

# Constructing and testing a genetic network for controlling seed germination in Arabidopsis

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## ABSTRACT

The funiculus (FUN) is continuous with the chalazal seed coat (CSC) during seed development. We previously reported that AUXIN SIGNALING F-BOX PROTEIN 1 and 5 (*AFB1* and 5) expressed in FUN and CSC suppress seed germination. To find the genes regulated by AFBs in the seed germination process, we first determined the expression patterns of AFBs in the FUN-CSC continuum based on the publicly available data. We found that *TIR1*, *AFB1*, and *AFB4* (the *AFB1* group) exhibit a down-expression gradient and *AFB2*, *AFB3*, and *AFB5* (the *AFB5* group) an up-expression gradient from FUN to CSC. The estimated mRNA concentrations of the three *AFBs* in each group are highly linearly correlated in FUN, CSC, and the distal (away from CSC) seed coat (DSC) region. We then searched for auxin-regulated genes that exhibited a down-expression or an up-expression gradient from FUN to CSC. We found 118 such genes that were assigned into four groups based on their response mode (positive or negative) to auxin and expression gradient direction (up or down) from FUN to CSC: 1) downregulated presumably by the *AFB1* group with an up-expression gradient, 2) upregulated presumably by the *AFB5* group with an up-expression gradient, 3) upregulated presumably by the *AFB1* group with a down-expression gradient, and 4) downregulated presumably by the *AFB5* group with a down-expression gradient. These four groups were further broken down into 12 subgroups based on linear correlation analysis of their mRNA concentrations in FUN, CSC, and DSC. Three of the 12 subgroups, including a total of 30 genes, were investigated further because 21 of the 30 genes are known or highly likely to function in the seed germination process. We have tested seed germination in the mutants of five of the nine genes (two alleles/gene) unknown for their involvement in seed germination, with the mutants of three genes exhibiting delayed germination and the mutants of the other two genes exhibiting hastened germination. The experimental results support the validity of the approach used to predict the involvement of these genes in seed germination. Based on the publicly available data and data from this investigation, we constructed a genetic network that consists of the 30 identified proteins, the six AFBs, *BES1* and *BZR1* involved in brassinosteroid signaling, and *ABI3*, 4, and 5 involved in ABA signaling. This genetic network should provide a valuable framework and new clues for future studies of the molecular mechanism controlling seed germination.

## INTRODUCTION

Plant hormones play influential roles in seed germination. Abscisic acid (ABA) is known for promoting seed dormancy whereas gibberellins (GAs) for breaking seed dormancy. An Auxin signaling pathway also interacts with the ABA signaling pathway to promote seed dormancy. It should be expected that a genetic network regulating seed germination should involve these hormones and possibly other hormones. This network from here on is referred to as the seed germination network (SGN).

We have found that maternal AUXIN SIGNALING F-BOX 1 (*AFB1*) and 5 (*AFB5*) inhibit seed germination (Wang et al., 2022). The expression domains of *AFB1* and *AFB5* overlap in the funiculus (FUN) in late seed development and in the hilum of the seed coat when the seed abscises from FUN, but *AFB1*, not *AFB5*, is transiently expressed in a small chalazal region of the seed coat during imbibition. Here we describe the process and outcome of developing the initial SGN, and the experimental validation of some of the components of the SGN. This initial SGN may help fully elucidate the SGN in the future.

## METHODS

### □ A strategy for identifying separate groups of coexpressed auxin-responsive genes in the FUN-CSC (chalazal seed coat) continuum

- Genes expressed in FUN and CSC  $\cap$  Auxin-responsive genes = Auxin-responsive genes in FUN and CSC (Belmonte et al., 2013; Khan et al., 2015) (Goda et al., 2004)
- Expression patterns of *AFBs* in FUN and CSC  $\cap$  Auxin-responsive genes in FUN and CSC = separate co-expressed auxin-responsive genes in the FUN-CSC continuum

### □ Linear regression analysis for assigning subgroups of coexpressed auxin-responsive genes to predict gene function

- Normalizing the available microarray signal values for distal seed coat (DSC) by dividing them by a factor of 26.8 that is the ratio of the volume of DSC to the volume of CSC
- Using the microarray signal values for FUN, CSC, and DSC to conduct linear regression analysis within the coexpressed auxin-responsive genes in the FUN-CSC continuum to further divide them into subgroups using  $R^2 > 0.9$
- If a subgroup contains genes known to function in seed germination, the remaining genes in the subgroup are predicted to also function in seed germination

### □ Testing mutants of predicted genes for seed germination phenotypes

- 10 mutants at five loci (two/locus) of the genes predicted to function in seed germination were tested in a germination assay—germination frequencies were determined at 50-hour imbibition on moist filter paper
- Germination frequencies were determined eight or 12 times for paired wild-type (Col-0) and mutant samples for each mutant allele, and they were statistically analyzed with paired t-test ( $p < 0.05$ ).

