Doctoral Dissertation

Searching for potential targets in selective control of root parasitic weeds in Orobanchaceae

（ハマウツボ科根寄生雑草選択的防除のための防除標的の探索）

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Contents

Chapter I Introduction

Weedy parasitic plants cause problems to agriculture and horticulture worldwide. “Parasitic plant” is a generic term for plants that parasitize other plants (host plants) and grow by absorbing nutrients from those. The total number of parasitic plant species is close to 4,500 in 270–275 genera, which accounts for about 1 % of all known seed plants (Govaerts, 2001; Heide-Jørgensen, 2013). The parasitic plants are classified according to capability of photosynthesis into hemiparasites and holoparasites. The hemiparasites have the ability to photosynthesize, by contrast, the holoparasites have lost this ability. Besides, a criterion for defining whether a parasitic plant is weedy or non-weedy is the importance of a host plant as a crop. If the host is an economically important crop species and affected by the infection of a parasitic plant species, this parasitic species is certainly regarded as a weed (Parker, 2013). Particularly, obligate root parasitic weeds, which parasitize roots of hosts and do not survive without hosts, in the Orobanchaceae are among the most destructive agricultural weeds. Within this family, the holoparasitic weedy broomrapes, *Orobanche* spp., *Phelipanche* spp., and the hemiparasitic weedy witchweeds, *Striga* spp., cause particularly devastating damage to agricultural crops worldwide. Food and Agricultural Organization of the United Nations (FAO) reported serious problems caused by these species (Elzein and Kroschel, 2003). *Orobanche* spp. and *Phelipanche* spp., are mainly distributed in the Mediterranean region, Southern and Eastern Europe, and West Asia, and cause damage to a wide range of vegetables, beans, and other agricultural crops. *Striga* spp., which are mainly distributed in Africa, are thought to be the largest biological cause of serious crop losses on the

continent. (Elzein and Kroschel, 2003; Aly, 2007; Heide-Jørgense, 2008; Parker, 2009). International Institute of Tropical Agriculture (IITA) researchers estimated that *Striga* spp. cause damage up to US$1.2 billion in every year to maize and cowpea in sub-Saharan Africa, and the Bill & Melinda Gates Foundation sponsored the project to help maize and cowpea farmers working in this region (International Institute of Tropical Agriculture, 2011). FAO estimated that *Striga* spp. cause financial losses of at least US$7 billion per year in African agriculture (Robson and Broad, 1989). There has been no case that these root parasitic weeds affect on food production in Japan. However, an alien species clover broomrape (*Orobanche minor*) (Fig. 1A) is spreading around Kanto area in Japan. Fortunately, harmful impact of *O. minor* on agricultural crops is still marginal even in the world, but there is no effective method to control its diffusion.

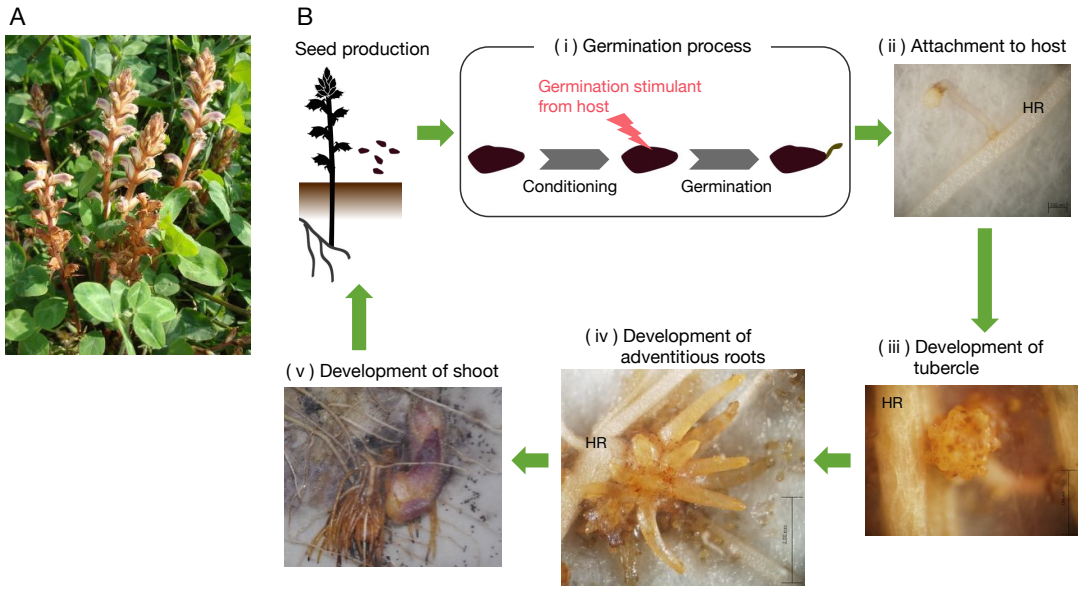


Figure 1. Parasitism of a root parasitic weed, clover broomrape (*Orobanche minor*). (A) *O. minor* infecting clovers (*Trifolium* spp.) at Chiba in Japan. (B) A life cycle of *O. minor*.

Figure 1. (continued) Tiny and numerous seeds germinate by perception of germination stimulants from host plants following a conditioning process at a suitable temperature (i). Attachment to hosts is established through a terminal haustorium (ii), and then, tubercle (iii) and adventitious roots (iv) are formed. Development of shoot occurs in the soil (v). HR, host root.

These obligate root parasitic weeds have evolved many parasitic adaptations and have unique life cycles that are tightly coupled with the ecological behavior of the host plants (Fig. 1B). For example, the seeds of root parasitic weeds in the Orobanchaceae require host-derived germination stimulants such as strigolactones to germinate (López-Ráez *et al*., 2009; Yoneyama *et al*., 2009). Recently, strigolactones have been shown to induce hyphal branching in arbuscular mycorrhizal fungi (Akiyama *et al*., 2005), and to function as a hormone to inhibit the branching of plant shoots (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Further studies have revealed that strigolactones might play a role in optimizing plant growth and development to cope with limiting resources (Brewer *et al*., 2013). Seeds of root parasitic weeds usually require a period of imbibition for several days at suitable temperatures (a preparatory step known as "conditioning") before they can respond to germination stimulants (Logan and Stewart, 1992). After germination, the radicles of root parasitic weeds attach to the host roots via specialized parasitic organs known as haustoria, and draw away water and nutrients from the hosts, causing serious reductions in crop growth and yields. The mature flowers of root parasitic weeds produce numerous (10,000–50,000 seeds per plant annualy) (Joel *et al*., 1995, 2007), tiny (around 0.2 mm), and long-lived (several decades) (Bekker and Kwak, 2005) seeds.

This complex life cycle of root parasitic weeds and their close association with the host plants make conventional weed control strategies virtually ineffective (Joel *et al*., 1995, 2007). In addition, root parasitic weeds have often caused irreversible damage by the time the infestation is detected from the aboveground emergence of their shoots. To date several potential methods for controlling root parasitic weeds have been developed (Hearne, 20009). As a non-chemical method for control, breeding of crop varieties resistant to root parasitic weeds should be an effective strategy and some resistant varieties have been identified (Rubiales *et al*., 2009; Yoder and Scholes, 2010). Application of strigolactone deficient mutants of host plants is also an effective strategy and low level of infection of root parasitic weeds was seen in the mutants (Koltai *et al*., 2010; Jamil *et al*., 2011). However, use of resistant varieties is limited to some crops (Rubiales, 2003). A chemical control method is one of the practical strategies (Hearne, 2009). Herbicides can control root parasitic weeds to some extent (Aly, 2007; Rubiales and Fernández-Aparicio, 2012). For example, glyphosate, an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), and imidazolinones and sulfonylureas, inhibitors of acetolactate synthase (ALS), are used to control root parasitic weeds. However, these herbicides are not fully selective for root parasitic weeds and may damage host plants.

To avoid damaging the host, the most effective strategy is to reduce the soil seed bank and/or inhibit the parasite at an early growth stage (e.g., germination and radicle elongation). In that sense, “suicidal germination” is one of the most attractive strategies to reduce the soil seed bank (Zwanenburg *et al*., 2009). In this approach, the seeds of the parasite are forced to germinate by applying a natural or synthesized

germination stimulant, generally a strigolactone, to fields without a host crop, resulting in the death of the root parasitic weeds. While this is a promising approach, it is expensive because it requires large-scale synthesis of structurally complex germination stimulants. Also, application of strigolactones could have adverse environmental effects, because they function as hormones and signaling molecules in plants and soil fungi. Recently new control approach combining strigolactone deficient mutants and a strigolactone mimic 4-Br debranone (4BD), which shows inhibitory activity against tiller bud outgrowth but less stimulating activity for seed germination of root parasitic weeds, is proposed (Fukui *et al.*, 2011, 2013). In this approach, strigolactone deficient mutants grows normally by applying 4BD, but germination of root parasitic weeds is not induced because of the low amounts of natural strigolactones from mutant plants and the less activity of 4BD as the germination stimulant. Although there are challenges of the cost and the broad environmental effects as described above when considering the practical use of strigolactones, this finding represents a major breakthrough in the production of target-selective strigolactone mimics and the application of them as agricultural chemicals.

Alternatively, inhibitors targeting the early growth stages of root parasitic weeds have been screened, and many natural compounds from fungi have been found (Vurro *et al*., 2009). Some compounds have been shown to strongly inhibit seed germination and radicle elongation; for example, some macrocyclic trichothecenes inhibited seed germination of *Orobanche* (*Phelipanche*) *ramosa* at 0.1 μM. However, their mode(s) of action (MOA) is unknown, and their negative

effects on the growth of other organisms (*e.g.*, host plants or environmental microorganisms) have not been fully evaluated. These situations call for new control strategies to reduce the soil seed bank and inhibit the early developmental stages.

In order to establish a new control strategy, we have focused on the unique germination process of root parasitic weeds in the Orobanchaceae to identify potential targets, which could be used to develop a selective control method. For example, if these seeds have a specific metabolic process that is essential for germination, then inhibitors of that process could specifically inhibit germination without affecting the hosts or other organisms. Previously, it was shown that an unknown trisaccharide decreased in response to germination stimulant in *O. minor* seeds by metabolomics approach (Figs. 2A–C) (Joseph, 2009). Furthermore, nojirimycin bisulfite (NJ) (Fig. 2D), a glycosidase inhibitor, inhibited germination of *O. minor* but had no effect on seed germination of Arabidopsis and red clover (*Trifolium pratense*), a host of *O. minor* (Fig. 2E) (Joseph *et al.*, unpublished). It was also revealed that germination of *Striga hermonthica* seeds was not affected (Fig. 2E), but the radicle elongation was inhibited by NJ treatment (Joseph *et al.*, unpublished). These pervious results suggested that sugar metabolism including the unknown trisaccharide is important for germination of *O. minor*, and NJ affects its metabolism resulting in inhibition of the germination. Therefore, the objective of this thesis is to elucidate the role of the unknown trisaccharide in an early stage of seed germination process of *O. minor* and other root parasitic weeds in the Orobanchaceae, and to evaluate the relationship between the inhibitory effect of NJ on the germination and the metabolism of unknown trisaccharide. Furthermore,

investigation of molecular mechanism of the inhibitory effect of NJ and discovery of other targets regulating the germination process using NJ as a germination inhibitor via transcriptome analysis of *O. minor* seeds were attempted. In Chapter 2, I identified the unknown trisaccharide as planteose by nuclear magnetic resonance (NMR) analysis, and surveyed its metabolism by gas chromatography-mass spectrometry (GC-MS) analysis. Subsequently, in Chapter 3, the effects of NJ on the germination and planteose metabolism were evaluated by comparing the sugar contents in the germinating seeds and the NJ-treated seeds. Finally, transcriptome analysis of *O. minor* seeds using *de novo* assembly of reads obtained by next-generation sequencing of expressed mRNAs (RNA-Seq) was conducted in Chapter 4, in order to identify genes associated with the inhibition of germination by NJ and further explore potential targets other than the planteose metabolism by understanding gene expression changes in the early stage of germination.

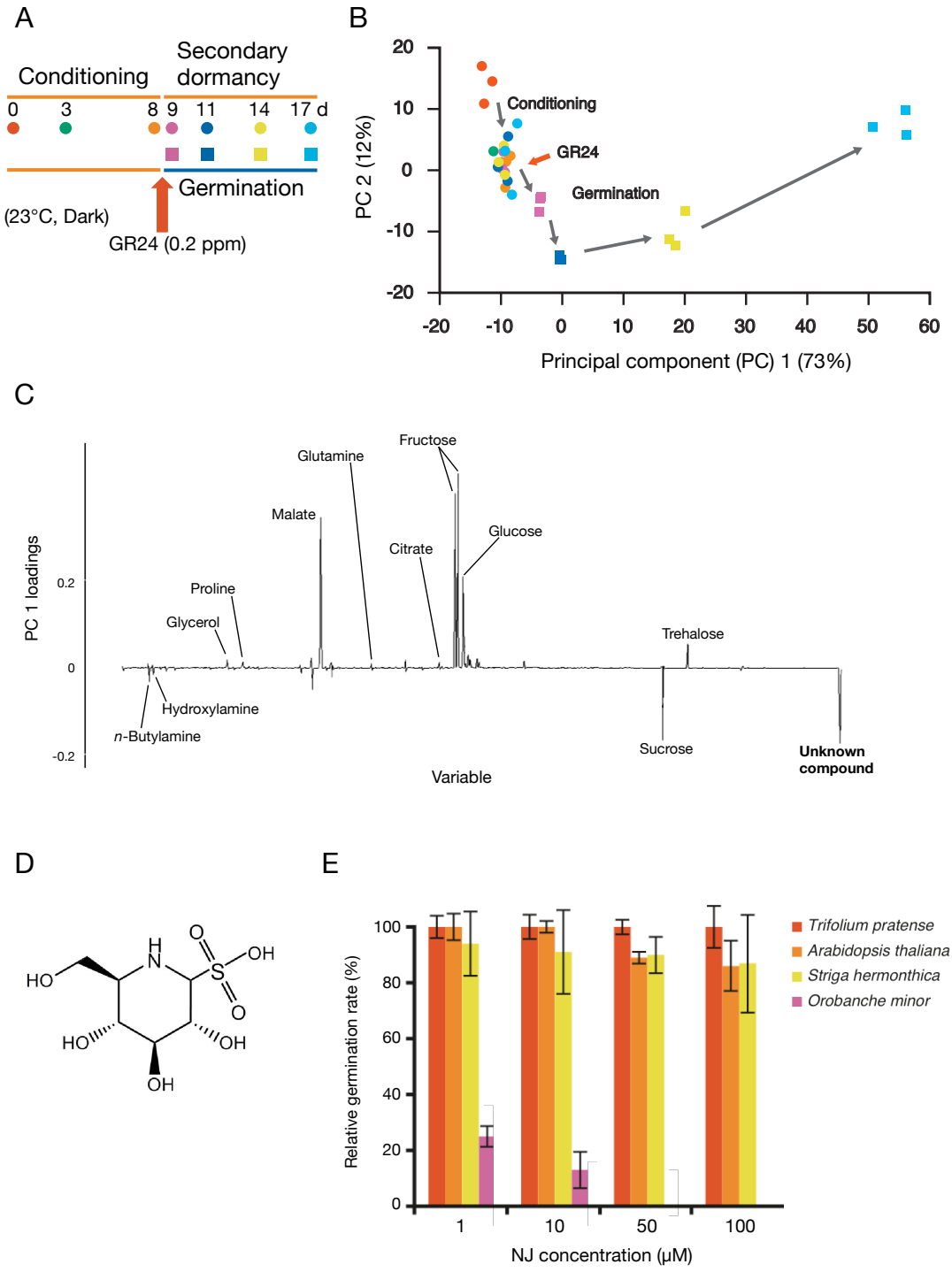


Figure 2. Metabolic profiling of germinating seeds of *O. minor* (Joseph, 2009) and inhibition of the germination by nojirimycin bisulfite (NJ) (Joseph *et al.*, unpublished). (A) Squares (with GR24 treatment) or circles (without GR24 treatment) show time points of sample collection. GR24 was applied on day 8 (arrow). (B) Principal component analysis of metabolite profiles of seeds at different stages during conditioning and germination, shown as the combination of the first two principal components (representing 85% of metabolite variance).

Figure 2. (continued) Each data point is an independent sample. (C) Loading plot showing weight for each data point of the total ion current chromatogram in calculating principal component 1. (D) Structure of NJ. (E) Effect of NJ on seed germination rates in parasitic and non-parasitic plant species (mean ± SD, $n = 3$).

