taken together, the data indicate that maternally carried FBP mutations have a dramatic effect on chalazal endosperm development. At 5 and 7 DAP, *tt10* × Col4x crosses collapse of chalazal endosperm was evident along with nodule formation on the peripheral endosperm (Figure 4 (E)). In *tt19/14* × Col4x, the number of nodules was lowest at 5 DAP, with a mean of 0.3. Moreover, the number of nodules in tt10 × Col4x was smallest at 7 DAP, with a mean of 0.1 (Table 1).

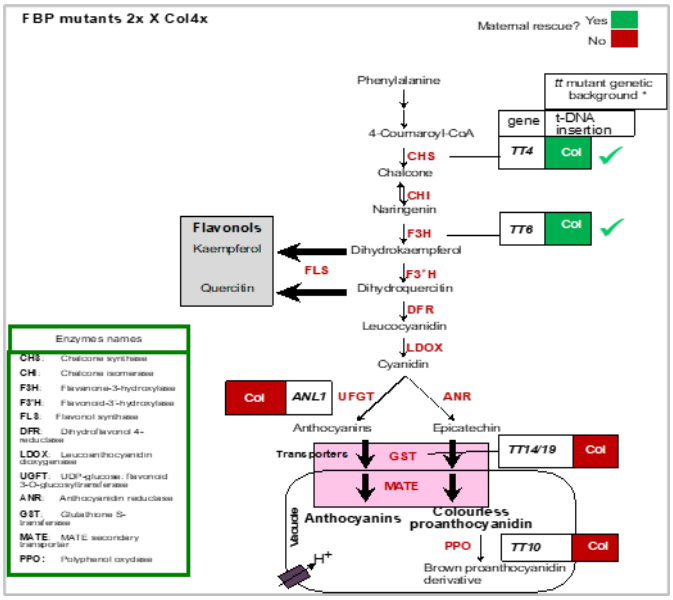

Figure 3. The FBP and its potential role controlling endosperm cellularisation in interploidy hybrids. An updated model of FBP in the *A. thaliana* seed coat. The pink box shows transporters into the vacuole. The white box indicates the vacuole. Mutant alleles of genes with green backgrounds rescue Col4x-induced seed lethality (paternal excess). Genes with red backgrounds do not rescue seed lethality. The presence of an active pathway can be proposed to disrupt the transport of a cellularisation factor into the endosperm. Enzymes are shown with a red colour, and their names are listed in the green box to the left (modified from [16, 19, 20, 21]).

