Generation of *Arabidopsis* Double and Triple Mutants in *BES1*, *BZR1*, and *BEH1* Transcription Factors for Plant-Nematode Interaction Studies

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Background

- The beet cyst nematode (BCN) *Heterodera schachtii* was used as a model to study how nematodes manipulate *Arabidopsis* developmental pathways into forming feeding cells (syncytia) within roots.
- Infective second-stage juvenile nematodes secrete effector peptides into selected root cells to change gene transcription.
- The CLE-like peptide effectors are molecular mimics of plant CLAVATA3/EMBRYO SURROUNDING REGION (CLE) peptides.
- Plant B-type CLE TRACHEARY ELEMENT INHIBITORY DIFFERENTIATION FACTOR (TDIF), which is also secreted by cyst nematodes, can suppress xylem formation by activating GLYCOGEN SYNTHASE KINASE 3 (GSK3), which in turn deactivates BES1 (BRI1-EMS-Suppressor 1).
- BES1 and and its homologues BZR1 and BEH1 are a family of transcription factors that function in promoting xylem differentiation from procambial cells.
- In this study, we set out to determine whether the GSK3-BES1 pathway is involved in plant nematode parasitism to develop a better understanding of how cyst nematodes may be using B-type CLE peptide effectors to facilitate infection.

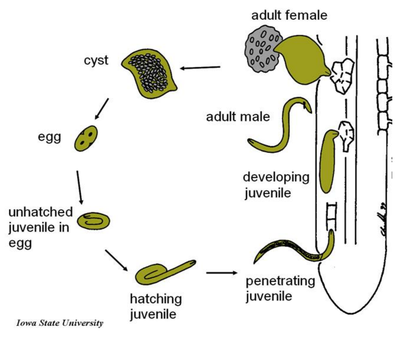


Figure 1. Life cycle of the beet cyst nematode. Infective juveniles penetrate into an *Arabidopsis* root where they select a procambial cell for feeding cell formation.

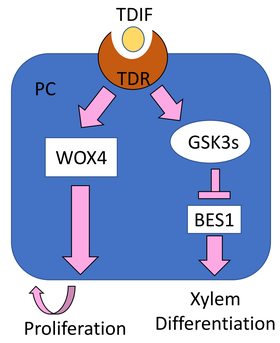


Figure 2. TDIF-GSK-BES1 signaling pathway is involved in xylem differentiation in *Arabidopsis* vasculature. PC: Procambial Cell.

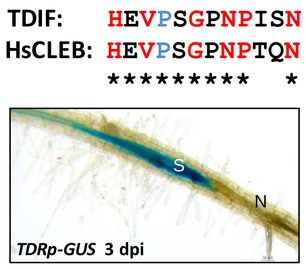


Figure 3: Amino acid sequence alignment between plant and nematode B-type CLE peptides. A cyst nematode forming a feeding cell. Expression of TDR is tagged with a blue marker.

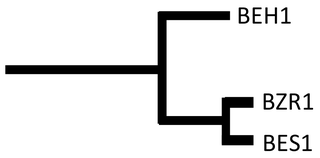


Figure 4: Phylogenetic tree showing the relationship between BES1 and its homologues based on sequence similarity.

Objective

BES1 (*BRI1 EMS SUPPRESSOR1*), *BZR1* (*BRASSINAZOLE RESISTANT 1*), and *BEH1* (*BZR1/BES1 HOMOLOG*) are closely related homologues in *Arabidopsis*. They may play redundant roles in mediating xylem differentiation and nematode infection. The goal of this study was to generate *bes1 beh1*, *beh1 bzr1* double mutants and *bes1 bzr1 beh1* triple mutants.

Single mutants of *BES1*, *BZR1*, and *BEH1* (Figure 5) were obtained from ABRC or other labs. The mutants were crossed to generate F2 populations for double/triple mutant screening using PCR and gel electrophoresis.

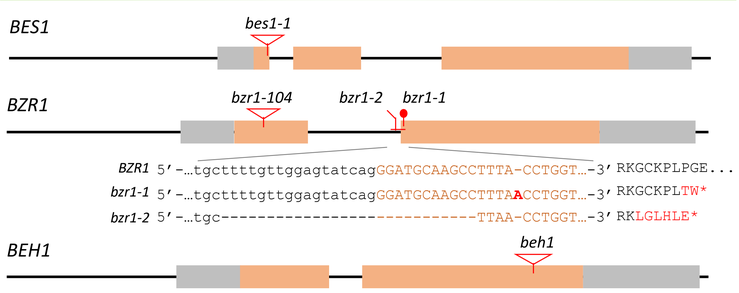


Figure 5: BES1, BZR1, and BEH1 null alleles showing position of T-DNA insertion or CRISPR/Cas9 modification. Grey box: UTRs; Orange box: exons. Vertical red line: site of CRISPR mutation; red triangle: mutated by T-DNA insertion. *bzr1-1* has one nucleotide insertion, causing pre-mature termination; and *bzr1-2* has 29-bp deletion, also causing pre-mature termination.