Chapter 2 Identification of planteose as a storage carbohydrate required for early stage of germination of *Orobanche minor*

2-1. Introduction

Root parasitic weed species in the Orobanchaceae have the unique germination process as described in Chapter 1. The control strategy based on the root parasitic weed-specific germination can be a promising approach, and an understanding of this specific biological event is important for developing selective control strategies. Metabolomics has proved to be a powerful technology in identifying the MOA of bioactive compounds (Aliferis and Chrysayi-Tokousbalides, 2011; Aliferis and Jabaji, 2011), in identifying novel metabolic pathways, and in evaluating the cellular responses of plants in detail (Weckwerth and Fiehn, 2002). Metabolomics using gas chromatography-time-of-flight mass spectrometry (GC-TOF-MS) in the early stage of germination process of *O. minor* found out previously a specific metabolism in this process (Joseph, 2009). The experiment was conducted as follows. The seeds were collected at various times during conditioning and germination that was induced by the synthetic strigolactone, GR24 (Thuring *et al*., 1997), after 8 days of conditioning (Fig. 2A). Subsequently, derivatized hydrophilic metabolites and fatty acids were analyzed according to previous reports (Jumtee *et al.*, 2008, 2009). Principal component analysis (PCA) of the GC-TOF-MS total ion current chromatograms provided a detailed view of the characteristic metabolic changes associated with the gradual transition of dormant seeds to germinating seeds via conditioning (Fig. 2B) (Joseph, 2009). In the PCA, principal component (PC) 1

accounted for 73% of the variation in metabolite contents among samples during conditioning and germination. The loading of PC 1 indicated that the levels of amino acids, organic acids, and some sugars (glucose, fructose, and trehalose) increased during germination, while the levels of sucrose and an unknown compound decreased (Fig. 2C) (Joseph, 2009). This unknown compound was predicted to be a trisaccharide, based on its retention time and mass spectrum. However, there was no corresponding spectrum in our in-house mass spectral library nor in the NIST MS database. The decreases in sucrose and the unknown trisaccharide implied that they play roles in the germination of *O. minor* seeds.

Therefore, I made an attempt to purify the unknown trisaccharide from the extract of *O. minor* dry seeds by isocratic high-performance liquid chromatography (HPLC), and then identify its structure by GC-MS and NMR analyses. After the structural determination of the compound, its metabolism during the germination was evaluated.

2-2. Materials and methods

2-2-1. *Germination test*

The seed germination assay for parasitic weeds was conducted as described previously (Chae *et al*., 2004), with some modifications. Seeds of root parasitic weeds were surface-sterilized with a solution containing 1% (v/v) sodium hypochlorite and 0.1% (v/v) Tween 20 for 2 min, rinsed with distilled water, and dried under vacuum. Approximately 50 surface-sterilized seeds were conditioned on a filter disk (10 mm, Whatman GF/D; GE Healthcare Bio-Sciences AB, Uppsala,

Sweden) placed on another filter paper (47 mm, Whatman GF/D) in a Petri dish (50 mm) with 1.5 ml distilled water in the dark at 23°C for 1 week for *O*. *minor*, *Orobanche crenata*, and *Phelipanche aegyptiaca*, or 2 weeks for *S. hermonthica*. Four filter disks with seeds were placed in each Petri dish. After conditioning, the GF/D disks with the seeds were transferred to a new Petri dish containing fresh filter paper (47 mm, Whatman GF/D) to remove surplus water. Germination was induced by adding 1.5 ml GR24 solution [0.1–1.0 $mg \cdot l^{-1}$ (w/v)]. GR24 was kindly supplied by Dr. Yukihiro Sugimoto from Kobe University, Japan. After the germination stimulation treatment, seeds were observed under a microscope to count germinated seeds. The germinating seeds were stained with crystal violet to facilitate detection of radicles if necessary.

2-2-2. *Sample preparation for sugar analysis*

For sugar analysis, around 50 mg of seeds of root parasitic weeds were conditioned and germination was induced as described above. Samples were collected at various times during conditioning and after the GR24 treatment, and were stored at –80°C until use.

Seeds of *O*. *crenata*, *P*. *aegyptiaca*, and *S*. *hermonthica* were collected at different days in each because their germination rates were different. It is known that seeds of *Striga* spp. can germinate faster than those of *Orobanche* spp. and *Phelipanche* spp. (Wigchert *et al*., 1999; Matusova *et al*., 2005).

2-2-3. *Sugar analysis by GC-MS*

The frozen seeds were disrupted by ball milling (20 Hz, 2 min) with a MM 301 mixer mill (Retsch GmbH, Haan, Germany) and then extracted in 300 µl distilled water at 95°C for 30 min. The solution was centrifuged at 12,000 *g* for 10 min, and the supernatant was collected in a new Eppendorf tube. Proteins were removed from the extract by ultrafiltration with an Amicon Ultra-0.5 10 K centrifugal filter (Merck KGaA, Darmstadt, Germany). The solution was passed through a Chromatodisk filter (Type: 4A, pore-size: 0.2 µm, GL Sciences Inc., Tokyo, Japan) and then freeze-dried. The sample was dissolved in 100 µl pyridine, and an aliquot of the sample was derivatized with the same volume of *N*-trimethylsilylimidazole (Sigma-Aldrich, St. Louis, MO, USA) at room temperature.

GC-MS analysis was performed using a JMS-AMSUN200 quadrupole mass spectrometer (JEOL Ltd., Tokyo, Japan) coupled to a gas chromatograph (6890A; Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies, Inc.). Analyses were carried out in the splitless mode with a 1-µl injection volume. The injector temperature was 250°C, and the helium gas flow rate through the column was 1 $ml \cdot min^{-1}$. The column temperature was held at 70°C for 2 min, then raised by 10°C$\cdot min^{-1}$ to 325°C and held at that temperature for 10 min. The interface temperature and ion source temperature were set at 280°C and 250°C, respectively. The ions were generated by a 70 eV electron beam, and two scans per second were recorded in the mass range of *m*/*z* 100–750. For each sample, chromatographic peaks were identified by comparing their retention time with those of authentic standards. Compounds were quantified from the peak areas using the

external standard method.

2-2-4. *Purification of planteose*

Sugars were extracted from dry seeds of *O. minor* as described above. The extract was concentrated by a centrifugal concentrator, and then the trisaccharide fraction was purified by isocratic HPLC with a COSMOSIL Sugar-D column (20 × 250 mm, 5 μm, Nacalai Tesque, Inc., Kyoto, Japan). Eluted compounds were detected with a Shimadzu RID-10A refractive index detector (Shimadzu Corp., Kyoto, Japan). The mobile phase was 65% acetonitrile. The column oven was set at 30°C, and the flow rate was 9.0 ml·min^{-1}. HPLC was performed using an LC workstation (Shimadzu Corp.) with CLASS-VP ver. 6.1 software. The HPLC system consisted of a system controller (SCL-10Avp), a column oven (CTO-10A), an auto-sampler (SIL-10Axl), and a pump (LC-10AT).

2-2-5. *NMR analysis*

NMR spectra (^{1}H, ^{13}C, COSY, HMBC, HSQC, TOCSY, HSQC-TOCSY, and NOESY) of the purified trisaccharide were recorded on a JMN ECA-500 system (JEOL Ltd.) in D_2O. The internal standard was 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt.

2-2-6. *Sugar analysis by UPLC-ELSD*

The dried sugar extracts were dissolved in 80% acetonitrile and analyzed with the ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters Corp.,

Milford, MA, USA) using a Waters ACQUITY UPLC BEH AMIDE column (2.1 mm × 100 mm, 1.7 μm). Eluted compounds were detected using a Waters evaporative light scattering detector (ELSD). The detector conditions were as follows: gain, 200; gas pressure, 50 psi; drift tube temperature, 55°C; nebulizer mode, cool. The mobile phase was acetonitrile with 0.2% triethylamine (TEA) (solvent A) and distilled water with 0.2% TEA (solvent B). Separations were performed using a gradient program with a mixture of solvents A and B, as follows: 20–30% B for 0–2.8 min, 30–50% B for 2.8–4.5 min, 50–80% B for 4.5–5.0 min, 80–20% B for 5.0–6.5 min, and 20% B for 6.5–7.0 min (re-equilibration). The flow rate was set as follows: 0.25 $ml \cdot min^{-1}$ for 0–4.5 min, 0.25–0.10 $ml \cdot min^{-1}$ for 4.5–5.0 min, 0.10–0.25 $ml \cdot min^{-1}$ for 5.0–6.5 min, and 0.25 $ml \cdot min^{-1}$ for 6.5–7.0 min. The column oven was set at 35°C and the sample injection volume was 5 μl. For each sample, chromatographic peaks were identified by comparing the retention time with those of authentic standards. The chromatograms were analyzed with the Waters Empower 2 data processing program.

2-3. Results

2-3-1. *Structure identification of the trisaccharide*

The unknown trisaccharide in seeds of *O. minor* was extracted from the dry seeds and purified by HPLC (Fig. 3). GC-MS analysis of the acid hydrolysate of the purified trisaccharide revealed that its constituent monosaccharides were glucose, fructose and galactose because released monosaccharides from the trisaccharide were consistent with those released from raffinose,

(α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside),

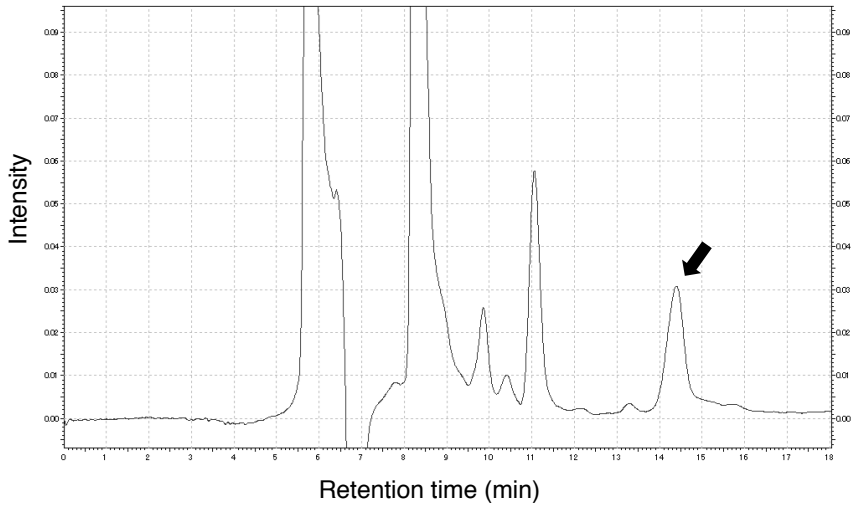


Figure 3. Purification of unknown trisaccharide from dry seeds of *O. minor*. Sugars were extracted from dry seeds of *O.* minor; unknown trisaccharide was purified from seed extract by HPLC-RID. The arrow marks unknown trisaccharide.

and a subset of the hydrolytic product of trisaccharide was consistent with the constituent monosaccharides of sucrose (β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) (Fig. 4). Following the constituent analysis, NMR analysis using ^{1}H-NMR, ^{13}C-NMR, COSY, HMBC, HSQC, TOCSY, HSQC-TOCSY, and NOESY was conducted to identify the structure, and Table 1 showed the assigned ^{1}H and ^{13}C NMR spectral data of the trisaccharide (Fig. 5, Table 1). To distinguish the structure of glucose or galactose in the trisaccharide, ^{1}H–^{1}H spin–spin coupling constant of vicinal protons at the C4 position of each monosaccharide moiety is important because, in principle, the coupling constant in the axial-equatrial relation in galactose (0–5 Hz) is different from that in the axial-axial relation in glucose (6–14 Hz). Therefore, the structures of galactose and glucose were distinguished by the observed coupling constants (3.4,

1.3 Hz and 9.8, 9.0 Hz, repectively). It was also revealed that glucose and galactose were linked via α-glycosidic bonds to fructose moiety based on the coupling constants at the C1 position (4.0 Hz in glucose and 3.9 Hz in galactose, respectively). Moreover, the observed NOE (blue dashed arrows in Fig. 5) and HMBC correlations (red solid arrows in Fig. 5) supported the estimated steric structure. Consequently, the structure of unknown trisaccharide was determined as a planteose (α-D-galactopyranosyl-(1→6)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside).

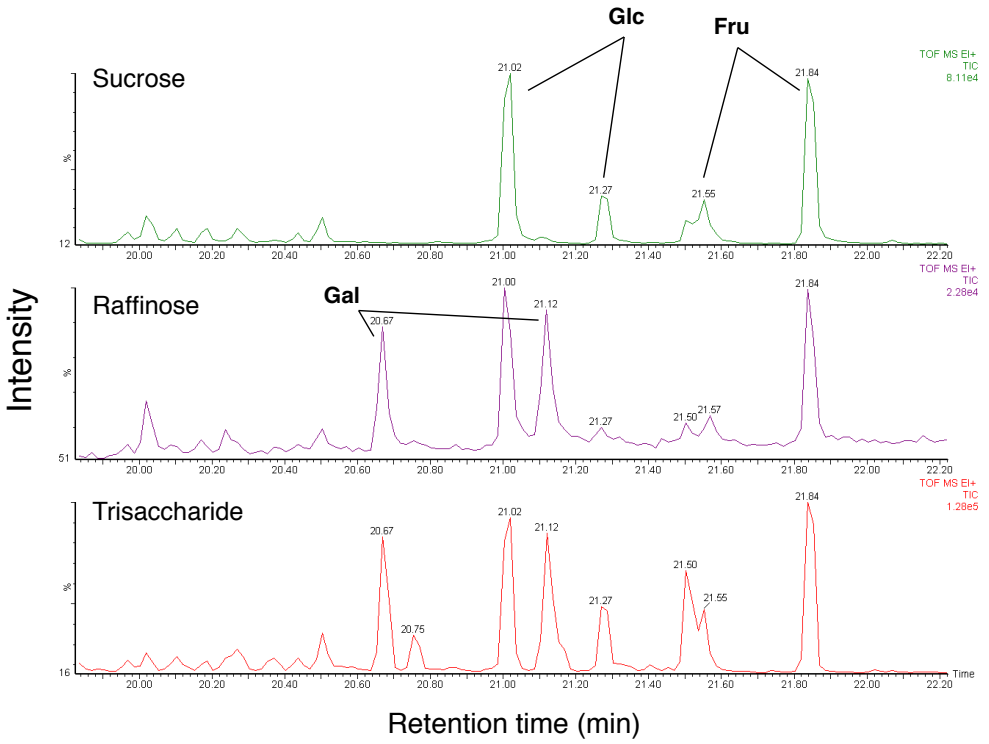
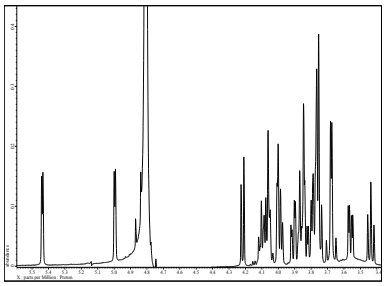


Figure 4. GC-MS analysis of acid hydrolysates of sucrose, raffinose, and unknown trisaccharide. Sucrose, raffinose, and purified trisaccharide were acid-hydrolyzed in 1 N HCl at 95°C for 1 h. TMS derivatization forms multiple peaks for each monosaccharide because of the multiple conformers in the solution (*e.g.*, α- and β-pyranose and α- and β-furanose). Monosaccharides released from the unknown trisaccharide by acid hydrolysis were consistent with those released from raffinose. Glc, glucose; Fru, fructose; Gal, galactose.

