The effect of nojirimycin on the transcriptome of germinating Orobanche minor seeds

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Orobanchaceae root parasitic weeds cause serious agricultural damage worldwide. Although numerous studies have been conducted to establish an effective control strategy for the growth and spread of root parasitic weeds, no practical method has been developed so far. Previously, metabolomic analyses were conducted on germinating seeds of a broomrape, Orobanche minor, to find novel targets for its selective control. Interestingly, planteose metabolism was identified as a possible target, and nojirimycin (NJ) selectively inhibited the germination of O. minor by intercepting planteose metabolism, although its precise mode of action was unclear. Here, transcriptome analysis by RNA-Seq was conducted to obtain molecular insight into the effects of NJ on germinating O. minor seeds. Differential gene expression analysis results suggest that NJ alters sugar metabolism and/or signaling, which is required to promote seed germination. This finding will contribute to understanding the effect of NJ and establishing a novel strategy for parasitic weed control.

Keywords: broomrape, nojirimycin, Orobanche minor, parasitic weed, sugar signaling, transcriptome.

Electronic supplementary materials: The online version of this article contains supplementary materials (Supplemental Tables S1–S6, Fig. S1), which are available at https://www.jstage.jst.go.jp/browse/jpestics/
SL receptor, Striga HYPOSENSITIVE TO LIGHT/KARRIKIN INSENSITIVE2 7 (ShHTL/KAI2 7). It was shown that 100 pM SPL7 reduced the emergence of *S. hermonthica* artificially infested with maize in pot experiments.9

Since SL-dependent germination is a unique feature of root parasitic weeds, the investigation of the physiological and biochemical processes in the germination process will contribute to the identification of novel molecular targets for the selective control of root parasitic weeds. In line with this concept, metabolomic analyses were conducted on the germinating broomrape, *Orobanche minor*, and planteose metabolism was identified as a possible target for its control.10 Planteose, a trisaccharide composed of galactose, glucose, and fructose, is present in a limited number of plant species, including the seeds of several mints,11 tobacco,12 sesame (*Sesamum indicum*),13 and chia (*Salvia hispanica*).14 Neither the physiological role of planteose nor its metabolic pathway in plants has been well studied. In the seeds of *O. minor*, planteose is accumulated as a storage sugar and, after the detection of SLs, is metabolized rapidly via sucrose to the monosaccharides, glucose and fructose. Our previous study revealed that nojirimycin (NJ) selectively inhibited the germination of *O. minor* by intercepting planteose metabolism.10 When NJ was applied to synthetic SL (GR24)-treated seeds, the second step of the planteose metabolic pathway, the hydrolysis of sucrose by invertase, was significantly inhibited. Because NJ, an iminosugar, was originally isolated as a potent inhibitor of glucosidase,15 it was postulated that NJ directly inhibited invertases in *O. minor* seeds. However, *in vitro* assays revealed that NJ was not a potent inhibitor of invertases, but the activity of invertase in NJ-treated *O. minor* seeds was significantly decreased as compared to a control.10 Thus, we hypothesized that NJ could inhibit the post-translational modifications of invertases required for their activation through an unknown mode of action (MoA).

Invertase, one of the key enzymes involved in sugar metabolism, irreversibly hydrolyzes sucrose into glucose and fructose; thus, it has a pivotal role in plant development.16–19 Invertases are classified into three classes according to their subcellular localizations and optimal pHs; neutral/alkaline cytoplasmic invertases (CINs), acid vacuolar invertases (VINs), and acid cell wall invertases (CWINs).19 Because the hydrolysis of sucrose occurs in many important physiological processes, such as osmotic regulation and phloem unloading during sugar translocation, the regulation of invertase activity is crucial for plant seed germination. In *Arabidopsis thaliana*, the expression of genes encoding VINs and CWINs is induced by gibberellin synthesized after the ovule initiation through sugar signaling, possibly mediated by the extracellular receptor-like-kinases (RLKs) and hexose transporters.20

Here, transcriptome analysis was conducted to obtain molecular insights into the effect of NJ on germinating seeds of *O. minor*. The transcriptome in germinating *O. minor* seeds was assembled *de novo* by RNA-Seq and was found to reflect the loss of photosynthesis. Interestingly, differentially expressed genes (DEGs) in NJ-treated seeds involved homologues of invertase inhibitors, sugar transporters, phosphatases, and kinases, suggesting that NJ treatment affects sugar signaling during germination in *O. minor*.