### □ Constructing a genetic network for controlling seed germination

- The core structure of the SGN is postulated to include the auxin, ABA, and brassinosteroid signaling pathways.
- By search the genes in the subgroups of interest in the datasets from Okamoto et al. (2010), Tian et al. (2020), and Liu et al. (2020), the connections among these hormonal pathways were found.
- A genetic network showing these connections was drawn first in Cytoscape\_3.9.1 and then, for simplicity, in Microsoft PowerPoint.

## RESULTS AND DISCUSSION

### □ Identification of auxin-down- or upregulated coexpressed genes in FUN and CSC

- TIR1*, *AFB1*, and *AFB4*, (the *AFB1* group) were expressed in a downward trend (Fig. 1A) whereas *AFB2*, *AFB3*, and *AFB5* (the *AFB5* group) in an upward trend (Fig. 1B) from FUN to CSC

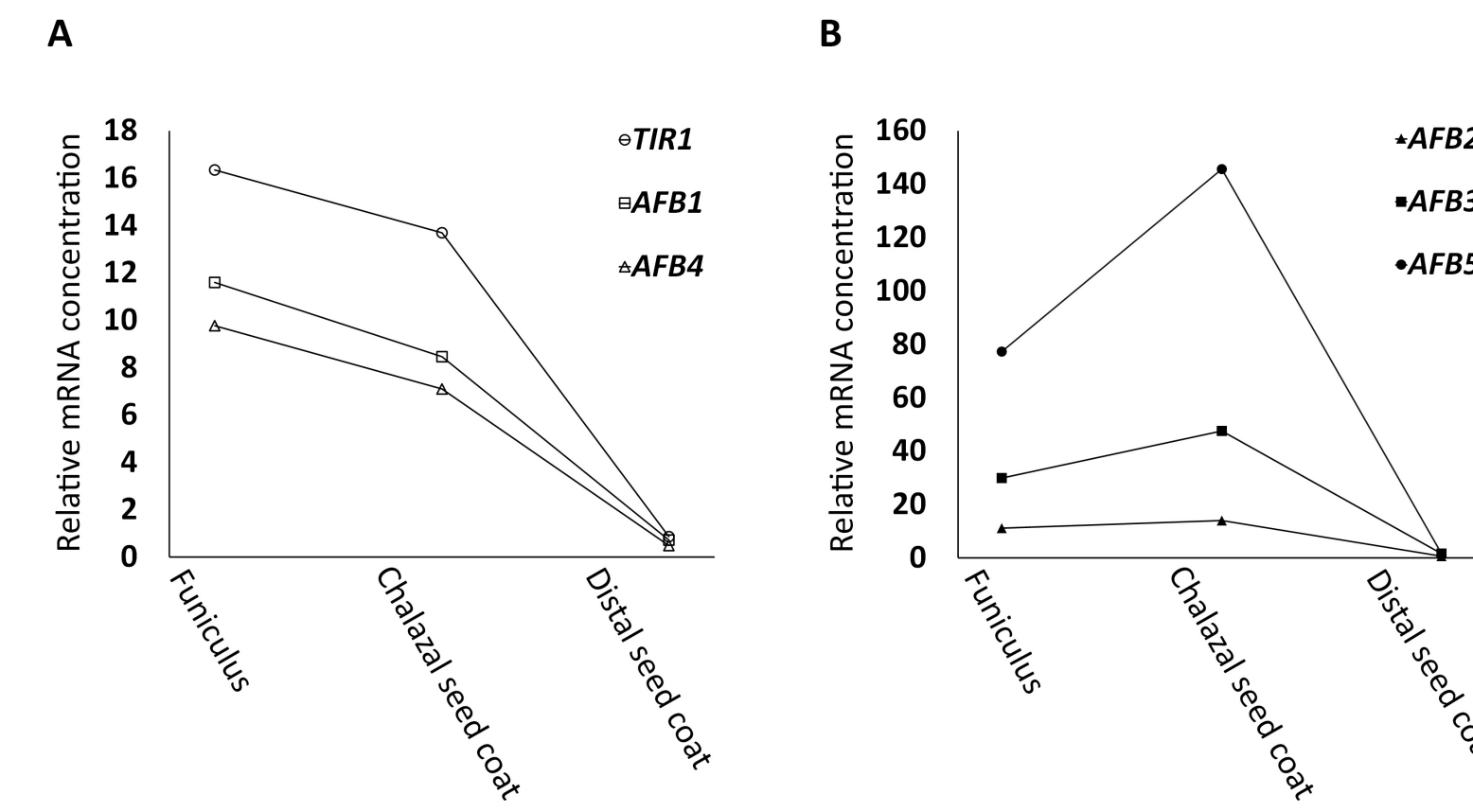


Fig. 1. Relative mRNA concentrations of *AFBs* in FUN, CSC, and DSC of nearly mature seeds. (A) *TIR1*, *AFB1*, and *AFB4* exhibit a trend of downregulation from FUN to CSC. (B) *AFB2*, *AFB3*, and *AFB5* exhibit a trend of upregulation from FUN to CSC.

- 118 genes, either down- or upregulated by auxin were also identified, which were assigned into four groups based on whether they had a downward or an upward expression trend from FUN to CSC and whether they were down- or upregulated by auxin

### □ Identification of genes that are highly correlated in expression with genes known to be involved in seed germination

- The *AFBs* in the *AFB1* group were highly correlated ( $R^2 = 0.99$ , *TIR1* vs. *AFB1*;  $R^2 = 1$ , *AFB4* vs. *AFB1*; Fig. 2A), and so were the *AFBs* in the *AFB5* group ( $R^2 = 0.99$ , *AFB3* vs. *AFB5*;  $R^2 = 0.92$ , *AFB2* vs. *AFB5*; Fig. 2B). However, between *AFB1* and *AFB5*,  $R^2 = 0.57$ , supporting the separation of the two groups