A



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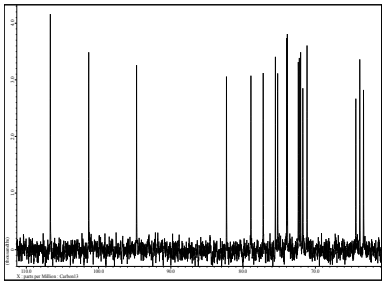

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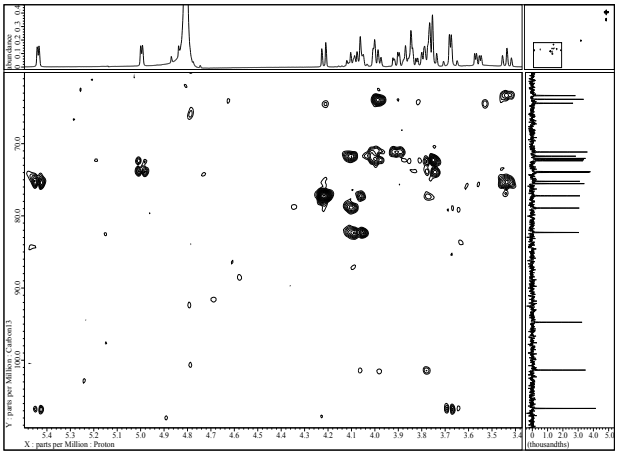

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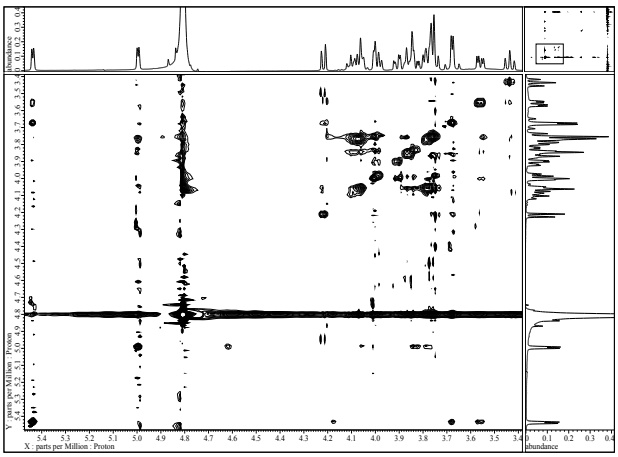

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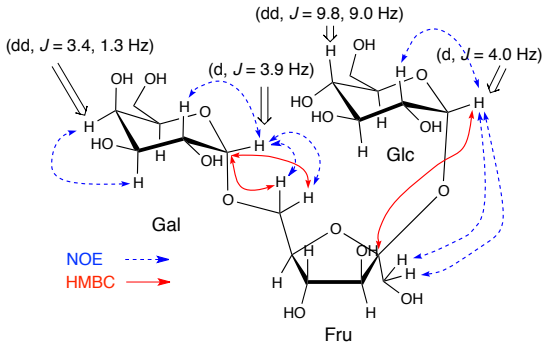

Figure 5. Structural identification of the unknown trisaccharide by NMR. (A) ^{1}H and (B) ^{13}C NMR signals. (C) HMBC and (D) NOESY spectra of trisaccharide. (E) Structure of identified trisaccharide, planteose (α-D-galactopyranosyl-(1→6)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) in which C1 of galactose is attached to C6 of fructose moiety of sucrose via an α-glycosidic linkage. Figure shows selected long range correlations observed in HMBC spectrum and NOE of planteose.

Table 1. ^{1}H and ^{13}C NMR spectral data of the trisaccharide.

		δ_C	δ_H		J_{HH}
Glc	1	94.7	5.44	*d*	4.0
	2	74.0	3.56	*dd*	10.0, 3.9
	3	75.5	3.76	*dd*	10.2, 8.7
	4	72.4	3.44	*dd*	9.8, 9.0
	5	75.2	3.86	*ddd*	9.8, 3.7, 1.5
	6	63.3	3.86	*dd*	13.0, 2.3
			3.78	*m*	
Fru	1	64.4	3.68	*d*	4.1
	2	106.7			
	3	78.9	4.22	*d*	8.5
	4	77.2	4.10	*t*	8.5
	5	82.3	4.06	*m*	
	6	71.7	3.78	*m*	
			4.06	*m*	
Gal	1	101.4	5.00	*d*	3.9
	2	71.2	3.84	*dd*	10.4, 3.8
	3	72.2	3.91	*dd*	10.4, 3.2
	4	72.0	4.03	*dd*	3.4, 1.3
	5	73.9	3.99	*m*	
	6	63.9	3.75	*m*	

2-3-2 *Profiling of sugars in germinating seeds of* O. minor

Next, I quantified each sugars in *O. minor* seeds during germination by GC-MS analysis to investigate planteose metabolism (Fig. 6). In the dry seeds, the main sugars were planteose (3.48 ± 0.17 nmol·mg^{-1} seeds), sucrose (0.73 ± 0.29 nmol·mg^{-1} seeds), glucose (3.54 ± 1.06 nmol·mg^{-1} seeds), and fructose (2.82 ± 1.30 nmol·mg^{-1} seeds) (Fig. 6A). The amounts of glucose and fructose significantly reduced to about one-third shortly after imbibition, and, the levels of these sugars including sucrose and planteose did not change for two weeks without GR24 treatment (Fig. 6B). After the GR24 treatment on the 7th day of imbibition, germination rate of *O. minor* seeds gradually increased and reached maximum rate 86.2% at 7 days after GR24 treatment (DAG) (Fig. 6C). Planteose metabolism proceeded with a parallel increase in the amounts of glucose and fructose during germination (Figs. 6D–G). At 3 DAG, when the radicle had emerged, the planteose level began to decrease significantly, and the amounts of glucose and fructose gradually increased. The amount of sucrose, which is a intermediate of planteose metabolism, significantly decreased from 5 DAG subsequent to the decrease of planteose (Figs. 6F, G). Hydrolysis of the glucose moiety of planteose can release planteobiose (α-D-galactopyranosyl-(1→6)-β-D-fructofuranose). Therefore, planteobiose is a possible intermediate in planteose metabolism, but this compound was not detected in any of the samples. From these results, a metabolic pathway of planteose was predicted as follows. Hydorolysis of planteose might produce sucrose followed by production of glucose and fructose by hydrolysis of sucrose. Planteose and sucrose were almost completely consumed by 5 DAG while germination rate

increased drastically from 3 to 5 DAG (Figs. 6C, F and G). Galactose, a constituent of planteose, was not detected in any seed samples.

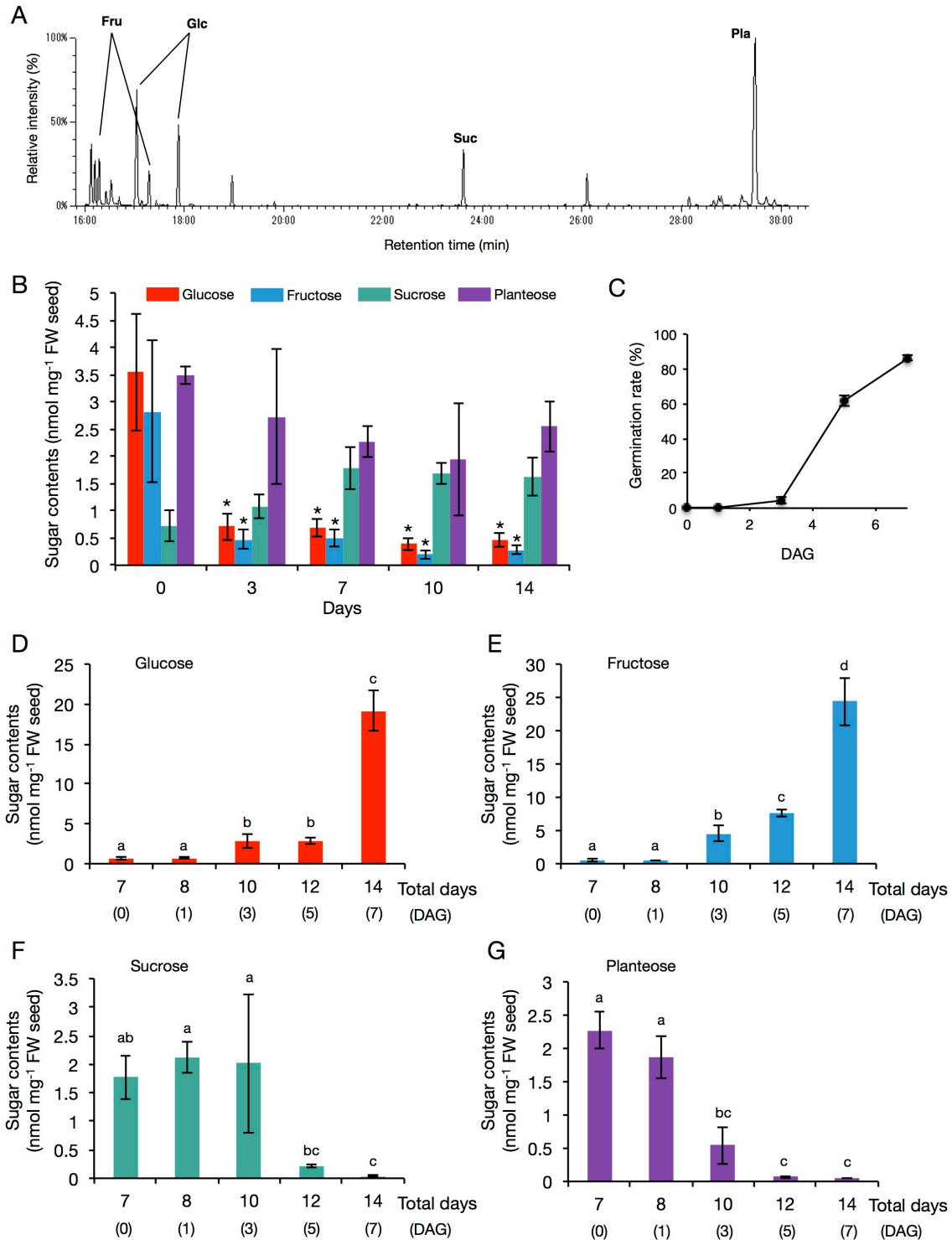

Figure 6. Sugar profiles in *O. minor* seeds during conditioning and germination. Galactose was not detected in any samples. (A) Total ion current chromatogram of sugars in dry seeds obtained by GC-MS analysis. Glc, glucose; Fru, fructose; Suc, sucrose; Pla, planteose. (B) Changes in the amounts of sugars in seeds without GR24 treatment (mean ± SD, $n = 3$). Conditioning period was 7 days and distilled water was applied on the final day of conditioning, therefore germination was not induced. Asterisks indicate significant differences in each sugar contents between dry seeds and during conditioning seeds ($P < 0.05$, Tukey-Kramer). (C) Germination rate of *O. minor* seeds after GR24 treatment. Conditioning period was 7 days; 10 $mg \cdot l^{-1}$ (w/v) GR24 was applied on the final day of conditioning. Seeds did not germinate without GR24 treatment. Seeds were observed and germinated seeds were counted under a microscope (mean ± SD, $n = 3$). DAG; days after GR24 treatment. (D–G) Changes in the amounts of sugars in seeds with GR24 treatment (mean ± SD, $n = 3$). Conditioning period was 7 days and 10 $mg \cdot l^{-1}$ (w/v) GR24 was applied on the final day of conditioning. Different letters indicate significant differences in sugar contents during germination ($P < 0.05$, Tukey-Kramer). DAG; days after GR24 treatment.

2-3-3. *Planteose metabolism in weedy broomrapes and a witchweed*

Planteose metabolism was also evaluated in weedy broomrapes and a witchweed including *O. crenata*, *P. aegyptiaca*, and *S. hermonthica*, which actually cause serious crop losses in the world (Parker, 2013). I detected planteose in the seeds of these species (Fig. 7). The sugar composition in seeds of other broomrapes (*O. crenata* or *P. aegyptiaca*) was similar to that in the seeds of *O. minor*, and the amounts of planteose and sucrose (Fig. 7, black and gray arrows, respectively) decreased during germination. However, there was less amount of planteose in *S. hermonthica* seeds compared to those in the seeds of the three broomrapes. The sucrose level in *S. hermonthica* seeds drastically decreased during germination, and

there was a significant accumulation of monosaccharides at the later stage of germination, similar to that observed in the broomrapes.

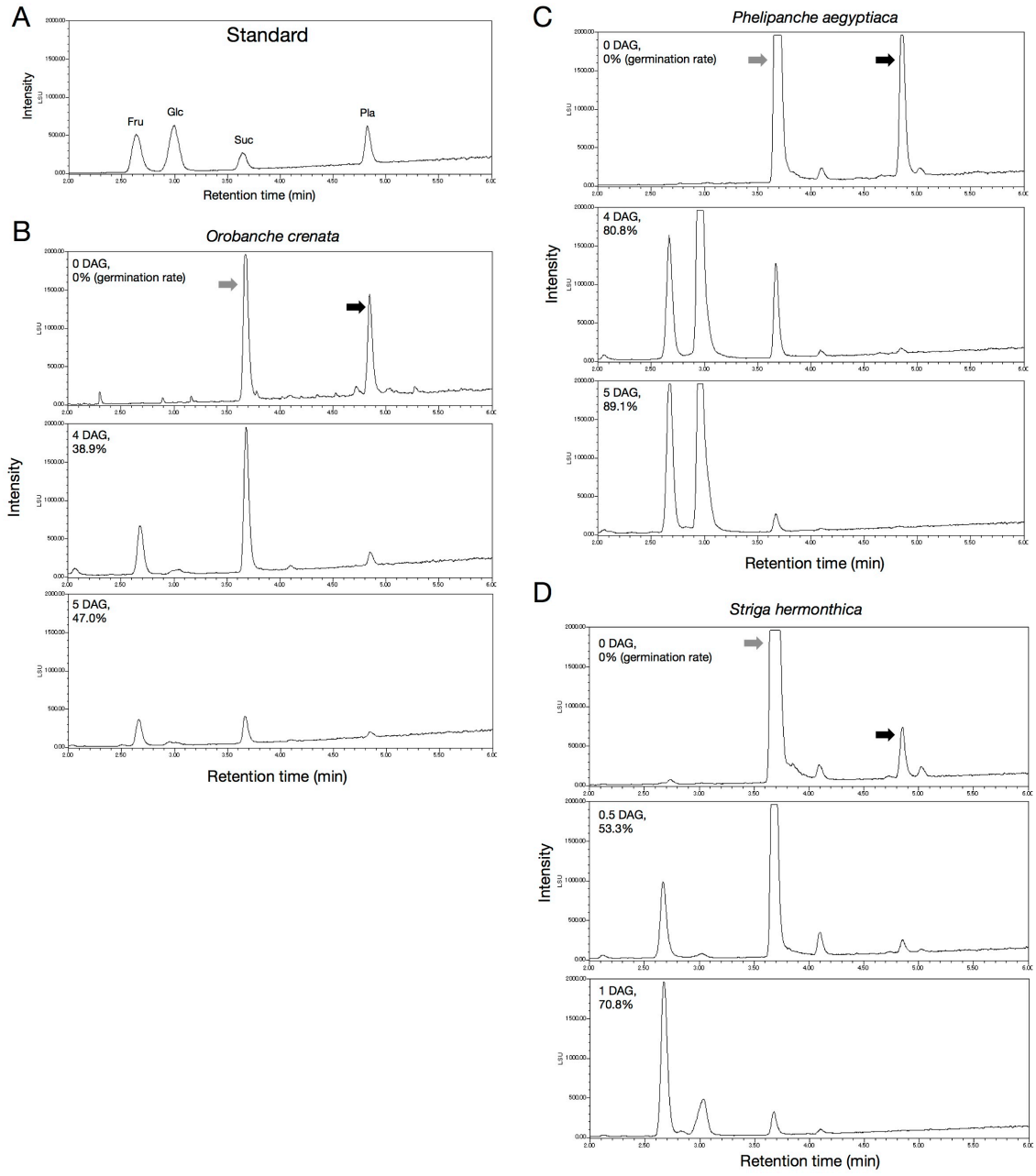

Figure 7. UPLC-ELSD analysis of sugars in seeds of various root parasitic weeds. (A) Authentic standard compounds. (B) *O. crenata*. (C) *P. aegyptiaca*. (D) *S. hermonthica*. Black and gray arrows indicate planteose and sucrose, respectively. Days after GR24 treatment (DAG) and germination rate at that time are shown in upper left of chromatograms. Seeds of each parasitic weed were collected immediately after conditioning and at two time points during germination before reaching maximum germination rate. The dates of collection of seeds were different among species because germination rate of each plant species is different.

2-4. Discussion

In the present study, the results show that planteose metabolism is involved in the early stage of germination of root parasitic weeds in the Orobanchaceae. To my knowledge, this is the first report of the presence of planteose in seeds of members of the Orobanchaceae. Planteose was first identified in *Plantago* seeds and has since been found in the seeds of a number of other plants (Amuti and Pollard, 1977). It has been reported that planteose exists mainly in seeds and functions as a storage carbohydrate because its amount increases during seed ripening and it is consumed during germination (Kandler and Hopf, 1982; Peterbauer and Richter, 2001; Downie *et al*., 2003). However, it is still unknown whether planteose metabolism is essential for seed germination or not (Peterbauer and Richter, 2001). Planteose is an isomer of raffinose, a member of the raffinose family oligosaccharides (RFOs), which are composed of sucrose and chains of α-galactosyl residues attached to the glucose moiety of sucrose via an α-(1→6) galactosidic linkage. Planteose has an α-galactosidic linkage at the fructose moiety. In some plant species, RFOs might function as an easily available source of energy during the early stages of seed germination (Blöchl *et al*., 2007). The most common RFO, raffinose, was not detected in *O. minor* seeds. Alternatively, there was approximately five times more planteose than sucrose in the dry seeds of *O. minor*. These findings suggest that planteose is the main storage carbohydrate providing energy required for the early stages of germination of *O. minor* seeds.