**Materials and Methods**

1. **Plant material and germination treatment**

*O. minor* seeds were collected from mature plants grown in colonies in Yokohama, Japan, in June 2013 and stored at 4°C. Seed germination was induced as reported previously.10 The seed surface was sterilized with a solution containing 1% sodium hypochlorite and 0.1% (w/v) Tween 20 for 2 min, rinsed with distilled water, and dried under a vacuum. Then, the seeds were conditioned on two layers of glass filters (Whatman GF/D; GE Healthcare, Chicago, IL, USA) fully moistened with distilled water in a Petri dish in the dark at 23°C for one week. After conditioning, the upper layer of the glass filter with the seeds was transferred to a new Petri dish containing a fresh glass filter (Whatman GF/D). Germination was induced by the application of SL solution (1 mg/L rac-GR24) with or without NJ (10 µmol/L). Seeds were collected just after the conditioning, and 0.5, 3, 24, and 48 hr after treatment (HAT) with GR24. Seeds were frozen in liquid nitrogen and stored at −80°C until use.
2. RNA isolation and sequencing
Total RNA was isolated using the PureLink® RNA Mini Kit (Thermo Fischer Scientific, Waltham, MA, USA) in accordance with the manufacturer’s instructions. Preparation of RNA-Seq libraries and sequencing using the HiSeq 2000 (Illumina, San Diego, CA, USA) were performed at BGI Japan (Kobe, Japan). Raw paired-end reads (90 bp each, ca. 50 million reads in each library) were submitted to the DNA Data Bank of Japan (DDBJ) (Accession number: DRA10691).

3. RNA-Seq data processing
Transcriptome assembly was performed using DDBJ Read Annotation Pipeline.29 The trinity software package (version r2013-02-25) was used to construct the transcriptome.30,31 The quality of the assembled transcriptome was assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO) version 3.1.022 with the lineage dataset embryophyta-odb9. The DAVID bioinformatics tool33,34 was used to evaluate the results of the BUSCO analysis. Open reading frame (ORF) prediction in the assembled transcriptome was conducted using TransDecoder (https://github.com/TransDecoder/TransDecoder/wiki). Read mapping to the reference transcriptome and differential expression analysis were conducted with OmicsBox software (BioBam Bioinformatics, Valencia, Spain). The Pairwise Differential Expression Analysis (Without Replicates) module, based on the software package NOISeq, was used with the default parameters.35,36 Genes with a probability higher than 0.9 were considered to be differentially expressed between NJ-treated and non-treated seeds.

Results

1. De novo assembly of O. minor seed RNA-Seq data
The total RNAs were extracted from O. minor seeds after conditioning and at 0.5, 3, 24, and 48 HAT with or without NJ and purified. After quality control of the RNA samples, RNA-Seq libraries were constructed and sequenced using HiSeq 2000. De novo assembly of the transcriptome was performed using reads obtained from RNA samples after conditioning and at 48 HAT without NJ. A total of 119,181 contigs (length > 200 bp) with an average size of 951 bp and N50 of 1.5 kbp were obtained. The assembled transcriptome contained 68.9% of complete and 6.0% of fragmented BUSCOs, while 25.1% (361 of 1440 orthologs total) were missing. The high number of missing BUSCOs could represent the loss of photosynthesis in the holoparasitic O. minor. To evaluate this hypothesis, gene ontology (cellular compartment) enrichment analysis was conducted on representative A. thaliana orthologs corresponding to the missing BUSCOs in the O. minor transcriptome (Supplemental Table S1).