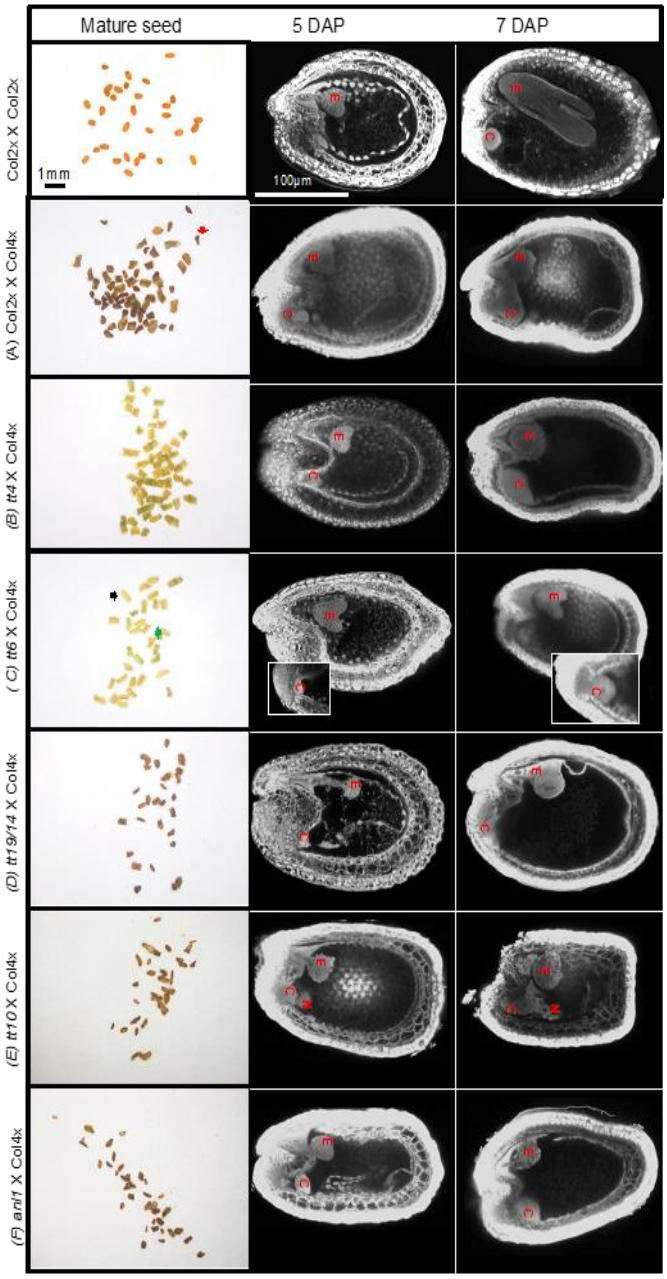

Figure 4. Effect of some mutations in the FBP on seed development (the Col-0 ecotype) following interploidy crosses. The first row: diploid crosses (2x × 2x) in the Col-0 ecotype. Column 1 (left): mature seeds (having paternal excess) derived from interploidy crosses (2x × 4x). Columns 2 (middle) and 3 (right): Confocal laser scanning photomicrographs of tt mutants in interploidy crosses at 5 and 7 DAP. Seeds were classified and counted as plump (indicated using a black arrow), burst (indicated using a green arrow) or shrivelled (indicated using a red arrow). Abbreviation: E: embryo; C: chalazal endosperm and N: nodules. All magnifications: 20x. Scale bars, column 1: 1 mm; columns 2 and 3: 100 µm

These results support previous research in 2x × 4x interploidy crosses that the seed has an embryo (3x) containing twice the normal number of paternal genomes (1m: 2p). The endosperm resulting from these crosses (4x) was affected by paternal genome dosage (2m: 2p), which led to abnormal seed development. This was characterised by accelerated endosperm mitosis and a delay in, or failure of, endosperm cellularisation; this excessive over-proliferation was linked to embryo abortion [7, 8].

Table 1 shows a variation in seed weight amongst seeds derived from the various FBP mutant crosses. Mature seeds arising from *tt4* × Col4x were the heaviest (mean weight: 25 µg). Subsequently, seeds derived from *tt6* with Col4x cross-produced heavy seeds in comparison to seeds derived from the Col2x × Col4x control had a mean weight of 22 µg. However, several mutants (*anl1* and *tt10*) produced seeds which were lighter than the control cross with *anl1*, creating the lowest seeds weight (6 µg).

In general, most FBP mutant's mature seeds have an irregular shape and in the same crossing line. There are some plump and burst seeds (large seed size) and shrivelled seeds (small seed size). Notably, seeds derived from *tt4* and *tt6* crossed to Col4x are noticeably more angular and larger than Col2x × Col4x seeds, most having angular, plump and burst shape seeds (rescue–viable category) (Figure 4 (B) and (C)). Mature seeds of *tt14/19*, *tt10*, and *anl1* with Col4x are different from the control cross; Col2x × Col4x being smaller and irregularly shaped almost all have shrivelled seeds (lethal–unviable category) (Figure 4 (D), (E), and (F)).

Furthermore, the seed-coating phenotype of the *tt4* and *tt6* mutants was yellowish. *TT4* works at the first stage of the FBP and encodes chalcone synthase (CHS) (Figure 3); therefore, its disruption should, in theory, have a substantial influence on the FBP. Significantly, the *tt6* mutant could affect good 'rescue' which encodes F3H, the next enzyme in the FBP. *tt14/19* had a tan brown seed coat. Kubo *et al.* (2007) reported lower levels of anthocyanin in plant tissues, which reflects the importance of *TT14/19* in anthocyanin transport [22]. *tt10* seeds had a very pale brown phenotype. *TT10*, the last gene of the FBP, whose product operates inside the vacuole of the endothelium layer, provided useful information on the potential role of proanthocyanidins (PAs) in the rescue of Col4x-induced seed lethality. *TT10* has a role in the oxidation of PAs [23] and catalyses the change to PAs from procyanidin. *anl1* seed coat colour was darker than that of the control cross.