I also detected planteose in the seeds of other root parasitic weeds in the Orobanchaceae; *O. crenata*, *P. aegyptiaca*, and *S. hermonthica*. In all of these

species, the amount of planteose in seeds decreased during germination (Fig. 7). Also, the change in sugar composition in seeds during germination was quite similar among the three broomrapes (*O. minor*, *O. crenata*, and *P. aegyptiaca*) (Fig. 6, Fig. 7). These results suggest that sugar metabolism in germinating seeds is conserved among broomrapes. Planteose was also detected in the seeds of *S. hermonthica.* These findings indicate that planteose may function as a storage carbohydrate for use during the early stages of seed germination of root parasitic weeds in the Orobanchaceae.

Plants generally store starch or lipids in seeds as a source of energy for germination. However, seeds of *S. hermonthica* lack detectable amount of starch (Vallance, 1951), suggesting that the dry seeds of root parasitic weeds may contain little or no starch. In *S. asiatica* seeds, triacylglycerol were not hydrolyzed during or after germination (Menetrez *et al*., 1988), indicating that this species does not rely on lipids to provide energy for germination. Previously, the levels of starch and total fatty acids in *O. minor* seeds were analyzed to evaluate contributions of their metabolism to the germination. The amount of starch (10.4 $\mu g \cdot mg^{-1}$ dry weight) in the seeds of *O. minor* gradually decreased during conditioning, but increased again after GR24 treatment (Fig. 8A) (Joseph, 2009). The amount of total fatty acids in the dry seeds of *O. minor* increased after GR24 treatment and was greater than that in GR24 untreated seeds at any time points (Fig. 8B) (Joseph *et al*., unpublished). These results indicate that starch and lipids are not significant energy sources for the seed germination of root parasitic weeds and support my hypothesis that planteose is the main storage compound for the germination.

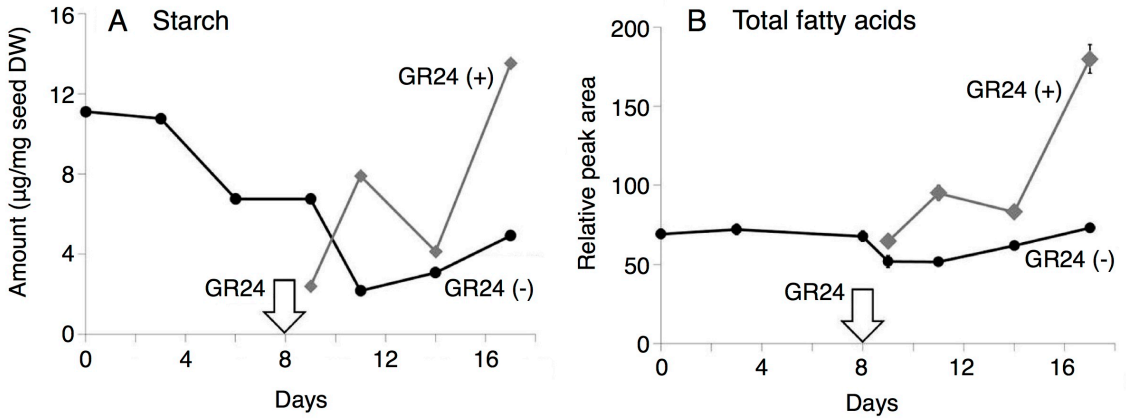

Figure 8. Profiles of starch and total fatty acids in germinating *O. minor* seeds. Amounts of starch (A) (Joseph, 2009) and total fatty acids (B) (Joseph *et al*., unpublished) were measured in seeds with (+) or without (-) GR24 treatment (mean ± SD, $n = 3$). Arrows indicate the day of GR24 treatment.

Chapter 3 Effect of nojirimycin on germination and planteose metabolism in the early stage of germination of *O. minor*

3-1. Introduction

NJ was firstly identified in metabolic products of *Streptomyces* spp. as an antibiotics showing remarkable biological activity against *Sarcina lutea*, *Xanthomonas oryzae* and *Shigella flexneri* (Inouye, 1968). The effect of NJ on plant growth was studied in *Avena sativa*, pea (*Pisum sativum*) and rice (*Oryza sativa*) (Nevins, 1975; Labrador and Nicolas, 1982). NJ, at concentrations of 0.1 to 3.0 mM, inhibited auxin-induced elongation of the coleoptile of *A. sativa* and the stem internode of pea (Nevins, 1975). Furthermore, NJ at 1.0 mM decreased autolytic activity of the cell wall in rice coleoptile *in vitro* (Labrador and Nicolas, 1982). However, there is no report showing inhibitory effect of NJ on germination of plant seeds. These results suggest that NJ has a small effect on plant growth, especially seed germination, and the inhibitory effects require high concentrations of NJ. Interestingly, the previous study showed that NJ strongly and selectively inhibited germination of *O. minor* at low concentrations (Fig. 2E) (Joseph *et al*., unpublished). This result suggested that NJ specifically inhibits a metabolism involving glycosidase(s) in the germination process of *O. minor*. As described in Chapter 2, planteose metabolism was considered to be characteristic and important for germination of root parasitic weeds in the Orobanchaceae, therefore, there was a possibility that planteose metabolic enzymes are inhibited by NJ. In this chapter, detailed effect of NJ and other glycosidase inhibitors on germination of *O. minor* seeds was studied and

subsequently, the relationship between the effects of NJ on germination of *O. minor* and on the planteose metabolism was investigated. Additionally, the effect of NJ on germination of non-parasitic seeds containing planteose was evaluated.

3-2. Materials and methods

3-2-1. *Germination test*

The seed germination assay for parasitic weeds was conducted as described in Chapter 2, 2-2-1.

Phtheirospermum japonicum seeds were kindly supplied by Dr. Satoko Yoshida at RIKEN Center for Sustainable Resource Science, Japan. The seeds were surface-sterilized, vernalized at 4°C for 2 days and then incubated at 25°C in the dark. The root length was measured at 5 days after imbibition.

Seeds of planteose containing plants, tomato (*Solanum lycopersicum*) (Amuti and Pollard, 1977; Gurusinghe and Bradford, 2001), sesame (*Sesamum indicum*) (Hatanaka, 1959), and spearmint (*Mentha spicata*) (French *et al*., 1959) were surface-strilized, vernalized at 4°C for 2 days, and then incubated at 23°C under a 16-h light/8-h dark photoperiod (sesame and spearmint) or at 25°C in the dark (tomato). Root lengths of tomato and sesame seeds were measured 4 days after imbibition, and that of spearmint was measured 10 days after imbibition.

3-2-2. *Glycosidase inhibitor assay*

The effects of glycosidase inhibitors; NJ (Niwa *et al*., 1970; Reese *et al*., 1971; Dale *et al*., 1985; Kodama *et al*., 1985), castanospermine (CS) (Saul *et al*., 1983),

1-deoxynojirimycin (DNJ) (Saunier *et al*., 1982) and 1-deoxygalactonojirimycin (DGJ) (Legler and Pohl, 1986), were tested by mixing them with GR24 solution. For the germination recovery test, sugar or UDP-glucose (final concentration 10 mM) was mixed with GR24 (final concentration 1.0 $mg \cdot l^{-1}$) and NJ (final concentration 10 μM). Radicle and root lengths were measured using ImageJ 1.47v software (http://rsbweb.nih.gov/ij/).

3-2-3. *Sample preparation for protein extraction*

For protein extraction, 50 mg of seeds of *O. minor* were conditioned as described in Chapter 2, 2-2-2. The samples were prepared by addition of GR24 solution (1.0 $mg \cdot l^{-1}$) without NJ, with NJ (10 μM), or with NJ (10 μM) and glucose (50 mM), and then collected at 5 DAG. The samples were stored at –80°C until use.

3-2-4. *Invertase extraction and enzymatic assay*

Protein extraction and the assay for activities of invertases (INVs) were conducted as described previously (Draie *et al*., 2011), with some modifications. The frozen seeds were disrupted by ball milling (20 Hz, 2 min) and extracted at 4°C in 1.5 ml extraction buffer (pH 7.0) composed of 50 mM HEPES, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 0.5 $mg \cdot ml^{-1}$ polyvinylpolypyrrolidone (PVPP), and 1% (v/v) Protease inhibitor cocktail (Sigma-Aldrich). The homogenate was centrifuged at 12,000 *g* for 10 min, and the supernatant was collected in a new Eppendorf tube. The pellet was re-extracted in the extraction buffer without PVPP and the extract was collected in same Eppendorf tube after centrifugation. The

collected enzyme solution was used for soluble acid invertase (SAI) and soluble neutral invertase (SNI) assays. The residue was rinsed with 1.0 ml extraction buffer without PVPP and Protease inhibitor cocktail and the supernatant was discarded after centrifugation. To extract cell wall invertase (CWI), the pellet was extracted in the extraction buffer containing 1.0 M NaCl. After centrifugation, the supernatant was used for CWI assay.

Before the assay, the enzyme extract was desalted on a PD-10 desalting column (GE Healthcare Bio-Sciences AB) previously equilibrated with 50 mM HEPES buffer (pH 7.0), and then concentrated with an Amicon Ultra-15 10 K centrifugal filter (Merck KGaA, Darmstadt, Germany). The enzymatic reaction (200 μl reaction mixture) was initiated by mixing the extracted enzyme (20 μg) and sucrose (final concentration 100 mM) in 50 mM sodium phosphate buffer (pH 5.0 for SAI and CWI or PH 7.5 for SNI). When required, NJ was added to the reaction mixture at a final concentration of 100 μM. The reaction mixture was incubated at 30°C for 2 h, and the reaction was stopped by heating at 95°C for 3 min. The amount of released glucose was determined using Glucose (HK) assay reagent (Sigma-Aldrich).

3-2-5. *Enzymatic assay of α-galactosidase*

Protein extraction was carried out as described in 3-2-4, and soluble and NaCl-soluble fractions were prepared. NaCl-soluble fraction may contain cell wall bound proteins. The enzymatic reaction (200 μl reaction mixture) was initiated by mixing the extracted enzyme (20 μg) and 4-nitrophenyl α-D-galactopyranoside (final

concentration 2 mM) in 50 mM sodium phosphate buffer (pH 5.0). The reaction mixture was incubated at 30°C for 1 h, and the reaction was stopped by addition of 50 μl of Na_2CO_3 (0.2 M). The absorbance at 400 nm was measured to calculate released 4-nitrophenol, and the activity was calculated as follows.

$$\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1} = \frac{\Delta A_{400} \times V_t}{18.4 \times l \times t \times 0.02}$$

V_t: total volume (μl), 18.4: molar extinction coefficient of 4-nitrophenol ($\mu l \cdot nmol^{-1} \cdot cm$), l: light path length (cm), t: reaction time (min), 0.02: amount of protein (mg).

3-2-6. *RT-PCR analysis*

Total RNA was extracted from approximately 50 mg conditioned, germinating and NJ-treated seeds of *O. minor* and purified using PureLink® RNA Mini Kit (Life Technologies, Grand Island, NY, USA) according to the manufacture's instruction. cDNA synthesis was performed from total RNA (0.5 μg) using PrimeScript™ RT-PCR Kit (Takara Bio Inc., Shiga, Japan) according to the manufacture's instruction. INV genes of *O. minor* were searched in our in-house EST library provided by Dr. Masaharu Mizutani (Kobe Univ., Japan), and three cDNA sequences encoding INVs expressed in the germinating seeds (*OmSAI*, *OmSNI* and *OmCWI*) were found. PCR was performed using primers OmSAI_F (CTCGATGGTGAAAAGTTATCCA) and OmSAI_R (AAAGTTGATCCAACGGGAAA) for *OmSAI*, OmSNI_F (GACCAGAGTTGGCCAGAAAG) and OmSNI_R (TGCCGTGTTTGGATTGTCTA) for *OmSNI*, and OmCWI_F

(AGTATGGCTCGATCGCAGAG) and OmCWI_R (CGGACCTAATCCACCTTCAA) for *OmCWI*, for 23 (for *OmSAI* and *OmCWI*) or 25 (for *OmSNI*) cycles of 95°C (15 s), 55°C (15 s) and 72°C (30 s), and a single final step at 72 °C for 30 s.

3-3. Results

3-3-1. *Effects of nojirimycin, a glycosidase inhibitor, on germination and radicle elongation of root parasitic weeds*

To determine the importance of planteose metabolism during germination, some glycosidase inhibitors were applied together with GR24. Among the tested inhibitors, NJ showed a strong and selective inhibitory effect on germination of *O. minor* seeds in a dose-dependent manner (Fig. 9A). NJ at 1 and 3 μM decreased the germination rate by 48.8% and 61.8%, respectively, and NJ at 10 μM or higher completely inhibited the seed germination (Fig. 9A). In the case of *S. hermonthica*, NJ did not inhibit the seed germination, but caused a dose-dependent reduction in radicle elongation (Figs. 9B, C). The radicle lengths of *S. hermonthica* with 10 μM and 100 μM NJ were nearly one-half and one-fifth of that of the control, respectively (Fig. 9C). To investigate whether NJ affects the growth of diverse plants in the Orobanchaceae, I analyzed the effect of NJ on *P. japonicum* which is a facultative hemiparasite closely related to *Orobanche* and *Striga* (Bennett and Mathews, 2006). Similar to the case in *S. hermonthica*, NJ did not affect the germination rate of *P. japonicum* seeds, but it inhibited the root elongation at concentrations of 10 μM or higher (Fig. 10).

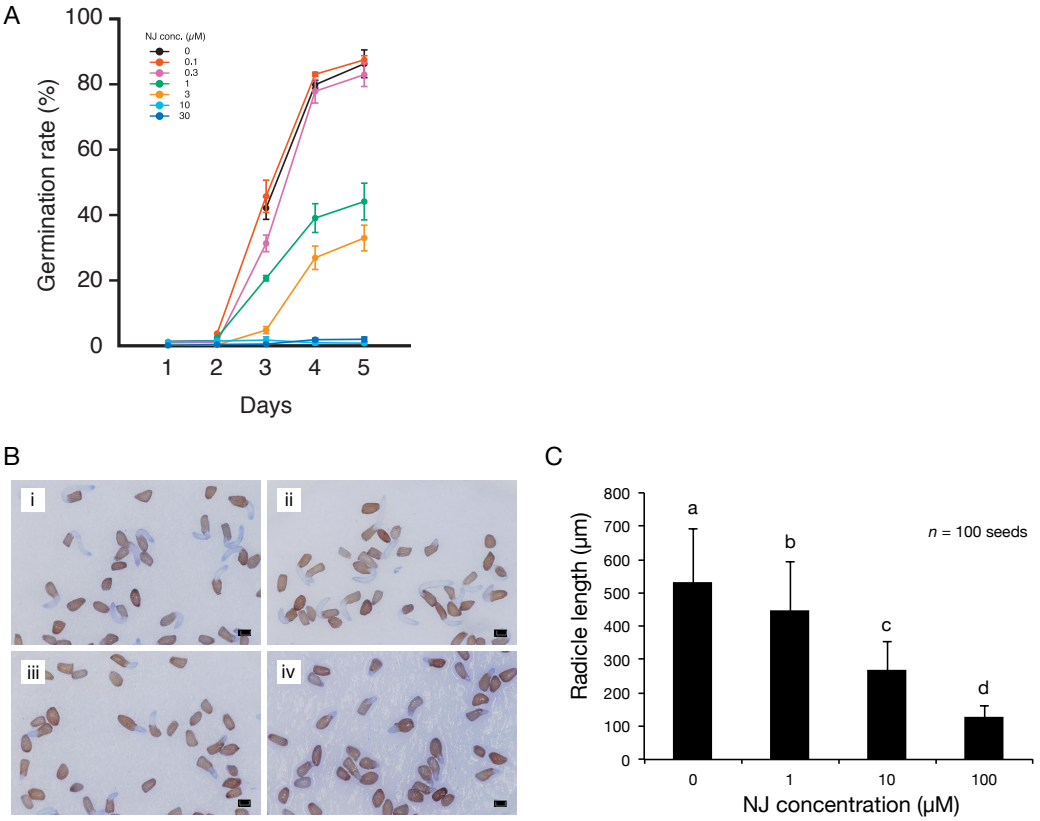

Figure 9. Effect of NJ on seed germination of root parasitic plants in the Orobanchaceae. (A) Effect of NJ at various concentrations on germination rates of *O. minor* seeds (mean ± SD, $n = 3$). Germination rate of *O. minor* seeds decreased in a dose-dependent manner with NJ. (B, C) Effect of NJ on radicle elongation of *S. hermonthica.* (C) Morphological changes in radicle elongation in the presence of NJ. Staining by crystal violet facilitated visualization of the radicles. Treatments were as follows: (i) 10 $mg \cdot l^{-1}$ (w/v) GR24 only; (ii) GR24 + 1 μM NJ; (iii) GR24 + 10 μM NJ; and (iv) GR24 + 100 μM. Bar: 200 μm. (D) Radicle lengths of *S. hermonthica* treated with NJ at various concentrations. Radicle lengths of 100 germinating seeds were determined using ImageJ software. Radicle elongation was inhibited by NJ in a dose-dependent manner. Different letters indicate significant differences in radicle lengths ($P < 0.05$, Tukey-Kramer).