Table 1. Gene Ontology term (cellular component) enrichment analysis of representative A. thaliana orthologs corresponding to the missing BUSCOs in O. minor transcriptome

| GO term | Counta | %b | P value | Fold enrichment |
|---------|---------|----|---------|-----------------|
| Chloroplast | 171 | 51.0 | 1.6E-55 | 3.4 |
| Chloroplast thylakoid membrane | 48 | 14.3 | 7.9E-31 | 9.2 |
| Chloroplast thylakoid | 27 | 8.1 | 2.0E-18 | 10.2 |
| Chloroplast thylakoid lumen | 18 | 5.4 | 2.2E-16 | 17.5 |
| Mitochondrion | 100 | 29.9 | 9.6E-16 | 2.3 |
| Chloroplast stroma | 38 | 11.3 | 4.5E-14 | 4.5 |
| Thylakoid lumen | 13 | 3.9 | 1.7E-12 | 19.8 |
| Thylakoid | 20 | 6.0 | 2.5E-11 | 7.5 |
| Chloroplast envelope | 28 | 8.4 | 2.5E-09 | 4.0 |
| Plastid chromosome | 6 | 1.8 | 5.1E-06 | 25.9 |
| Nucleoid | 7 | 2.1 | 2.5E-06 | 15.5 |
| Photosystem II oxygen evolving complex | 6 | 1.8 | 1.2E-05 | 19.4 |
| Chloroplast membrane | 11 | 3.3 | 1.2E-05 | 6.2 |
| Extrinsic component of membrane | 6 | 1.8 | 4.1E-04 | 9.5 |
| Plastid-encoded plastid RNA polymerase complex | 3 | 0.9 | 1.6E-03 | 46.6 |
| Chloroplast inner membrane | 6 | 1.8 | 3.0E-03 | 6.1 |
| NAD(P)H dehydrogenase complex (plastoquinone) | 3 | 0.9 | 1.2E-02 | 17.9 |
| Plastoglobule | 4 | 1.2 | 7.2E-02 | 4.1 |

a) Number of the orthologs related to the GO term. b) Percentage of the orthologs in the total representative A. thaliana orthologs (Supplemental Table S1).

Table 2. Number of genes differentially expressed in NJ-treated seeds of O. minor compared to non-treated seeds

| Time after GR24 treatment (hr) | Number of up-regulated genes | Number of down-regulated genes |
|-------------------------------|------------------------------|-------------------------------|
| 0.5                           | 35                           | 10                            |
| 3                             | 13                           | 28                            |
| 24                            | 17                           | 14                            |
| 48                            | 320                          | 196                           |
ed, more than half of the missing BUSCOs were genes encoding chloroplast-related proteins (Table 1), indicating that the high number of missing BUSCOs represents the loss of photosynthesis. Based on this result, we judged that the quality of the assembled transcriptome was acceptable for further analysis.

2. Effect of nojirimycin on the transcriptome of O. minor seeds

Using TransDecoder, 86,857 ORFs were predicted in the assembled transcriptome. Functions of the proteins encoded by the predicted ORFs were annotated using OmicsBox with Blast and InterProScan using default parameters. After removing duplicated sequences, 16,672 contigs with annotations were used as a reference transcriptome for differential expression analysis. Cleaned reads obtained from each sample were mapped against the reference transcriptome, and a count table was created with OmicsBox using default parameters. Differential expression analysis was performed for each pair of transcripts (NJ-treated vs. non-treated) in the seeds at the same sampling point. As a

![Differentially up-regulated genes](image)

![Differentially down-regulated genes](image)

Fig. 1. Venn diagram illustrating the number of differentially (A) up-regulated and (B) down-regulated genes in NJ-treated seeds of O. minor as compared to non-treated seeds. The genes differentially expressed at multiple time points are listed in Supplemental Table S6.