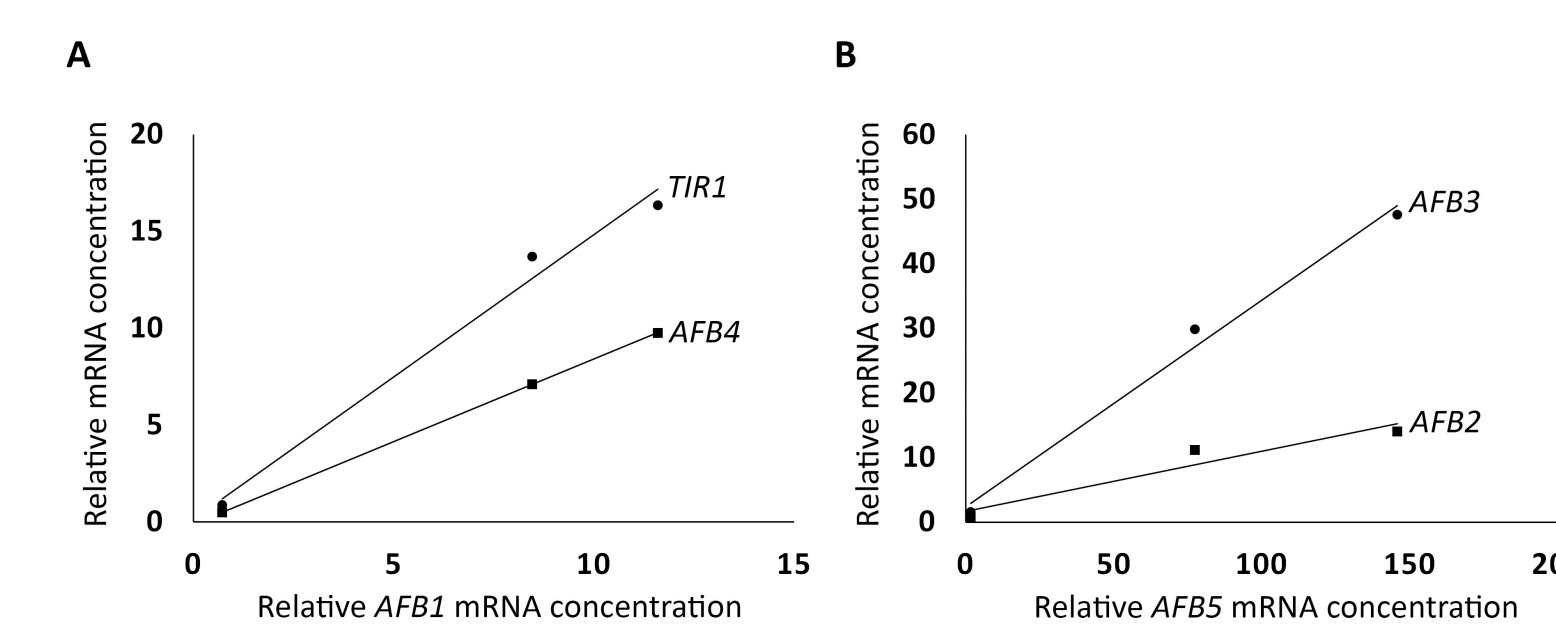


Fig. 2. Linear correlations in relative mRNA concentrations in FUN, CSC, and DSC among *TIR1*, *AFB1*, and *AFB4*, and among *AFB2*, *AFB3*, and *AFB5*. (A) *TIR1* and *AFB4* were plotted against *AFB1*, respectively. (B) *AFB2* and *AFB3* were plotted against *AFB5*, respectively.

- 12 subgroups were formed similarly as the *AFB1* group and *AFB5* group above
- Three of them, Group 1A, Group 1B, and Group 4A, contained genes functioning in seed germination (Figs. 3 and 4; data not shown)