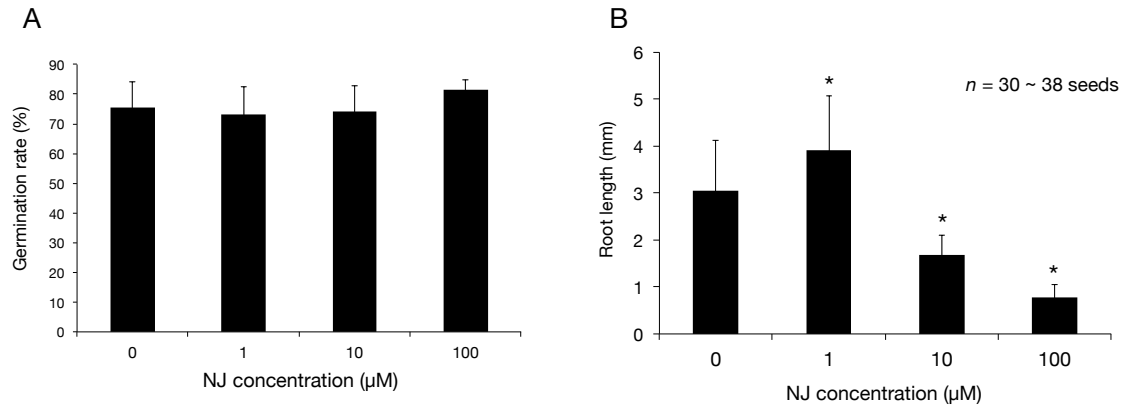

Figure 10. Effects of NJ on seed germination (A) and root elongation (B) of *P. japonicum*. (A) NJ, even at 100 μM, did not affect germination rate of *P. japonicum*. Number of germinated seeds (out of 20) was counted (mean ± SD, $n = 3$). (B) Effects of NJ on root elongation became visible at 10 μM; root length of seedlings treated with 100 μM NJ was approximately one-quarter that of control. Radicle lengths of 30 to 38 germinated seeds were measured using ImageJ software. Asterisks indicate significant differences in the root lengths between control and NJ-treated seeds ($P < 0.05$, Student's *t* test).

The glycosidase inhibitor CS inhibited *O. minor* seed germination at concentrations of 1 μM or higher, however its inhibitory effect was weaker than that of NJ (reduction of 7.2% at 1 μM and 15.6% at 10 μM) (Fig. 11A). Germination was not affected by two other iminosugars, DNJ and DGJ, at concentrations up to 100 μM (Fig. 11B), but DNJ inhibited radicle elongation of *O. minor* (Figs. 11C, D) in a similar way to NJ on *S. hermonthica*.

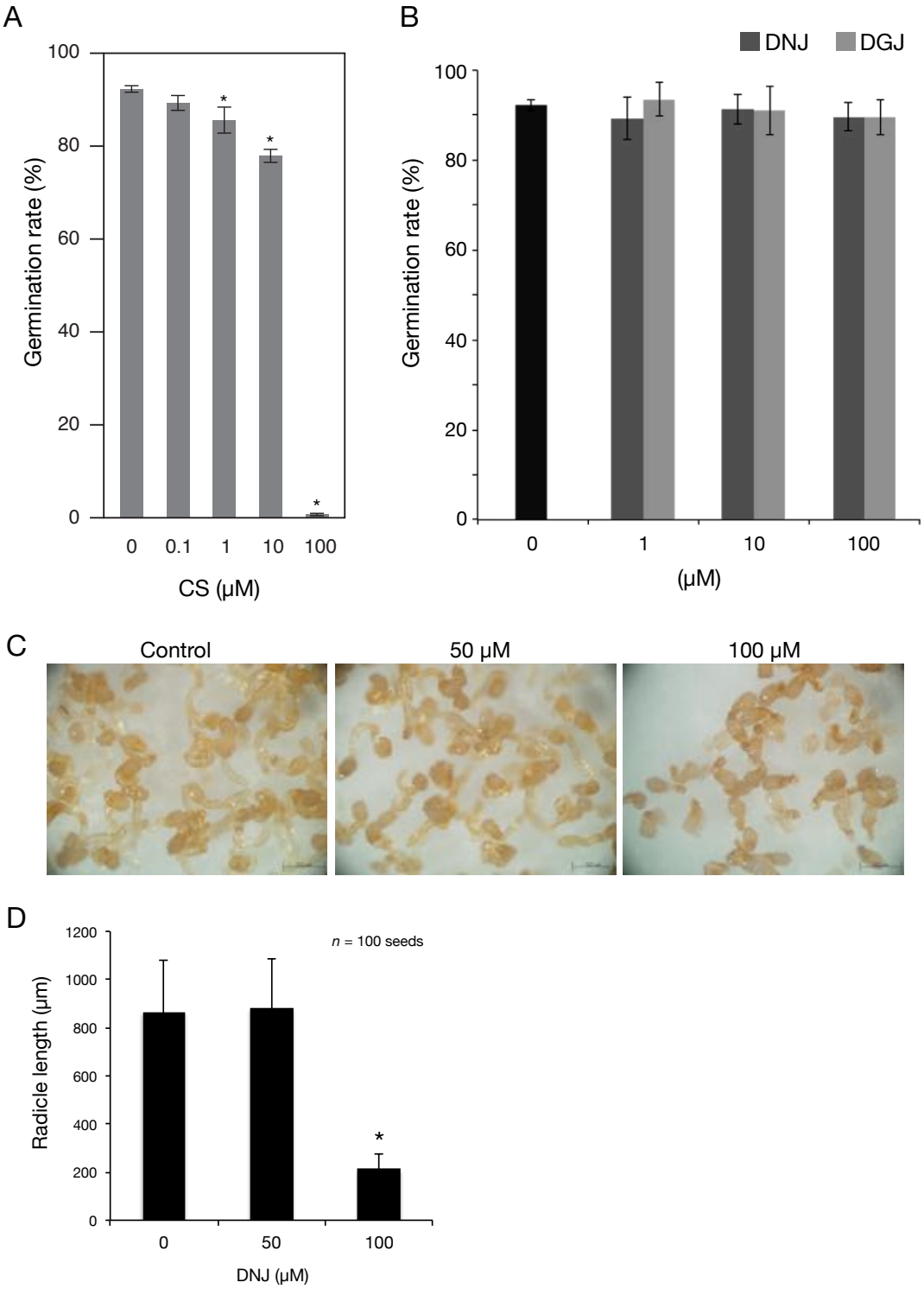

Figure 11. Effects of glycosidase inhibitors on germination of *O. minor*. Germination rates of *O. minor* seeds in the presence of various concentrations of CS (A) and DNJ and DGJ (B). Number of germinated seeds (out of 200) was counted (mean ± SD, $n = 3$). Asterisks indicate significant differences in the root lengths between control and glycosidase inhibitor-treated seeds ($P < 0.05$, Student's t test). (C, D) Effect of DNJ on radicle elongation of *O. minor* seeds.

Figure 11. (continued) Conditioning period was 7 days; 10 $mg \cdot l^{-1}$ (w/v) GR24 with or without DNJ was applied on the final day of conditioning. Effect of DNJ was observed 7 days after GR24 treatment. (C) Images of control seeds (left), 50 μM DNJ-treated seeds (middle), and 100 μM DNJ-treated seeds (right). Bar: 500 μm. (D) Radicle lengths of *O. minor* treated with DNJ. Radicle lengths of 100 germinating seeds were determined using ImageJ software. The radicle length treated with 100 μM DNJ was approximately one-quarter that of control radicle length. Asterisk indicates significant difference in the root lengths between control and DNJ-treated seeds ($P < 0.05$, Student's *t* test).

3-3-2. *Effect of nojirimycin on planteose metabolism in* O. minor

I speculated that NJ caused changes in sugar metabolism in *O. minor* seeds, ultimately inhibiting their germination. To investigate the MOA of NJ, I quantified the sugars in germinating *O. minor* seeds in the presence of NJ. In seeds at 3 DAG, the sugar compositions were almost same both in non-treated and NJ-treated seeds. However, compared with non-treated seeds at 7 DAG, the NJ-treated seeds at 7 DAG showed increased levels of sucrose and severely decreased levels of glucose and fructose (Fig. 12). The amounts of glucose and fructose in NJ-treated seeds were about one-fourth of those in non-treated seeds, and, the amounts of sucrose and planteose in NJ-treated seeds were about hundred and five times higher than those in non-treated seeds, respectively.

To investigate the relationship between the inhibitory effect of NJ on germination and sugar metabolism in *O. minor* seeds, I applied exogenous sugars or a nucleotide sugar together with GR24 and NJ. In plants, sucrose degradation is catalyzed by INVs (EC3.2.1.26, sucrose ↔ glucose + fructose) and sucrose synthases (SUSs) (EC2.4.1.13, sucrose + UDP ↔ UDP-glucose + fructose). To

determine whether the restricted supply of sucrose degradation products affects germination, I added UDP-glucose or the constituent sugars of planteose. The germination rate was significantly recovered to 71.2% by addition of glucose with NJ (Fig. 13). Exogenous galactose also recovered the germination rate of the NJ-treated seeds to 13.2%, but with a much lower efficiency than that of glucose. Fructose and UDP-glucose did not recover the germination rate of NJ-treated seeds. Sucrose recovered the germination rate to 4.8%, however, the degree of the recovery was significantly lower than that of glucose or galactose.

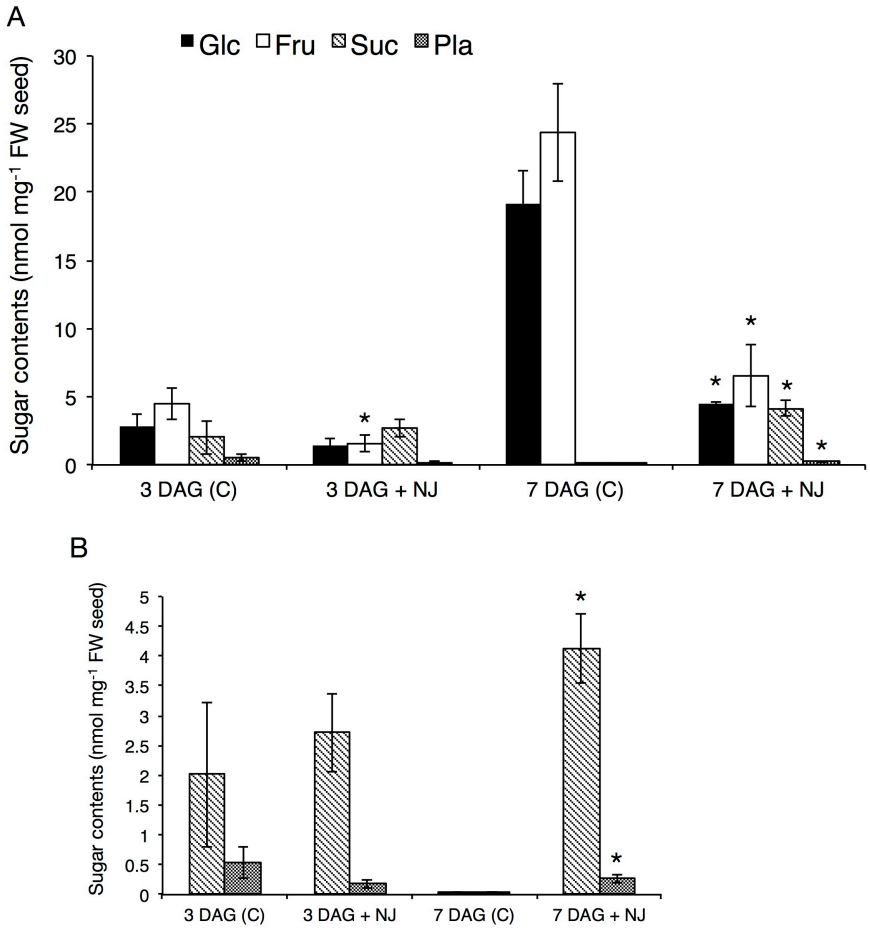

Figure 12. Changes in sugar contents in *O. minor* seeds in the presence of NJ. (A) Amounts of sugars in seeds at 3 days after GR24 treatment (DAG) and at 7 DAG with (+NJ) or without (C: control) NJ treatment (mean ± SD, n = 3).

Figure 12. (continued) NJ (10 μM) was added along with GR24 after the conditioning period. (B) Magnification of graph showing amounts of sucrose and planteose. Asterisks indicate significant differences in amounts of sugars between control and NJ-treated seeds on the same day ($P < 0.05$, Student's *t* test). Glc, glucose; Fru, fructose; Suc, sucrose; Pla, planteose.

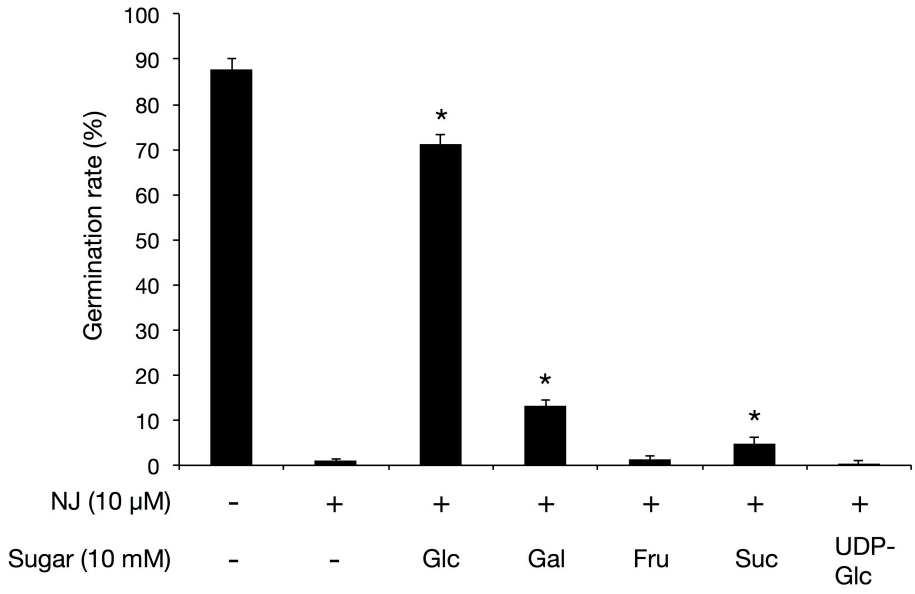

Figure 13. Recovery of seed germination rate by simultaneous addition of exogenous sugars and NJ. Seed germination rates were measured at 7 DAG (mean ± SD, $n = 3$). Asterisks indicate significant differences in germination rates between seeds treated with NJ and those treated with NJ + exogenous sugars ($P < 0.05$, Student's *t* test). Glc, glucose; Fru, fructose; Gal, galactose; Suc, sucrose; Pla, planteose; UDP-Glc, UDP-glucose.