### Table 3. Representative up-regulated genes in the NJ-treated seed of O. minor

| HAT | Contig | Fold change | Description |
|-----|--------|-------------|-------------|
| 0.5 | comp34798_c0_seq1 (down-regulated at 3 HAT) | 8.7 | SEC61 BETA1, COBRA-LIKE PROTEIN-7 PRECURSOR, putative membrane-anchored cell wall protein |
|     | comp65219_c0_seq4 (up-regulated at 48 HAT) | 3.0 | PP2C CLADE D 5, protein phosphatase 2C family protein |
|     | comp33755_c0_seq1 (down-regulated at 3 and 48 HAT) | 9.2 | Plant invertase/pectin methylesterase inhibitor superfamily protein |
|     | comp59432_c0_seq2 (down-regulated at 3 HAT) | 5.7 | GARACTURONOSYL TRANSFERASE-LIKE 9, encodes a protein with putative galacturonosyltransferase activity |
|     | comp68818_c0_seq1 (down-regulated at 3 HAT) | 6.2 | SUGAR TRANSPORTER 1, encodes a H+/hexose cotransporter |
|     | comp232524_c0_seq1 (down-regulated at 3 HTA) | 4.9 | Protein kinase superfamily protein |
|     | comp68094_c0_seq1 (down-regulated at 3 HTA) | 5.4 | RECEPTOR LIKE PROTEIN 54 |
| 3   | comp92283_c0_seq1 (up-regulated at 48 HAT) | 6.7 | MYB55, encodes a putative transcription factor |
| 48  | comp34269_c0_seq1 | 3.2 | DROUGHT-INDUCED 8, RESPONSIVE TO ABA 18, belongs to the dehydrin protein family, ABA- and drought-induced glycinic-acid dehydrin protein |
|     | comp68110_c0_seq1 | 5.3 | LATE EMBRYOGENESIS ABUNDANT 1, encodes an ABA-induced protein that accumulates during seed maturation |
|     | comp64405_c0_seq5 | 2.2 | RNA-BINDING PROTEIN 25, an alternative splicing factor involved in mediation of abiotic stress and ABA responses |
|     | comp33799_c0_seq1 | 2.4 | Leucin-rich repeat (LRR) family protein |
|     | comp47352_c0_seq1 | 2.2 | KEEP ON GOING, encodes a RING E3 ligase involved in ABA signaling |
|     | comp7868_c0_seq5 | 2.6 | Leucin-rich repeat (LRR) family protein |

* a) Full list of the differentially expressed genes is available as supplemental materials. b) Hours after GR24 treatment. c) Ratio of normalized count of contig in the NJ-treated O. minor seed to that in the non-treated seed. d) Description for homolog in A. thaliana in TAIR database.
result, NJ was revealed to alter the expression of a small number of genes at all time points until 24 HAT (Table 2, Supplemental Tables S2–S4). On the other hand, a total of 516 genes were differentially expressed in the NJ-treated seeds at 48 HAT, which might reflect the secondary effects of NJ (Table 2, Supplemental Table S5). A few genes were differentially expressed at multiple time points (Fig. 1, Supplemental Table S6).

Among the DEGs, candidate genes for the MoA of NJ are listed in Tables 3 and 4. Some genes were differentially expressed at multiple time points, such as comp33755_c0_seq1, whose expression was up-regulated at 0.5 and down-regulated at 3 and 48 HAT (Table 3, Supplemental Table S6). Interestingly, this gene encodes a plant invertase/pectin methylesterase inhibitor superfamily protein. Another gene (comp68818_c0_seq1) encoding a sugar transporter that might be involved in sugar utilization was also up-regulated at 0.5 and down-regulated at 3 HAT (Table 3). A gene encoding protein phosphatase 2C family protein (comp65219_c0_seq4) was up-regulated at 0.5 and 48 HAT (Table 3, Supplemental Table S6), and genes encoding protein kinase superfamily protein (comp232524_c0_seq1) and receptor like protein 54 homolog (comp68094_c0_seq11) were also up-regulated at 0.5 HAT (Table 3), indicating that the expression of these signaling-related genes could be directly induced by NJ treatment. Genes encoding proteins involved in cell wall assembly or modification, such as COBRA-LIKE PROTEIN-LIKE 7-PRECURSOR homolog (comp34798_c0_seq1) and GARACTURONOSYL TRANSFERASE-LIKE 9 homolog (comp59432_c0_seq2), also showed increased expression at 0.5 HAT.