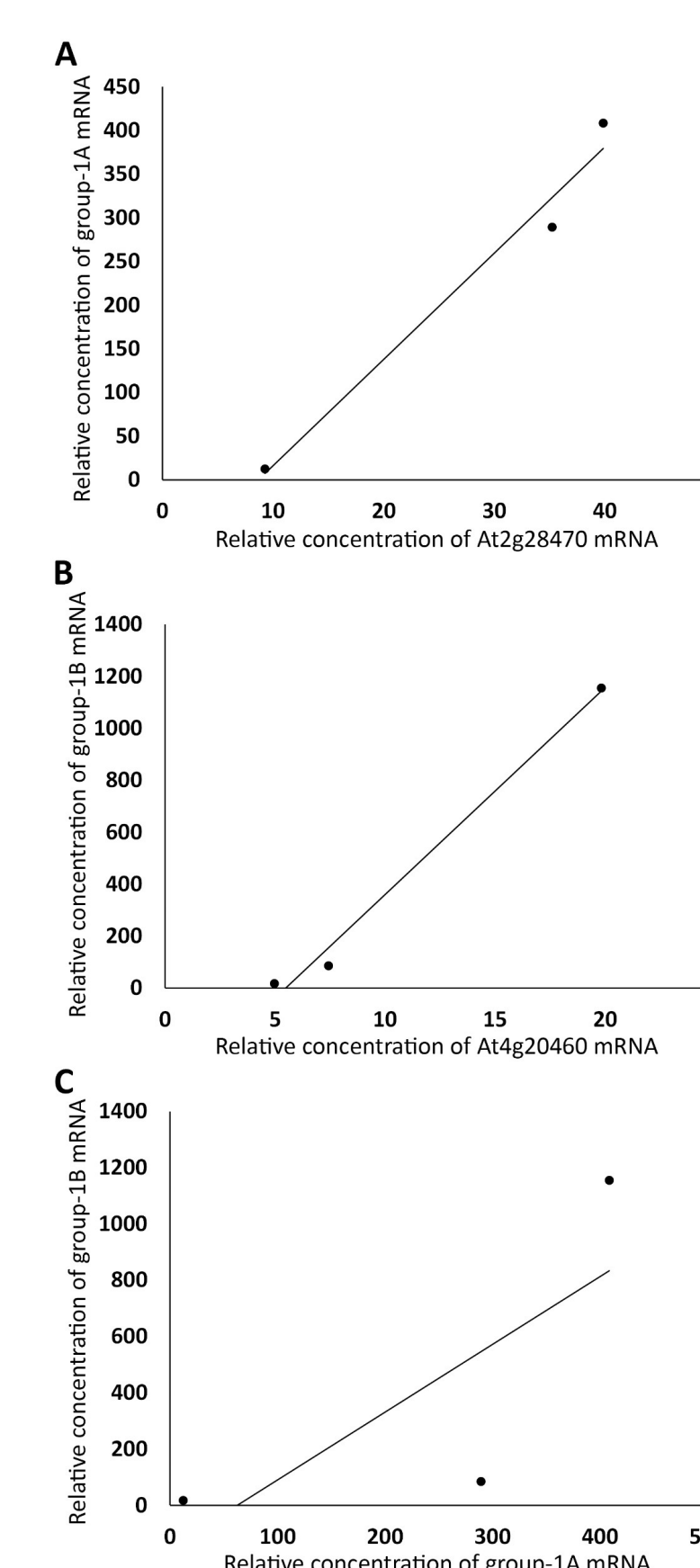


Fig. 3. Grouping auxin-downregulated genes by their linear correlations in relative mRNA concentrations in FUN, CSC, and DSC. (A) The sums of relative expression levels of all genes in group-1A, excluding those of *At2g28470*, were plotted against those of *At2g28470*. (B) The sums of expression levels of all genes in group-1B, excluding those of *At4g20460*, were plotted against those of *At4g20460*. (C) The sums of relative expression levels of all genes in group-1A, excluding those of *At2g28470*, were plotted against the sums of expression levels of all genes in group-1B, excluding those of *At4g20460*.