According to effects from the Col-0 FBP mutant study (Figure 4 and Table 1), several mutants, but not all, were capable of rescuing Col4x-induced seed lethality. Seed weight and the percentage of plump/burst seeds were consistent with one another as indicators of 'rescue' and that was in *tt4* and *tt6*. *tt4* and *tt6* are enzymatic steps operating early in the FBP (Figure 2) that would potentially block the production of all main classes of flavonoids [24]. Notably, seeds caused from maternal *anl1* (having less anthocyanin and potentially more PAs), as confirmed by Kubo *et al.* (2007), had higher seed lethality than the control Col2x × Col4x. Furthermore, they stated that a possible description of *anl1* seed colour phenotype, even with a reduced anthocyanin level, were faults in a) the regulatory gene of the anthocyanin pathway or b) a synthetic gene in the late step of anthocyanin biosynthesis in *A. thaliana* [22].

The Col-0 ecotype produces pollen/sperm that is especially aggressive in favouring endosperm proliferation and repressing endosperm cellularisation, an effect that has been hypothesised to be due to differences in genomic imprinting of as yet uncharacterised loci in this ecotype [8, 18, 25]. Clearly, the Col-0 ecotype is highly sensitive to the effects of paternal excess and FBP mutations that affect the good rescue in ecotypes.

The main hypothesis that rescue of Col4x-induced seed lethality is affected by timely cellularisation of the endosperm [8] remains plausible and is supported by some of the data presented in this study. In most of the crosses studied for the *tt* mutants in the Col-0 ecotype, evidence of cellularisation was not found by 7 DAP. Thus, further research is required to clarify if and when cellularisation occurs in these crosses [8].

Overall, following interploidy crosses, seed survival is strongly affected by genetic ecotype in *A. thaliana* as well as getting influenced by the FBP; indeed, the L*er* ecotype has decreased expression of *TTG2* [8]. In addition, the outcomes showed that FBP mutants in the Col-0 ecotype crossed with Col4x had relatively low mature seed weights even though the seed cavity was much bigger than that in controls (Col2x × Col4x). The likely reason behind this apparent discrepancy is that the number of endosperm nuclei increased dramatically during seed development in these crosses. However, the low level of endosperm cellularisation led to a failure of endosperm to feed the growing embryo sufficiently in many seeds, and subsequently, these seeds collapsed, thus affecting the mean seed weight values. On the basis of the above discussion, further investigation is warranted to recognise the signalling molecule(s) that are crucial for endosperm cellularisation and factors influencing the movement of the cellularisation 'signal'. Moreover, a role for auxin should be considered because certain flavonols are known to be crucial for auxin

Table 1: Summary data for a range of FBP mutants crossed with Col4x in the Col-0 ecotype. All plants in column 1 are the maternal origin of the cross with paternal Col4x background. The mean weight of the mature seeds could be a reliable measure to indicate 'rescue' of Col4x-induced seed lethality by crosses involving maternally carried mutations in the FBP. Thus, the series of FBP mutant crosses comprehensive in this table are ordered by seed weight running from heaviest at the top.

Genotype	Seed development at 5 and 7 DAP								Mature seeds	
	Embryo stage		Embryo sac area (µm ^{2})		Chalazal endosperm area (µm ^{2})		Nodules number		Seed weight (µg)	%Plump and burst seed
	5 DAP	7 DAP	5 DAP	7 DAP	5 DAP	7 DAP	5 DAP	7 DAP		
Col 2x × Col2x (reference cross)	Heart	Torpedo	nd	nd	nd	nd	0	0	17± 3 (250)	nd
Col2x (Background ecotype)	Early heart	Overgrown globular retarded	40026	50771	2645	6312	0.8	0.5	14 ± 2 (517)	6 ± 1
tt4	Early heart	Overgrown globular retarded	34534	130296 ***	1716	14663 ***	1.2	0.8	25 ± 1(483) **	21 ±2 **
tt6	Heart	Heart	38077	103324 ***	994 *	4604	0.5	1.2	22 ± 1 (243) **	27 ±5 **
tt19/14	Globular	Overgrown globular retarded	23655 **	109010 **	873 *	6471	0.3	0.2	17 ± 2 (245)	20 ± 5
tt10	Globular	Overgrown globular retarded	38261	108284 ***	1662	11190	0.9	0.1 *	12 ± 2 (286)	7 ± 2
anl1	Globular	Globular	40931	80717 ***	764 **	52555	0.7	1.9 **	6 ± 1 (210) *	5 ±2

transport and because auxin is vital in regulating plant growth and seed growth [26]