In later stages, the effect of NJ treatment was remarkable on...
abscisic acid (ABA) signaling. Genes encoding ABA-related proteins, such as RESPONSIVE TO ABA 18 homolog (comp34269_c0_seq1), a homolog of RNA-BINDING PROTEIN 25, an alternative splicing factor involved in mediation of abiotic stress and ABA responses (comp64405_c0_seq5), and a homolog of KEEP ON GOING, encoding a RING E3 ligase involved in ABA signaling (comp7352_c0_seq1), had increased expression at 48 HAT. In contrast, a homolog of HIGHLY ABA-INDUCED PP2C GENE 2, a negative regulator of dormancy, (comp65908_c0_seq1) showed decreased expression at 24 HAT; and a homolog of CYP707A1, ABA 8′-hydroxylase, which is involved in ABA catabolism (comp58051_c0_seq1), and a homolog of PYL4, an ABA receptor (comp34691_c0_seq1), were down-regulated at 48 HAT (Table 4). Additionally, many signaling-related genes encoding kinases and leucine-rich repeat (LRR) family proteins, as well as cell wall genes encoding expansins, glycosyl transferases, and glycosylases, were down-regulated in the NJ-treated seeds from 3 to 48 HAT (Table 4).

**Discussion**

In this study, a total of 119,181 contigs were obtained by de novo assembly of RNA-Seq data. BUSCO analysis revealed that one quarter of plant BUSCOs (361 of 1440 orthologs total) were missing in the assembled transcriptome (Table S1), and one half of the missing BUSCOs were chloroplast genes (Table 1). Since O. minor is a holoparasite, photosynthetic machinery was lost during its evolution. In Orobanche cernua and Orobanche (Phelipanche) ramosa, the genes encoding the Rubisco large subunit (rbCL) had become pseudogenes. This could have been caused by mutations under relaxed selective pressure for photosynthesis, as indicated by reconfigured plastomes and altered chromosomal architectures in the holoparasitic broomrape family. The missing chloroplast-related BUSCOs in the assembled transcriptome in this study might also reflect global alterations in chromosomes caused by the loss of photosynthesis in O. minor.

Differential gene expression analysis revealed that NJ alters the expression of a restricted number (<50) of genes until 24 HAT, indicating that the MoA of NJ is not non-specific toxicity (Table 2, Fig. 1). Previously, the activity of acid invertases, VINs and CWINs, was shown to be lowered in NJ-treated O. minor seeds. Our transcriptomic data showed that the expression of comp33755_c0_seq1, encoding plant invertase/pectin methylesterase inhibitor superfamily protein, was decreased by GR24 in germinating seeds but increased by NJ (Table 3, Supplemental Fig. S1A). This result strongly suggests that NJ alters sugar signaling in O. minor seeds. Since invertase inhibitors are key regulators of invertase activities, their increase might be one reason for low invertase activity in NJ-treated O. minor seeds. A similar expression pattern was observed for comp68818_c0_seq1 (SUGAR TRANSPORTER 1, STP1, homolog) which also supports this hypothesis (Table 3, Supplemental Fig. S1B). Recently, ovule-specific CWIN2 and CWIN4 in A. thaliana were silenced by microRNAs, and RNA-Seq analysis was conducted on flower buds from the silenced plant. Gene expression of STP2, STP6, and STP9, together with other signaling molecules like protein kinases, was decreased in the silenced plant, suggesting that the status of sugars at apoplasts and/or CWINs influences the transcription of these genes.