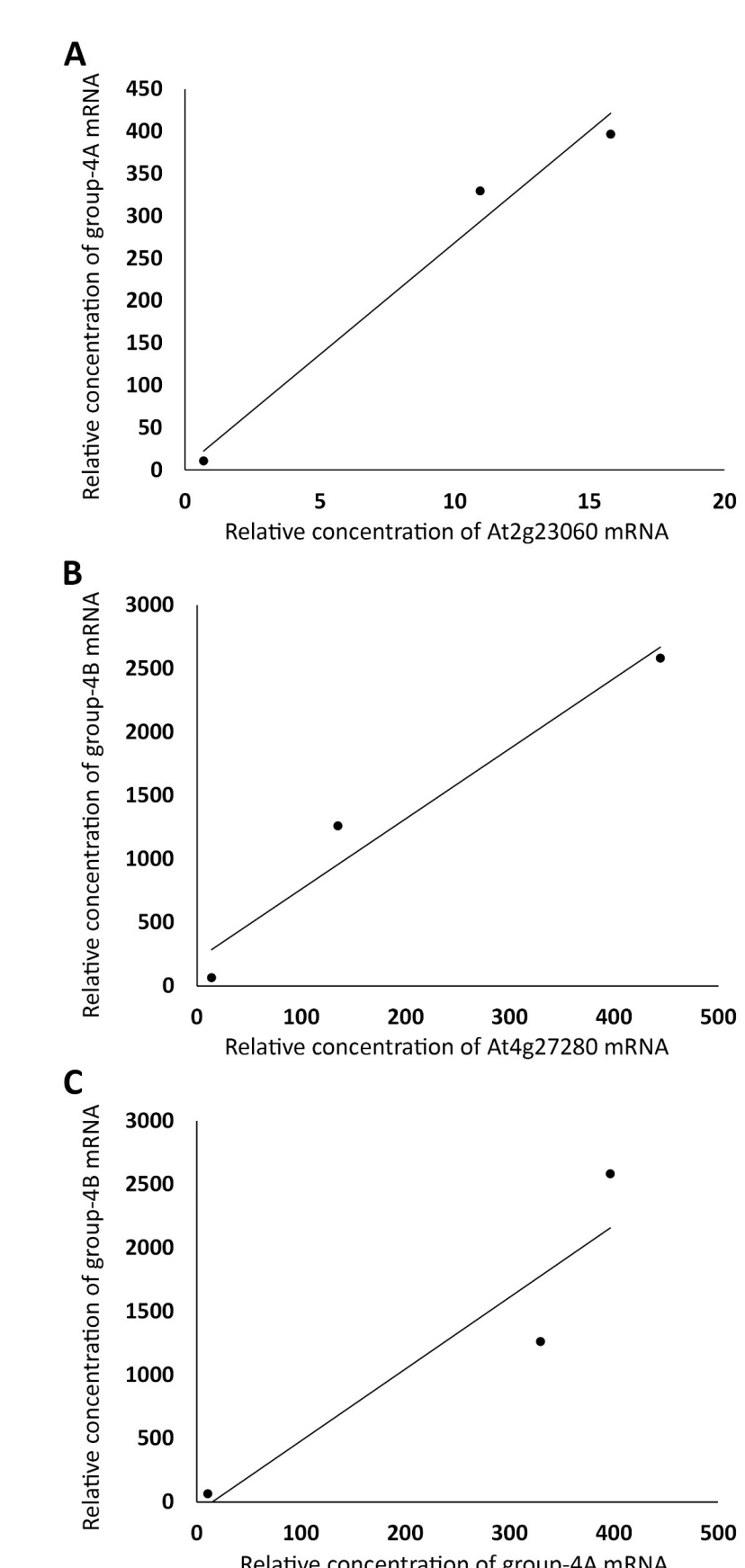


Fig. 4. Grouping auxin-upregulated genes by their linear correlations in relative mRNA concentrations in FUN, CSC, and DSC. (A) The sums of relative expression levels of all genes in group-4A, excluding those of *At2g23060*, were plotted against those of *At2g23060*. (B) The sums of expression levels of all genes in group-4B, excluding those of *At4g27280*, were plotted against those of *At4g27280*. (C) The sums of relative expression levels of all genes in group-4A, excluding those of *At2g23060*, were plotted against the sums of expression levels of all genes in group-4B, excluding those of *At4g27280*.