Methods

Three primers were used for genotyping plants containing T-DNA inserts. Two gene specific primers (LP and RP, Figure 6A) and one T-DNA specific primer (BP, Figure 6A) were used to create two amplicons of differing size. A three primer PCR in one mix is shown in the gel to the right. The expected banding patterns for plants that are wild type (WT), heterozygous (HZ), or homozygous (HM) for the mutation are shown.

dCaps primer (P1, Figure 6C) was used for genotyping *bzr1-1*. Due to a single nucleotide mismatch in P1, amplicon of P1 and P3 from *bzr1-1* contains a restriction site for *HpaI* (GTTAAC), but not for amplicon from *BZR1*. After *HpaI* digestion, *bzr1-1* band will be smaller and appears further down the gel.

Primers P2 and P3 shown in Figure 6C were used for genotyping *bzr1-2*. Due to the 29-bp deletion in *bzr1-2*, the amplicon from *bzr1-2* is smaller than that of *BZR1,* appearing further down the gel.

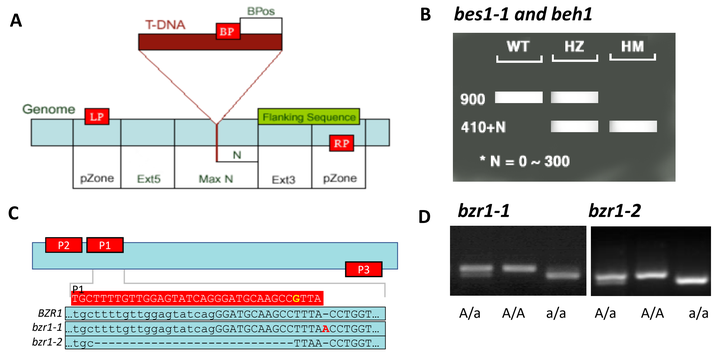


Figure 6. Methods of genotyping.

Results

Identification of a *bes1 beh1* double mutant

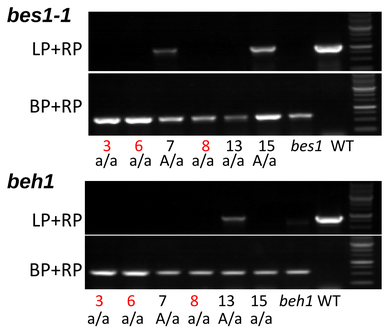
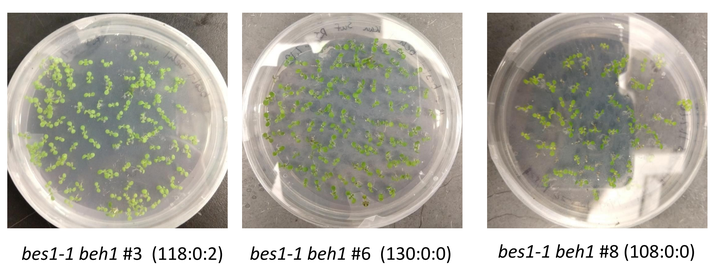


Figure 7. Gel electrophoresis results of *bes1-1 beh1* F2 plants. Plants #3, #6, and #8 are homozygous for both *BES1* and *BEH1* mutations. #13 is heterozygous for *BEH1*. #7 and #15 are both heterozygous for *BES1*. Positive (*bes1* or *beh1*) and negative (WT) controls are included. A marker for molecular weight is shown in the last lane.

Verification of the *bes1 beh1* double mutant using antibiotic selection



bes1-1 beh1 #3 (118:0:2) *bes1-1 beh1* #6 (130:0:0) *bes1-1 beh1* #8 (108:0:0)

Genotype [1]	Expected Ratio[2]
AaBb	9 : 3 : 4
Aabb	3 : 1 : 0
aaBb	3 : 0 : 1
aabb	16 : 0 : 0

1. A: BES1, B: BEH1
2. Green: Yellow: Dead

	A	a
A	AA	Aa
a	Aa	aa

	B	b
B	BB	Bb
b	Bb	bb

	AB	aB	Ab	ab
AB	AABB	AaBB	AABb	AaBb
aB	AaBB	aaBB	AaBb	aaBb
Ab	AABb	AaBb	AAbb	Aabb
ab	AaBb	aaBb	Aabb	aabb

Figure 8. Progenies of *bes1-1 beh1* double mutants (F3) were verified by kanamycin (Kan) and sulfonamide (Sul) double selection. *bes1-1* and *beh1* are associated with T-DNA inserts containing Kan and Sul resistance genes, respectively. Heterozygosity of either locus results in a F3 segregation ration on Kan/Sul double selection plates as shown in bottom panels. All 3 lines are double homozygous as neither of their progenies segregates on Kan/Sul double selection plates.