Genes comp65219_c0_seq4 (protein phosphatase 2C family protein), comp232524_c0_seq1 (protein kinase superfamily protein), comp68094_c0_seq1 (receptor like protein), and comp68094_c0_seq11 (MYB55 homolog), which also had altered expression, may also be involved in sugar signaling in germinating O. minor seeds. Although NJ is an iminosugar and is recognized as a β-glucosidase inhibitor, it is possible that NJ acts as a glucose mimic in the sugar signaling pathway through its close structural similarity. Taken together, we hypothesized that NJ inhibited the germination of O. minor by disrupting the sugar signaling pathway, a process essential for promoting germination.

As compared to the restricted effect of NJ on gene expression until 24 HAT, the expression of a wider range of genes was affected at 48 HAT (Table 2, Fig. 1). Since the germination process was suppressed in NJ-treated seeds, DEGs at this time point might be involved in promoting germination. Changing the balance of gibberellin and ABA doses during germination ends dormancy and promotes germination. The ABA catabolic enzyme PrCYP707A1 is a key component in Phelipanche ramosa germination. During conditioning, the DNA methylation status of the PrCYP707A1 promoter was modulated, enabling it to respond to SL. These observations indicate that ABA catabolism is a key process in broomrape germination. Our DEG analysis revealed that the gene expression of CYP707A1 homolog (comp58051_c0_seq1) and ABA receptor PYL4 homolog (comp34691_c0_seq1) was decreased in the NJ-treated seeds at 48 HAT (Table 4). This result indicates that sugar metabolism and/or signaling promotes ABA catabolism in germinating O. minor seeds. There is a close link between sugar status.

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**Fig. 2.** Hypothetical scheme of the germination process of O. minor and the MoA of NJ. SLs induce plantose metabolism to provide glucose required to promote the germination process. There might be a feedback regulation of plantose metabolism by products through sugar signaling. NJ, as a glucose mimic, disrupts sugar signaling and ongoing plantose metabolism. Accordingly, ABA catabolism and signaling, key processes in germination, are suppressed.
and ABA response in plants.\textsuperscript{45,46} Exogenous glucose suppresses the expression of CYP707A2, another ABA catabolic enzyme, in \textit{A. thaliana}.\textsuperscript{47} As shown previously,\textsuperscript{10} since glucose is depleted in NJ-treated seeds, NJ, as a glucose mimic, might suppress CYP707A1 expression in \textit{O. minor} seeds. Genes with increased expression were involved in ABA signaling (comp34269_c0_seq1, comp68110_c0_seq1, comp64405_c0_seq5, and comp47352_c0_seq1 in Table 4), which also suggests ABA catabolism is not induced in NJ-treated seeds. Taken together, a hypothetical germination process scheme of \textit{O. minor} and MoA of NJ is illustrated in Fig. 2.

Additionally, genes related to cell walls were differentially expressed in NJ-treated \textit{O. minor} seeds. During plant germination, cell wall degradation and synthesis are coordinately regulated for radicle emergence and cell expansion.\textsuperscript{49} Since polysaccharides are major components in the cell wall, the sugar status of seeds may affect cell wall metabolism. Because of the complex structure of the cell wall and the large number of components involved in its metabolism, the regulatory mechanisms of cell wall metabolism through sugar signaling have not been studied extensively in root parasitic weeds or in model plants. Our transcriptomic data indicates that sugar status affects the expression of diverse cell wall-related genes in \textit{O. minor}.

Our transcriptome analysis offers new insights into the inhibitory effect of NJ on the germination of \textit{O. minor}. The changes in the gene expression profile of NJ-treated seeds suggest that NJ acts as a glucose mimic, disrupting sugar signaling. Furthermore, ABA catabolism, a key process in germination, was suppressed in NJ-treated seeds (Fig. 2). Precise quantification of some key genes, together with metabolic profiling involving ABA and its catabolite, will increase the understanding of the effect of NJ on germination and help to establish a novel strategy for parasitic weed control.

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