### □ Experimental confirmation of the functions of five newly identified genes in seed germination

- Mutants of *At1g51170* and *BRL3* had increased germination frequencies (Fig. 5)
- Mutants of *At2g23060*, *HMP39*, and *At4g35060* had reduced germination frequencies (Fig. 5)

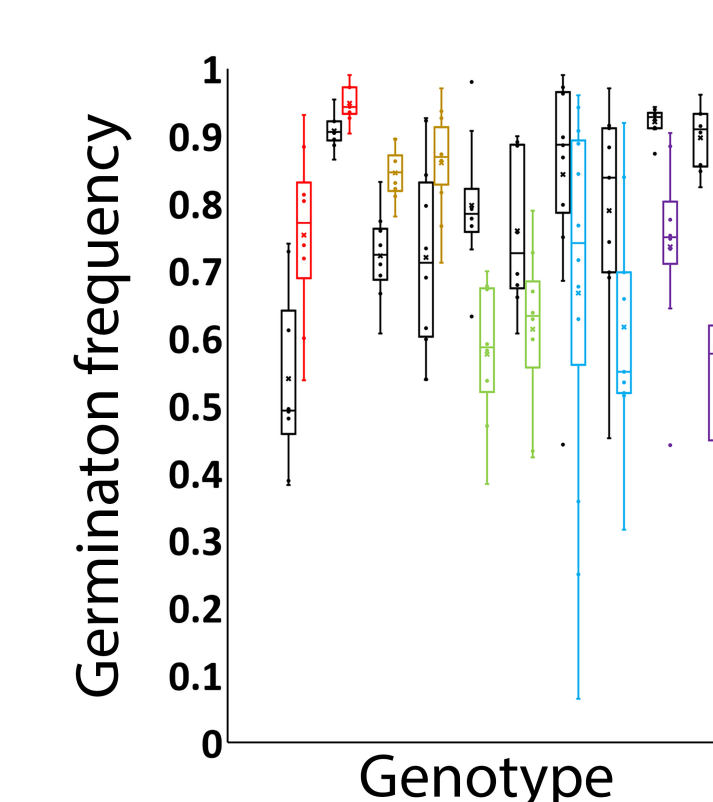


Fig. 5. Germination frequencies of the wild type and 10 mutants. Black: wild type; red: two mutant alleles of *At1g51170*; gold: two *brl3* mutants; green: two mutants of *At2g23060*; blue: two mutants of *At1g78090*; purple: two mutants of *hmp39* mutants.