3-3-3. *Effect of nojirimycin on activities of invertases*

The results described above indicated that the inhibition of germination of *O. minor* by NJ is caused by a lack of adequate supply of glucose. Therefore, the inhibition might be attributed to suppression of activities of INVs in the planteose metabolic

pathway. In plants, INVs can be classified into three types according to their solubilities, subcellular localizations, pH optima, and isoelectric points. SAIs localize in the vacuoles, SNIs localize in the cytosol, and CWIs are in the cell walls. These types of INVs were prepared as crude enzymes from germinating seeds of *O. minor* at 5 DAG. When NJ was added to the enzyme assays, it did not affect the activities of these INVs, even at 100 μM (Fig. 14A).

Next, I investigated the changes in the activities of INVs over time during germination, and the effects of NJ on them. I prepared crude enzyme extracts from germinating seeds and NJ-treated seeds. In the germinating seeds, the activities of INVs increased during germination (Fig.14B, solid line). The activities of INVs also gradually increased in the NJ-treated seeds, but they were significantly lower in the NJ-treated than in the non-treated seeds at 5 DAG (Fig. 14B, dashed line). The activities of acid INVs (SAI and CWI) in the NJ-treated seeds were about one-quarter those in the germinating seeds. The activity of SNI was less affected by NJ treatment, and the activity in the NJ-treated seeds was reduced by about 60% compared to that in the non-treated germinating seeds. On the other hand, comparable level of activity of α-galactosidase, which may catalyze the first step of planteose degradation, was observed in both non-treated and NJ-treated seeds at 5 DAG (Fig. 14C).

Activities of acid INVs can be regulated by invertase inhibitors (INHs), and INHs have been studied in some plant species (Weil *et al*., 1994; Link *et al.*, 2004; Privat *et al*., 2008; Reca *et al*., 2008; Tauzin *et al*., 2014). To test the possibility that NJ induces INHs causing the decrement of acid INV activities, the INV activities in

the mixture of equal amounts of crude proteins prepared from the germinating (10 μg) seeds and the NJ-treated seeds (10 μg) were measured. However, the activities did not decrease by addition of crude proteins prepared from the NJ-treated seeds (Fig. 14D). As described above, exogenous glucose recovered the germination of NJ-treated seeds. Therefore, INV activities in the NJ-treated seeds with exogenous glucose were evaluated (Fig. 14E). The acid INV activities in NJ-treated seeds with glucose were higher than those in the NJ-treated seeds without glucose, suggesting that exogenous glucose recovered acid INV activities.

Furthermore, I investigated gene expression levels of INVs during germination in the presence of NJ by semi-quantitative RT-PCR. The primers used for RT-PCR analysis were designed based on the sequences found in our in-house EST library of *O. minor*, however I could not prepare a suitable housekeeping gene to standardize the gene expression levels. The results showed that the expression levels of all INV genes of *O. minor* (*OmSAI*, *OmCWI* and *OmSNI*) were not significantly changed through the germination by NJ treatment (Fig. 14F).

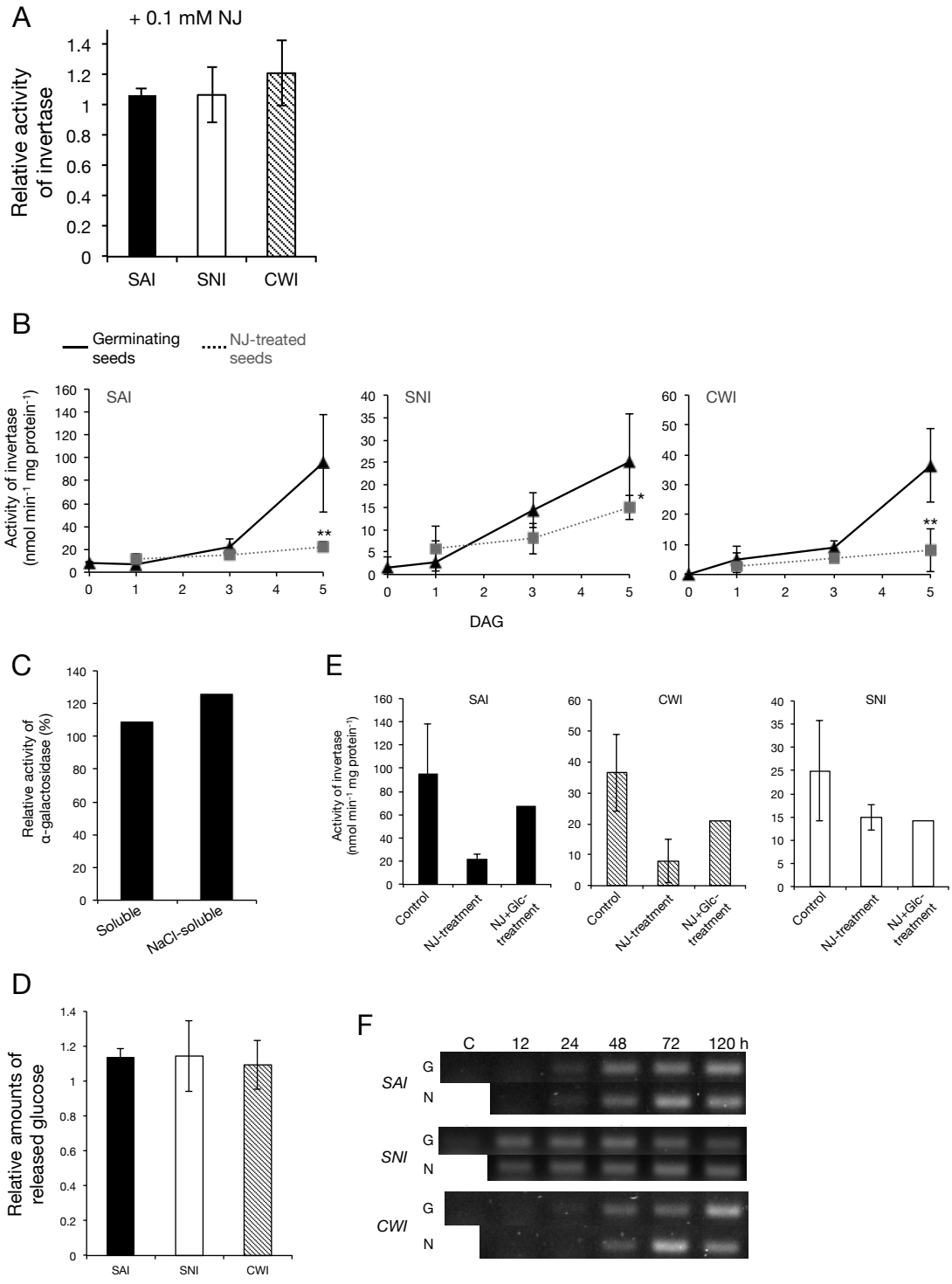

Figure 14. Effects of NJ on activities of INVs. (A) Effects of NJ on activities of INVs *in vitro*. A reaction mixture contained crude enzyme extract from germinating seeds at 5 DAG and NJ at a final concentration of 0.1 mM. NJ effect was calculated as a ratio of activity with NJ treatment to that without NJ (mean ± SD, $n = 3$). (B) Effects of NJ on activities of INVs *in vivo*. Crude enzyme extracts were prepared from germinating and 10 μM NJ-treated seeds of *O. minor*. Activities of SAI, SNI, and CWI were assayed.

Figure 14. (continued) Solid and dashed lines show mean values of enzyme activities in the non-treated and the NJ-treated seeds, respectively (mean ± SD, n = 6 to 8). Enzyme activity is expressed as nmol glucose·min^{-1}·mg protein^{-1}. DAG; days after GR24 treatment. Asterisks indicate significant differences in enzyme activities between non-treated and NJ-treated seeds at same time point (* $P < 0.05$, ** $P < 0.01$, Student's t test). (C) Effect of NJ on activity of α-galactosidase *in vivo*. Crude enzyme extracts were prepared from germinating and 10 μM NJ-treated seeds of *O. minor* at 5 DAG. The activity was assayed using a soluble fraction and a NaCl-soluble fraction. The activity was calculated as a ratio of activity in the germinating seeds to that in the NJ-treated seeds. The graph shows mean values (n = 2). (D) The INV activities of the mixture with crude enzymes from the germinating (10 μg) and the NJ-treated seeds (10 μg). The enzyme activity was calculated as a ratio of the amount of released glucose in the mixed enzyme solution to that in the control enzyme solution (10 μg crude protein derived from the germinating seeds) (mean ± SD, n = 3). (E) The activities of INVs in the NJ-treated seeds with or without exogenous glucose *in vivo*. Acid INV activities in the NJ-treated seeds with glucose were higher than those in the NJ-treated seeds without glucose. The graph shows mean ± SD (Control and NJ-treatment) or only mean value (NJ+Glc-treatment). Control, germinating seeds at 5 DAG (n = 6–8); NJ-treatment, NJ-treated seeds at 5 DAG (n = 6); NJ+Glc-treatment, NJ-treated seeds with glucose at 5 DAG (n = 2). (F) RT-PCR analysis of *OmSAI*, *OmSNI* and *OmCWI* in the germinating (G) and the NJ-treated (N) seeds after GR24 treatment (12–120 h after; C, conditioned seeds).

3-3-4. *Effect of nojirimycin on germination of planteose-containing seeds of non-parasitic plants*

The results shown so far indicated that planteose degradation might be involved in the early stage of germination, and that sucrose degradation and the supply of glucose after planteose degradation might be key steps in seed germination of *O. minor*. NJ might inhibit seed germination in *O. minor* by interfering with activities

of INVs, thereby inhibiting the degradation of sucrose in the planteose metabolic pathway. Therefore, NJ may also inhibit germination in seeds of other non-parasitic plants that contain the planteose metabolic pathway. To investigate the effects of NJ on planteose containing seeds, I conducted germination assays and root length measurements using seeds of tomato (*Solanum lycopersicum*), sesame (*Sesamum indicum*), and spearmint (*Mentha spicata*). The germination rates of these seeds were not affected by NJ, even at 1 mM (Fig. 15A). NJ inhibited root elongation of sesame and spearmint, but not tomato (Fig. 15B). However, the concentration of NJ required to inhibit the root elongation was higher for sesame and spearmint than for *S. hermonthica* and *P. japonicum* (Fig 9, Fig 10). Sugar analysis of NJ-treated and non-treated tomato seeds showed that they had the same sugar composition. In the NJ-treated and the non-treated tomato seeds, planteose was degraded, but the amount of sucrose remained constant throughout the germination process (Fig. 15C).

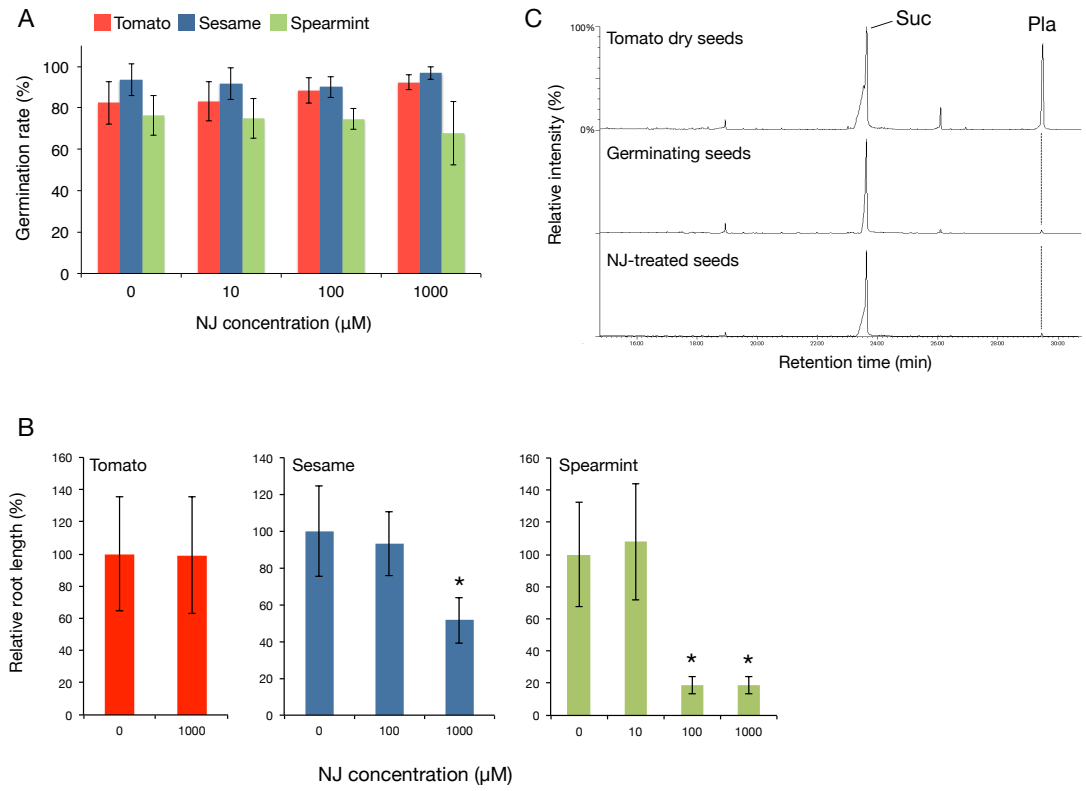

Figure 15. Effects of NJ on germination, root elongation, and planteose metabolism in planteose-containing seeds. (A) Effect of NJ on germination of seeds containing planteose. Number of germinated seeds (out of 20) was counted. Analysis was carried out in triplicate (mean ± SD, $n = 3$). (B) Effect of NJ on root elongation. Germinated seeds were selected and radicle lengths were determined using ImageJ software. Numbers of counted seeds were as follows: 22 (control) and 29 (1 mM NJ) for tomato; 29 (control), 31 (100 μM NJ), and 21 (1 mM NJ) for sesame; and 24 (control), 26 (10 μM NJ), 33 (100 μM NJ), and 33 (1 mM NJ) for spearmint. Asterisks indicate significant differences in the root lengths between control and NJ-treated seeds ($P < 0.05$, Student's t test). (C) GC-MS analyses of sugar compositions in dry, germinating, and NJ-treated tomato seeds. There were no differences in sugar compositions between the non-treated germinated seeds and the NJ-treated seed.

3-4. Discussion

In the NJ-treated *O. minor* seeds at 7 DAG, highly accumulated level of sucrose was observed (Fig. 12), which suggested that NJ might inhibit sucrose degradation. The sum total of sucrose and planteose in the dry seeds (4.2 nmol·mg^{-1} FW seed) was almost equal to the amount of sucrose (4.1 nmol·mg^{-1} FW seed) in the NJ-treated seeds at 7 DAG. This result implied that the accumulated sucrose was a result of the inhibited degradation of the sucrose in the planteose metabolism and of that originally present in the dry seeds. Glucose recovered the germination rate in the presence of NJ but other sugars did not show high activity of germination recovery (Fig. 13), which indicates that glucose is required for seed germination of *O. minor*. Whereas it was concerned that the difference of the extents of germination recovery by addition of different sugars (Fig. 13) was attributed to the difference of efficiencies of uptake of sugars into the seeds, it is thought that the efficiencies are almost equal because applications of different sugars at same concentration (*e.g.*, glucose, galactose and sucrose) delayed Arabidopsis seed germination in a similar way (Dekker *et al.*, 2004). There was significantly less glucose in the NJ-treated *O. minor* seeds than in the non-treated seeds (Fig. 12). This decreased glucose level may have a fatal effect on seed germination. There was also less fructose in NJ-treated seeds than in non-treated seeds. However, exogenous fructose could not fully recover the germination rate (Fig. 13), suggesting that there is a much stronger requirement for glucose than for fructose during *O. minor* seed germination. The weak recovery of the germination rate by sucrose indicated that *O. minor* seeds were unable to metabolize sucrose in the presence of NJ. In addition, galactose recovered

the inhibited germination rate, however, it was not detected in any seed samples. These results suggest that galactose is immediately converted to another compound (e.g., galactose-1-phosphate) in the galactose salvage pathway upon its release (Blöchl, 2007), and the converted compounds involve the promotion of germination.

In general, two classes of enzymes, INVs and SUSs, are involved in sucrose degradation. Both types of enzymes have important roles in plant development at diverse stages (Koch, 2004). Recent studies on *P. ramosa* showed that the transcript levels of INV and SUS genes and the activities of INVs increased in germinating seeds (Draie *et al*., 2011; Péron *et al*., 2012). Therefore, these two classes of enzymes might have important roles in the germination of broomrape seeds. I assume that NJ affects the activities of INVs rather than SUSs because the inhibition of germination was fully recovered only by adding exogenous glucose which is a product of sucrose hydrolysis by INVs, and UDP-glucose, which is a product of sucrose hydrolysis by SUSs, did not recover the germination rate. In *O. minor* seeds, the activities of INVs gradually increased during germination, consistent with the results of previous studies on *P. ramosa* (Draie *et al*., 2011). The activities of INVs were significantly lower in the NJ-treated seeds than in the non-treated seeds at 5 DAG (Fig. 14B). On the other hand, the activity of α-galactosidase at 5 DAG seemed not to be changed in the presence of NJ (Fig. 14B). These results suggest that NJ specifically decreased the activities of INVs. Additionally, the activities of acid INVs at 5 DAG were recovered in the seeds treated with NJ and glucose (Fig. 14E). This recovery effect by glucose implies that the accumulation of glucose in the early stage of germination enhances acid INV activities.