3. Materials and Methods

3.1. Plant material

A. thaliana T-DNA (homozygous) insertion lines in the Col-0 ecotype are as follows: (*tt4-12*) GABI_304D03.02, (*tt6*) SALK_068963.53.50, (*tt14/tt19*) SALK_105779, (*anl1*) SALK_049338.56.00, and (*tt10*) GABI _146E10.02. T-DNA insertion lines were provided by the Nottingham Arabidopsis Stock Centre (NASC), UK. Columbia-0 (Col2x and Col4x) seeds were kindly provided by Prof. Rod Scott (University of Bath), UK.

3.2. Seed germination and plant growth

Seeds were immersed in 0.1% electrophoresis grade agarose (Invitrogen, UK) and incubated at 4 °C for 2–3 days. Seeds were germinated in trays that included Levingtons F2 + S (compost with sand; Scotts, UK). Trays (35 cm × 25 cm) were watered from the base with tap water accompanied by a surface area treatment with 0.2 g/L of insecticide (Intercept 70 WG; Scotts). Trays were in that case covered with a plastic cover and positioned in a Gallenkamp environment chamber under a 16 h/8 h light/dark cycle. Temperatures were 22 °C in the day and 18 °C at night, with 70% humidity. Plastic covers were removed after 1 week.

3.3. Crosses and controlled pollination

Emasculation and pollination were performed according to Scott *et al.* (1998) for developing and mature seeds [7]. The developing seeds were collected at 5 and 7 DAP to permit a study of confocal microscopy.

3.4. Image capture and treatment of homozygous t-DNA lines

Photos of the seeds were obtained utilising a Coolpix 4500 digital camera (Nikon). Images of dry seeds were captured using a Nikon SM2 1500 with a photonic PL2000 light source using NIS-Elements F2.30 software. Mean individual seed weight was calculated as follows.

$$\text{Mean individual seed weight} = \frac{Total\ weight - mean\ weight\ of\ weighing\ boat}{seed\ number}$$

On the basis of differentiating morphology and size of mature seeds, the seeds were classified into two classes predicated on their viability: a) 'plump and burst' seed phenotypes into the rescue category and b) 'shrivelled' seed phenotype into the lethal non-viable category.

3.5. Confocal laser scanning microscopy

The sample size in seed advancement at 5 and 7 DAP was between eight to ten seeds. The planning of specimens, including Feulgen staining, was performed as described by Braselton *et al.* [27], and images were taken with an argon-ion laser—488 nm excitation and 515-530 nm emission—by utilising a Nikon C1 confocal microscope system with a 90i Eclipse microscope and EZ-C1 software, 20x lens (Nikon UK). The images were saved as TIFF files after that prepared using Adobe Photoshop Elements (Adobe, USA). Maximum cross-sectional areas of the embryo sac and chalazal endosperm were measured as well as the number of nodules present in the central peripheral endosperm.

3.6. Statistical analysis

Data were analysed using non-parametric tests: the Kruskal–Wallis and then Mann–Whitney tests ($P < 0.05$ was set as significant) using the SPSS software package version 21 (IBM, USA).

4. Conclusions

This study showed that seed survival in 2x × 4x interploidy crosses is heavily influenced by the genetic background and by some maternal FBP mutations in *A. thaliana*. Further research should investigate the role of a putative signalling molecule in endosperm cellularisation and also whether auxin is a factor in endosperm cellularisation due to its role in regulating plant growth and seed growth. Future work will focus on the link between seed auxin and the FBP.

Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Maha Aljabri, James Doughty and Rod J. Scott. The first draft of the manuscript was written by Maha Aljabri and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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