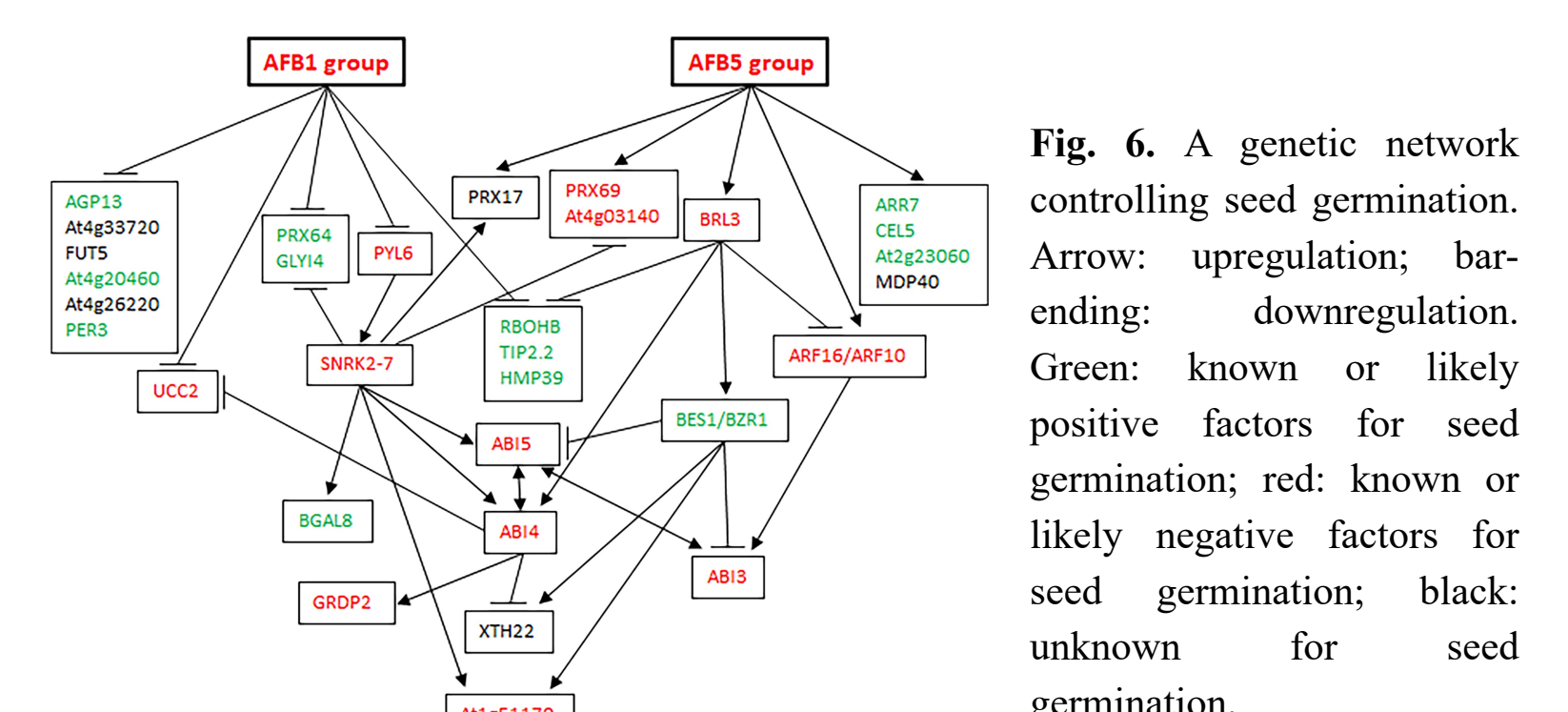


Fig. 6. A genetic network controlling seed germination. Arrow: upregulation; bar: downregulation. Green: known or likely positive factors for seed germination; red: known or likely negative factors for seed germination; black: unknown for seed germination.

### □ An integrated genetic network for controlling seed germination

- The network consists of the 30 identified proteins, six AFBs, *BES1* and *BZR1* involved in brassinosteroid signaling, and *ABI3*, 4, and 5 involved in ABA signaling (Fig. 6)
- Both the *AFB1* group and the *AFB5* group suppress several genes positive for seed germination and activate several genes negative for seed germination, and vice versa in the cases of other genes (Fig. 6)
- The *AFB1* group and the *AFB5* group both negatively and positively regulate ABA signaling (Fig. 6)

## References

Belmonte et al., 2013, Proc Natl Acad Sci USA 110: E435-44; Goda et al., 2004, Plant Physiol 134: 1555-1573; Khan et al., 2015, Plant J 82: 41-53; Liu et al., 2020, Plant Physiol 184: 1072-1082; Okamoto et al., 2010, Plant J 62: 39-51; Tian et al., 2020, Plant J 103: 1679-1694; Wang et al., 2022, Biosci Rep 42: BSR20221504