There should be other pathways producing monosaccharides during the late stages of germination because, the amounts of monosaccharides in the seeds at 7 DAG were higher than the estimated amounts produced from sucrose and planteose degradation. Additionally, the monosaccharides were significantly increased from 5 to 7 DAG, whereas sucrose and planteose were almost consumed by 5 DAG (Figs. 6D–G). This increment might indicate the involvement of other pathways to produce monosaccharides (e.g., cell wall polysaccharide and/or fructan). It is known that sugars act as important signaling molecules like phytohormones throughout all stages of plant development (Rolland *et al*., 2006; Eveland and Jackson, 2012). For example as a sugar-inducible carbohydrate related metabolism, a supply of sucrose, glucose or fructose to Arabidopsis plant induces expression of a gene for β-amylase (Mita *et al*., 1995). Because the substantial increase in monosaccharides was not observed in the NJ-treated seeds at 7 DAG (Fig. 12), it is likely that glucose provided from sucrose and planteose triggers the following processes required for germination including the degradation of other storage products.

NJ is an iminosugar, and some other iminosugars have been reported to inhibit seedling growth of some plant species. Seedling growth and root elongation in *Raphanus sativus* (kaiware radish) were inhibited by CS at 0.5 mM or higher, or DNJ at 5 mM or higher (Mega, 2004, 2005). Similarly, DNJ, *N*-butyl DNJ, and miglitol at 0.5 mM inhibited root elongation in germinating barley (Stanley *et al*., 2011). In both studies, the iminosugars did not inhibit seed germination. Additionally, the concentrations of iminosugars used in those studies were higher than those used in the present study (1–100 μM NJ for the inhibition of *O. minor*

germination). At present, the MOA of these iminosugars remains unclear. In the case of *O. minor*, DNJ at 0.1 mM inhibited the radicle elongation, but did not affect the seed germination, as is the case in other plants (Figs. 11B–D). Since NJ inhibited *O. minor* seed germination at a much lower concentration, the inhibitory mechanism of NJ likely differs from those of other iminosugars such as DNJ. CS also inhibited the germination of *O. minor*, but higher concentrations of CS than NJ were required for the inhibitory effect (Fig. 11A). This finding suggests that NJ and CS inhibit germination via the same mechanism, but that they have different affinities for their target site(s). NJ did not inhibit seed germination in species other than *O. minor,* and only inhibited the root and radicle elongation of other species at high concentrations. Therefore, NJ may inhibit root elongation of these plants via a similar mechanism to that of other iminosugars.

Aoki and Hatanaka (1991) reported that 1,4-dideoxy-1,4-iminoarabinitol (DIA), which is a class of iminosugar along with NJ, at concentrations of 0.1 to 10 mM inhibited growth of seedlings of rape, lucerne, castor bean, barley, and rice, but had little effect on their seed germination. As in the present study, the activities of SAI and CWI were lower in 0.5 mM DIA-treated lucerne seedlings than in non-treated seedlings. They also reported that DIA did not affect activities of acid INVs *in vitro* as NJ. Furthermore, I revealed that gene expression levels of INVs were not affected by NJ treatment (Fig. 14F). Together with the results of this and previous studies, it is suggested that NJ and DIA affect the activities of INVs indirectly by inhibiting translational or post-translational processes. Recently, heterologous expression of tomato SAI (TIV-1) in *Pichia pastoris* revealed that

N-glycosylation in TIV-1 was important for its activity and stability (Tauzin *et al*., 2014). Considering the role of NJ as a glycosidase inhibitor, NJ may affect glycosylation of INVs in *O. minor*.

NJ specifically inhibited the germination of *O. minor* seeds, but not those of the other tested plants. The plant species could be ranked in terms of their sensitivity to NJ, from the most sensitive to the least sensitive, as follows: *O. minor* (obligate holoparasite), *S. hermonthica* (obligate hemiparasite), *P. japonicum* (facultative hemiparasite), spearmint (Lamiaceae), sesame (Pedaliaceae), and Arabidopsis (Brassicaceae), red clover (Fabaceae) and tomato (Solanaceae) were not affected by NJ treatment (Fig. 9, 10 and 15). Evolutionary events of parasitism are thought to originate in an invasive haustorium formation in the Orobanchaceae (Westwood *et al*., 2010). Facultative hemiparasite species develop lateral haustoria on the sides of their roots. In obligate parasites including *Striga* spp., *Orobanche* spp. and *Phelipanche* spp., an evolutionary event subsequent to the development of lateral haustorium might be evolution of terminal haustorium formation, which develops at the apexes of the radicles soon after germination. Holoparasites evolved from hemiparasites accompanied with loss of plastid genes associated with photosynthesis (Krause, 2008; Westwood *et al*., 2010). Interestingly, the order of sensitivity of tested plants to NJ is the reverse order of the evolutionary process of parasitism (Fig. 16) (Stevens, 2001; Angiosperm Phylogeny Group, 2009; Westwood *et al*., 2010). These results imply that the difference in the sensitivity to NJ among the tested plants is related to the divergence of the way of sugar use during the evolutionary process of parasitism. In other words, sugar metabolism during germination may

differ between broomrapes, parasitic plants in the Orobanchaceae, and non-parasitic plants. In fact, sugar metabolism in germinating seeds of *S. hermonthica* may slightly differ from that in germinating seeds of broomrapes because there was less planteose in *S. hermonthica* seeds than in the broomrape seeds (Fig. 8) In tomato, the sucrose content in seeds did not change during germination (Fig. 15C), while the sucrose content in seeds of root parasitic weeds decreased. The differences in sugar use during germination of root parasitic weeds in the Orobanchaceae may be attributed to their ability to photosynthesize because hemiparasites are capable of fixing carbon themselves, while holoparasites obtain all of their reduced carbon from their hosts (Press *et al*.,1991; Irving and Cameron 2009). Since NJ inhibits only the germination of *O*. *minor,* some physiological events during its germination that are targeted by NJ may have arisen during evolution. Comparing the effects of NJ among a wide variety of plant species will clarify the relationship between NJ inhibition and the evolution of parasitism.

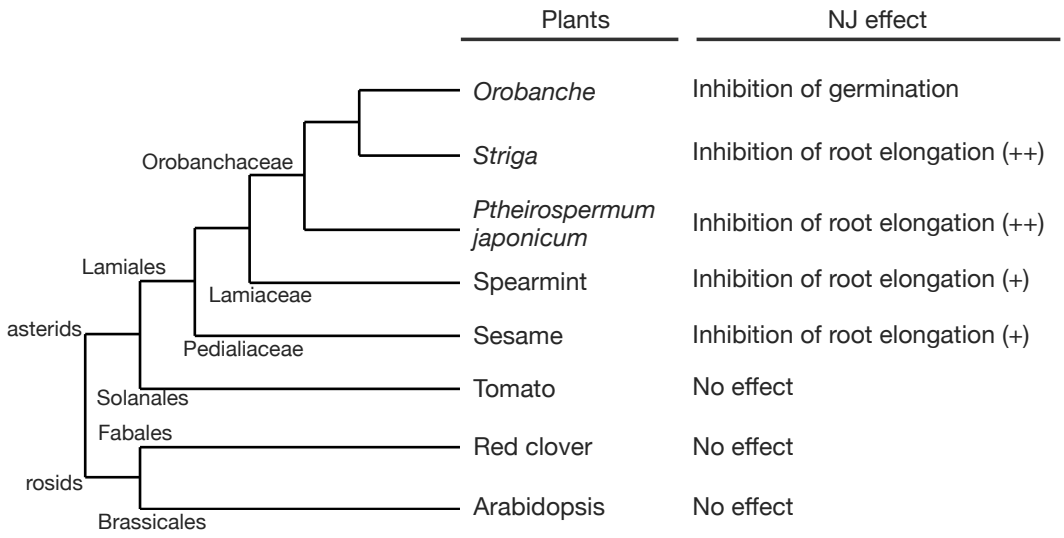

Figure 16. Relationship between plant evolution and NJ effect. The order and family were classified based on Angiosperm Phylogeny Group III system (Angiosperm Phylogeny Group, 2009), and parasitic plants in the Orobanchaceae were assigned by proposed evolutional process (Westwood *et al*., 2010). The plant species is closer to *O. minor*, the stronger the effect of NJ appeared. (++) indicates strong inhibitory effect; (+) indicates weak inhibitory effect.

Chapter 4 *De novo* assembly of transcriptome in *O. minor* germinating seeds and analysis of differential gene expression in the NJ treated seeds

4-1. Introduction

Transcriptome analysis is used to understand a genome-wide changes in gene expression in a wide range of biological processes. Next-generation sequencing (NGS) technology allows us to perform cost-effective genomic sequencing (DNA-Seq) and transcriptome sequencing (RNA-Seq) at an unprecedented high speed. RNA-Seq using the feature of NGS is a powerful way to survey gene expression levels with low background noise and large dynamic range (Wang *et al*., 2009). Another attractive advantage of RNA-Seq is that RNA-Seq can be utilized to functional genomics research in non-model organisms whose genomic sequences have not been fully determined by *de novo* transcriptome assembly (Haas and Zody, 2010; Surget-Groba and Montoya-Burgos, 2010; Martin and Wang, 2011; Zhao *et al.*, 2011). Therefore, RNA-Seq and *de novo* transcriptome assembly have been applied to several non-model plant species in order to characterize gene expression changes during plant development (Feng *et al.*, 2012; Alkio *et al.*, 2014), in response to biotic stress and pathogen infection (Rubio *et al.*, 2014; Yates *et al*., 2014), or to reveal specific genes related to biosyntheses of plant specialized metabolites (Liu *et al*., 2013; Jung *et al*., 2014).

The Parasitic Plant Genome Project (PPGP; http:// ppgp.huck.psu.edu/) has provided transcriptomic data, obtained by NGS technology, of key developmental

stages from seeds conditioning to anthesis in *S. hermonthica* and *P. aegyptiaca* to understand genetic changes associated with parasitism, and to contribute to the control of the parasitic weeds (Westwood *et al*., 2010; 2012). Moreover, mechanism of host plant–parasitic plant interaction has gradually become clear recently at genetic level through the *de novo* assembly of parasitic plant transcriptomes (Honaas *et al*., 2013; Ranjan *et al*., 2014). Thus, RNA-Seq analysis enables us to address a key question about parasitic plant evolution and mechanisms of unique developmental processes.

The planteose metabolism in early stage of germination of *O. minor* seeds was shown as a promising target for selective control of root parasitic weeds in the Orobanchaceae as described in Chapters 2 and 3. In this study, *de novo* assembly of transcriptome in *O. minor* seeds at an early stage of germination process and the subsequent differential gene expression analysis between the germinating seeds and the NJ-treated seeds were performed to understand the inhibitory mechanism of NJ against *O. minor* seeds at molecular level, which is helpful in establishing the control method targeting the planteose metabolism. Moreover, genes that affected by NJ treatment should be important for germination of *O. minor*, therefore, physiological events associated with functions of these genes might be other potential targets for the selective control.

4-2. Materials and methods

4-2-1. *Plant materials and purification of total RNA*

Seeds of *O. minor* were prepared as described in Chapter 2, 2-2-1. NJ-treated seeds

were prepared by addition of GR24 solution (1.0 mg·l^{-1}) with NJ (10 μM) at the final day of conditioning. The conditioned seeds, germinating seeds and NJ-treated seeds (0.5, 3, 24, 48 h after GR24 treatment) were collected. Total RNA was extracted as described in Chapter 3, 3-2-6. More than 20 μg of total RNA was pooled and kept at –80°C.

4-2-2. *RNA sequencing*

RNA-Seq was performed by HiSeq2000 (Illumina, San Diego, CA) at BGI (Beijing, China) and paired-end reads (2×90 bp in length) were obtained. The RNA-Seq data were provided as FASTQ format.

4-2-3. De novo *transcriptome assembly*

The Trinity software package (version r2013-02-25) was used for the construction of a reference transcriptome (Grabherr *et al.*, 2011; Haas *et al.*, 2013). Transcriptome assembly was performed via DNA Data Bank Japan (DDBJ) Read Annotation Pipeline (Nagasaki *et al*., 2013). The command line used for assembly was Trinity.pl --seqType fq --JM 160G --bflyHeapSpaceMax 20G --bflyGCThreads 1 --CPU 8 --left read_1.fastq --right read_2.fastq --output output_dir --min_contig_length 201. Subsequently, CD-HIT-EST with sequence similarity threshold of 95% was used to remove the redundant contigs (Huang *et al.*, 2010).

4-2-4. *Differential gene expression analysis*

Each read was aligned to the reference transcriptome by Bowtie (v 1.1.1) using

following command line, bowtie -aS -X 800 --offrate 1 --phred64-quals reference transcripts -1 left read_1.fastq -2 right read_2.fastq (Langmead *et al.*, 2009). Subsequently, alignment results obtained as Sequence Alignment/Map (SAM) format were converted to Binary Alignment/Map (BAM) format using SAMtools (Li *et al*., 2009).

Differential gene expression analysis was carried out using TCC, an R package (Sun *et al.*, 2013). The raw read counts were normalized with the iDEGES/DESeq method, and pairwise comparisons of gene expression were conducted with the DESeq program in R, which corresponds to the iDEGES/DESeq-DESeq pipeline in the previous report (Sun *et al.*, 2013) to analyze two-group count data without replicates. The differentially expressed genes were identified with a false discovery rate (FDR) threshold of 0.05.

4-2-5. *Functional annotation of the differentially expressed contigs*

The contigs that were determined as differentially expressed were compared to the National Center for Biotechnology Information (NCBI) non-redundant (nr) database using BLASTX with an e-value threshold of 1e-3.

4-3. Results

4-3-1. De novo *assembly of RNA-Seq data*

The scheme of *de novo* assembly and following analyses was described in Figure 17. Total RNAs from *O. minor* seeds at after conditioning (conditioned seeds), 0.5 h, 3 h, 24 h and 48 h after GR24 treatment, and 0.5 h, 3 h, 24 h and 48 h after GR24 with

NJ treatment were extracted and purified. RNA-Seq was conducted and libraries each containing about 50 million clean paired-end reads (90 bp) with a high Q20 percentage were obtained (Table 2).

Table 2. Summary of RNA-Seq data.

Sample Name	Total Reads	Total bases	Q20 (%)	GC (%)
Conditioning	48,828,104	4,393,809,360	98.18	47.24
GR0.5h	51,977,034	4,677,933,060	96.88	45.64
GR3h	51,683,432	4,651,508,880	96.82	45.8
GR24h	50,768,506	4,569,165,540	97.14	44.88
GR48h	484,469,10	4,360,221,900	98.25	46.9
NJ0.5h	51,394,680	4,625,521,200	96.96	45.7
NJ3h	51,634,450	4,647,100,500	96.82	45.69
NJ24h	50,140,132	4,512,611,880	97.09	45.03
NJ48h	48,478,574	4,363,071,660	99.12	45.43

A reference genome or transcriptome sequence of *O. minor* was not available therefore *de novo* assembly with Trinity software package (Grabherr *et al.*, 2011; Haas *et al.*, 2013) was utilized to generate a reference transcriptome sequence. A total of 97,275,014 reads from libraries of conditioned seeds and seeds 48 h after GR24 treatment were *de novo* assembled then a total of 119,181 contigs (length > 200 bp) with N50 of 1.5 kb, and an average length of 951 bp were obtained (Om-seed-transcripts). Subsequently, in order to reduce redundant contigs contained in Om-seed-transcripts, CD-HIT-EST software (Huand *et al.*, 2010) was used with a sequence similarity threshold of 95 %, then a total of 105,904 contigs with N50 of 1.4 kb, and an average length of 868 bp were obtained (Om-seed-'CD-HIT-EST'-transcripts) (Table 3).

Table 3. Assembly statistics of transcriptome of *O. minor* seeds.