Identification of a *beh1 bzr1* double mutant

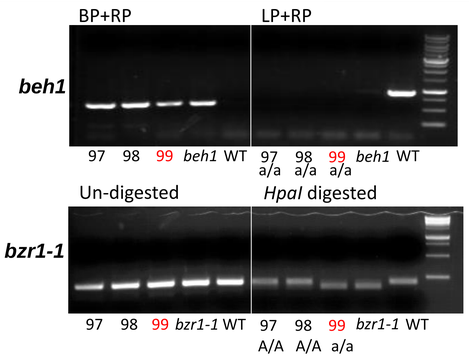


Figure 9. Genotyping results of *beh1 x bzr1-1* F2 plants. *bzr1-1* was genotyped by primers P1 and P2 shown in Figure 6C. Amplicons from *bzr1-1* allele contain an *HpaI* restriction site due to the combination of *bzr1-1* mutation and the single nucleotide mismatch in P1. After *HpaI* digestion, *bzr1-1* band is smaller than that of *BZR1.* As shown in above gels, plant #97, #98, #99 were shown to be homozygous for *beh1*, and plant #99 was homozygous for *bzr1-1.*

Verification of the *beh1 bzr1* double mutant in F3

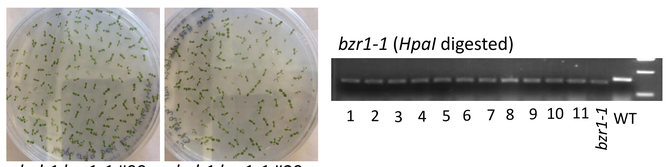


Figure 10. Verification of *beh1 bzr1-1* double mutant in F3. Progenies of *beh1 bzr1-1* #99 were verified with both sulfonamide selection (for *beh1*) and PCR (for *bzr1-1*). Neither locus shows indication of segregation, which means that *beh1 bzr1-1* #99 is homozygous for both *beh1* and *bzr1-1*. In contrast, progeny of *beh1 bzr1-1* #29, which is heterozygous for *beh1,* showed 3:1 segregation on sulfonamide selection plate.

Identification of a *bes1 bzr1 beh1* triple mutant

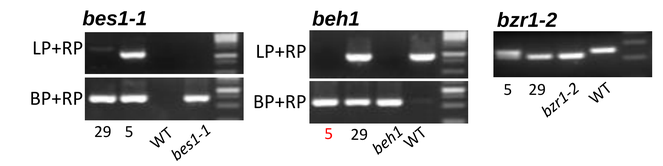


Figure 11: Representative genotyping results for *bes1-1 bzr1-2 x beh1* F2 plants. 72 F2 plants were genotyped but no triple homozygous was identified. Plant #5 and #29 were selfed as they were homozygous for one locus. Self crossed progenies gave a 1:2:1 ratio of plants for another locus. *BES1* and *BZR1* loci are tightly linked so they can be treated as one locus. *bzr1-2* was amplified using P2 and P3 shown in Fig. 6C and was distinguished based on the 29 bp deletion in the mutated plants.

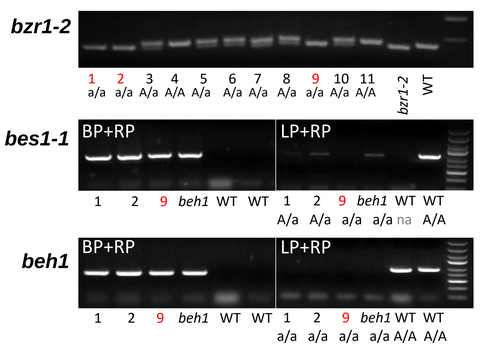


Figure 12: Genotyping results of *bes1-1 bzr1-2 x beh1* F3 plants. Since no triple mutant was able to be isolated from the F2 generation, plant #5 (F2) was selfed and F3 plants were genotyped. Plant #9 (F3) was homozygous for all three loci.

Conclusions and Future Work

- *bes1 beh1*, *beh1 bzr1* double mutants and a *bes1 bzr1 beh1* triple mutant was generated.
- These mutants will be used for future nematode infection assays to evaluate the potential role of this transcription factor family in feeding cell formation.

Acknowledgments

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