Parameters	Om-seed	Om-seed-'CD-HIT-EST'
Total length (Mb)	113	92
Number of contigs	119,181	105,904
Average length (bp)	952	868
Median length (bp)	600	525
Max length (bp)	13,486	13,486
Min length (bp)	202	202
N50 (bp)	1,542	1,422

4-3-2. *Analysis of differential gene expression in NJ-treated* O. minor *seeds*

As described in Chapters 2 and 3, NJ has the selective inhibitory effect on germination of *O. minor* affecting the metabolism in the early stage of germination. To reveal the molecular mechanism of the inhibitory effect of NJ, and to investigate the essential gene expression for the germination, short reads from the GR24-treated and the NJ-treated seed libraries were aligned to the reference transcriptome by Bowtie software (Langmead *et al.*, 2009). Tag count data obtained from mapping reads with Bowtie was used for identification of differentially expressed contigs by iDEGES/DESeq pipeline in TCC, an R package (Sun *et al.*, 2013). The differential gene expression analysis was conducted by comparing the tag count data of the GR24-treated and NJ-treated seed samples at the same time point using FDR < 0.05 as a cutoff value (Fig. 17).

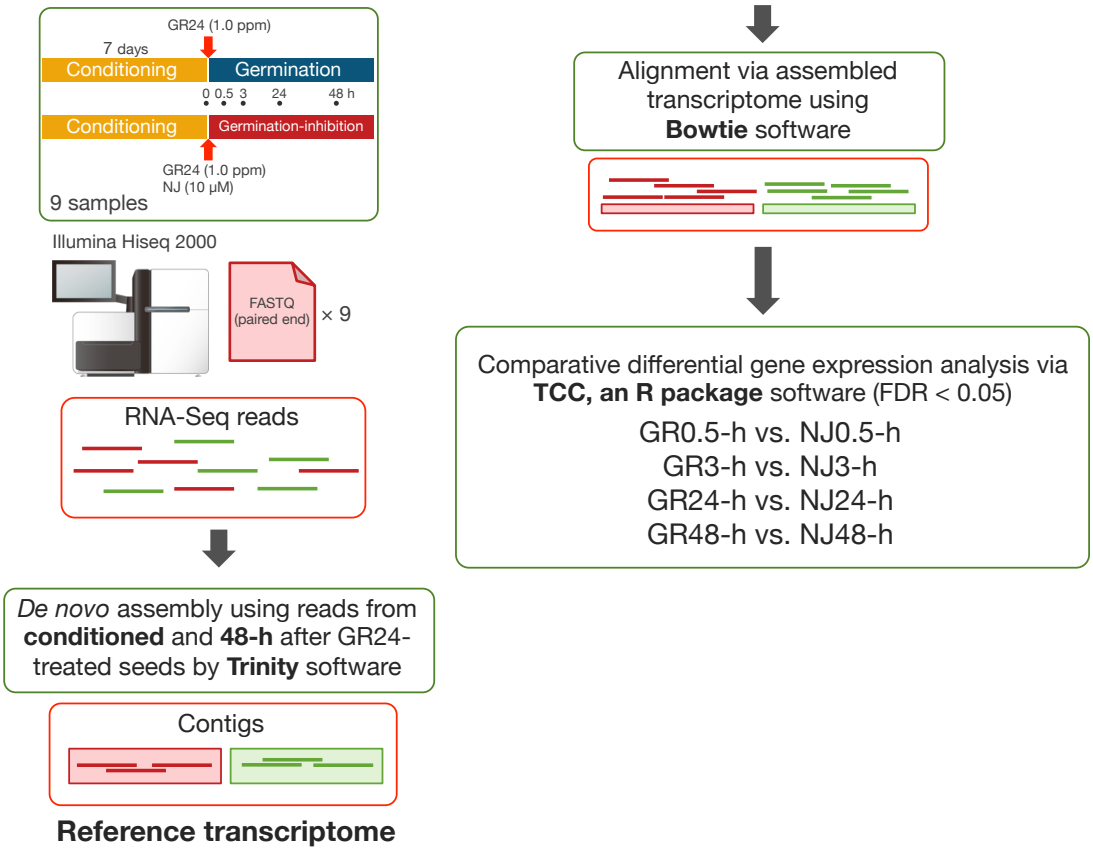

Figure 17. Scheme of *de novo* assembly and differential gene expression analysis.

The differentially expressed contigs were queried against the NCBI nr database with the BLASTX algorithm with an e-value cutoff of 1e-3. Consequently, 54 contigs among the contigs down-regulated significantly by NJ treatment and 24 contigs among the up-regulated ones were annotated (Table 4). As listed in Table 4, expressions of contigs encoding sugar transporters [hexose transporters and SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER (SWEET) proteins] were affected by NJ. These expressions were down-regulated or disrupt. For instance, expression of *Hexose transporter 1* was up-regulated 0.5 h after NJ treatment followed by down-regulation after 3 h. Other sugar metabolism-related contigs differently expressed in NJ-treated seeds included plant invertase/pectin methylesterase inhibitor superfamily proteins, β-1,4-xylosyltransferase IRX 14-like

protein, and β-amylase chloroplastic-like protein. In addition, the expressions of contigs encoding kinase (receptor-like cytosolic serine/threonine-protein kinases, LRR receptor-like serine/threonine-protein kinase, and CBL-interacting serine/threonine-protein kinase 23 isoform X1) and phosphatases (protein phosphatase 2C 29-like, and probable protein phosphatase 2C 63), which may be involved in signal transduction, were affected by NJ treatment. Notably, it was revealed that the expression of kinases promptly decreased in response to germination stimulant 3 h after GR24 treatment in the presence of NJ (Table 4).

Table 4. List of differentially expressed contigs in the NJ-treated seeds.

Down-regulated				
Contig ID	Time point	Length (bp)	Log (fold change)	Gene annotation
comp65741_c0_seq1	0.5 h (24 h-down, 48 h-up)	640	-2.817	Ycf1, partial
comp63347_c0_seq1	0.5 h (24 h-down, 48 h-up)	2441	-1.241	Ycf1, partial
comp67108_c7_seq21	0.5 h	3672	-0.985	Cytochrome oxidase subunit 1, partial (mitochondrion)
comp67108_c5_seq1	0.5 h	2145	-0.935	ATPase subunit 1 (mitochondrion)
comp61840_c0_seq1	0.5 h (24 h-down, 48 h-up)	351	-0.743	Ycf1 (chloroplast)
comp68209_c0_seq1	0.5 h	956	-0.683	Thioredoxin
comp167704_c0_seq1	3 h	394	-5.332	Receptor-like cytosolic serine/threonine-protein kinase RBK2
comp232524_c0_seq1	3 h	918	-5.182	Receptor-like cytosolic serine/threonine-protein kinase RBK2
comp67927_c3_seq78	3 h	701	-4.991	Probable LRR receptor-like serine/threonine-protein kinase At1g07650
comp46787_c0_seq1	3 h (0.5 h-up)	1180	-3.165	Axoneme-associated protein mst101 like
comp33755_c0_seq1	3 h (0.5 h-up)	899	-2.634	21-kDa protein, Plant invertase/pectin methylesterase inhibitor superfamily protein
comp34798_c0_seq1	3 h (0.5 h-up)	2566	-2.430	COBRA-like protein 7
comp55520_c0_seq1	3 h	1283	-2.233	Nucleic acid binding protein

Table 4. (continued)

comp68818_c0_seq1	3 h (0.5 h-up)	1839	-2.184	Hexose transporter 1
comp66368_c2_seq2	3 h	907	-2.166	GAG-protease-integrase-RT-R polyprotein
comp46528_c1_seq1	3 h	1019	-1.991	Dehydrin
comp66762_c1_seq66	3 h	749	-1.917	Phosphatidylserine synthase
comp70500_c0_seq1	3 h	407	-1.640	CDP-diacylglycerol—glycerol-3 -phosphate 3, partial
comp67904_c0_seq9	3 h	1563	-1.511	NAD-malic enzyme
comp64195_c0_seq6	3 h	1004	-1.289	Ribosomal RNA processing protein 36 homologue
comp56494_c1_seq2	3 h	1623	-1.217	GATA transcription factor 8-like
comp67639_c0_seq1	3 h	2272	-1.145	CBL-interacting serine/threonine-protein kinase 23 isoform X1
comp65305_c0_seq6	3 h (0.5 h-up)	1699	-1.092	Protein notum homologue
comp66745_c1_seq3	3 h	992	-1.066	Bidirectional sugar transporter SWEET7-like
comp66991_c0_seq14	3 h	1664	-1.049	HNH endonuclease domain-containing protein
comp71418_c0_seq1	3 h	1955	-1.024	beta-1,4-xylosyltransferase IRX 14-like
comp34670_c0_seq1	3 h	5257	-0.993	Myosin heavy chain family protein
comp67895_c0_seq11	3 h	745	-0.950	Protein phosphatase 2C 29-like
comp68092_c0_seq68	3 h	1381	-0.948	Phospholipase D
comp66650_c2_seq20	3 h	1623	-0.855	Peroxisomal fatty acid beta-oxidation multifunctional protein AIM1-like
comp33690_c0_seq1	3 h	1516	-0.814	Activating signal cointegrator 1
comp46425_c0_seq1	3 h	785	-0.784	Glutathione s-transferase
comp34256_c0_seq1	3 h	1886	-0.738	NRT1 PTR family protein
comp67192_c1_seq3	3 h	1612	-0.733	GRF-interacting factor 3-like

Table 4. (continued)

comp63809_c1_seq1	3 h	1180	-0.707	Dead-box ATP-dependent RNA helicase 46-like
comp69983_c0_seq1	3 h	1999	-0.686	Transcription initiation factor TFIID subunit isoform 1
comp36170_c0_seq2	3 h	1615	-0.682	Annexin D5-like
comp47044_c0_seq1	3 h	4025	-0.655	T-complex protein 11-like
comp65468_c1_seq2	3 h	1797	-0.643	NADAP-malic enzyme
comp65741_c0_seq1	24 h (0.5 h-down, 48 h-up)	640	-2.313	Ycf1, partial
comp60645_c1_seq1	24h	968	-1.616	U-box domain-containing protein 19-like
comp67258_c0_seq1	24h	888	-1.448	Histone-like
comp34108_c0_seq1	24h	1150	-1.330	Protein exordium-like 2
comp68369_c0_seq1	24h	1085	-1.089	Translocator homologue
comp63347_c0_seq1	24 h (0.5 h-down, 48 h-up)	2441	-0.974	Ycf1, partial
comp68183_c0_seq1	24h	1203	-0.938	Maturation protein pPM2
comp45446_c1_seq1	24h	1273	-0.869	Probable BOI-related E3 ubiquitin-protein ligase 3
comp69179_c0_seq1	24h	1497	-0.857	NINJA-family protein AFP3-like
comp61840_c0_seq1	24 h (0.5 h-down, 48 h-up)	351	-0.663	Ycf1 (chloroplast)
comp34638_c0_seq1	24h	3401	-0.644	F-box protein PP2-A15-like
comp60668_c0_seq1	48h	566	-7.021	MLP-like protein 328
comp65763_c1_seq1	48h	1480	-6.164	Methionine gamma-lyase-like
comp82286_c0_seq1	48h	674	-6.139	21-kDa protein, Plant invertase/pectin methylesterase inhibitor superfamily protein

Table 4. (continued)

comp35210_c0_seq1	48h	970	-6.034	Bidirectional sugar transporter SWEET12-like
comp62700_c2_seq1	48h	1560	-6.034	Beta-amylase chloroplastic-like
comp77546_c0_seq1	48h	1576	-2.818	Basic 7s globulin-like
comp68114_c0_seq1	48h	887	-2.429	40s ribosomal protein s30

UP-regulated				
Contig ID	Time point	Length (bp)	Log (fold change)	Gene annotation
comp46787_c0_seq1	0.5 h (3 h-down)	1180	3.259	Axoneme-associated protein mst101 like
comp45573_c0_seq1	0.5 h	699	3.200	Nudix hydrolase mitochondrial-like
comp34798_c0_seq1	0.5 h (3 h-down)	2566	2.842	COBRA-like protein 7
comp33755_c0_seq1	0.5 h (3 h-down)	899	2.782	21-kDa protein, Plant invertase/pectin methylesterase inhibitor superfamily protein
comp68818_c0_seq1	0.5 h (3 h-down)	1839	2.565	Hexose transporter 1
comp59432_c0_seq2	0.5 h	1440	2.552	Probable galacturonosyltransferase
comp65474_c0_seq3	0.5 h	1502	2.387	F-box protein At1g30790-like
comp70283_c0_seq1	0.5 h	627	2.061	Probable calcium-binding protein CML
comp67089_c1_seq3	0.5 h	1834	1.712	Nuclease HARBI 1
comp65219_c0_seq4	0.5 h	1317	1.627	Probable protein phosphatase 2C 63
comp64688_c0_seq3	0.5 h	250	1.120	Polyubiquitin-like protein
comp65305_c0_seq6	0.5 h (3 h-down)	1699	1.108	Protein notum homologue
comp55520_c0_seq1	0.5 h	1283	1.073	Nucleic acid binding isoform 2
comp70252_c0_seq1	0.5 h	2245	0.856	Probable transcription factor PosF21
comp46435_c0_seq2	0.5 h (3 h-up)	644	0.806	60s ribosomal protein L39
comp66369_c0_seq11	3 h (48 h-up)	282	1.182	Probable histone

Table 4. (continued)

comp46435_c0_seq2	3 h (0.5 h-up)	644	0.942	60s ribosomal protein L39
comp64239_c0_seq3	24 h	2111	0.754	Glycine dehydrogenase
comp68856_c0_seq1	24 h	676	0.675	LYR motif-containing protein
comp65741_c0_seq1	48 h (0.5 h and 24 h-down)	640	6.470	Ycf1, partial
comp61150_c0_seq2	48 h	338	6.422	Acetyl-CoA carboxylase carboxyltransferase beta subunit (chloroplast)
comp63347_c0_seq1	48 h (0.5 h and 24 h-down)	2441	3.189	Ycf1, partial
comp54688_c0_seq1	48 h	223	3.149	UDP-glycosyltransferase 86A1-like
comp61593_c3_seq1	48 h	1042	3.066	Ribosomal protein S14 (chloroplast)
comp61840_c0_seq1	48 h (0.5 h and 24 h-down)	351	2.684	Ycf1 (chloroplast)
comp66369_c0_seq11	48 h (3 h-up)	282	2.585	Probable histone

4-4. Discussion

The NGS technology contributes considerably to discover the genes regulating growth and development in non-model plants. In this chapter, a comprehensive transcriptome was built from RNA-Seq data of seeds of *O. minor* after conditioning and 48 h after GR24 treatment. The contig N50 is the length of the smallest contig in the set that contains the fewest (largest) contigs whose combined length represents at least 50% of the assembly (Miller *et al.*, 2010). The assembled reference transcriptome showed the remarkably higher N50, achieving 1,422, and the average length (Table 3) than those of the published unigene data of conditioned seeds (OrAe0GB1) and germinated seeds (OrAe0GB1) of *P. aegyptiaca* (obtained from PPGP; http:// ppgp.huck.psu.edu/), therefore, I established the comprehensive data of substantially longer transcripts in broomrape seeds, which is helpful for obtaining open reading frames and functional annotations (Table 5).

Table 5. Assembly statistics of transcriptome of *P. aegyptiaca* conditioned (OrAe0GB1) and germinated (OrAe1GB1) seeds obtained from PPGP.

Parameters	OrAe0GB1	OrAe1GB1
Total length (Mb)	28	40
Number of contigs	72,042	121,129
Average length (bp)	384	326
Median length (bp)	285	266
Max length (bp)	5,295	3,704
Min length (bp)	200	84
N50 (bp)	397	318

Previous transcriptomics with NGS technology in seeds of root parasitic weeds in the Orobanchaceae used completely germinated seeds (Westwood *et al.*, 2012). The assembled reference transcriptome in this study reflects the information of gene expression during a very short term after GR24 treatment including strigolactone-responsive genes associated with transition from conditioning to germination process. Moreover, it was considered that a comparative analysis of gene expression with the germinating and the NJ-treated seeds is useful to clarify key regulatory genes for the germination because NJ has the selective and strong inhibitory activity against the seed germination, which may result from inhibition of the specific germination mechanisms in the root parasitic weeds. The candidate genes involved in the seed germination of *O. minor* was found in the differentially expressed contigs in the NJ-treated seeds. It was predicted that expression of sugar metabolism related contigs should be changed because NJ interfered with sugar metabolism resulting inhibition of the germination as described in Chapter 3. NJ altered the expression of contigs encoding sugar transporters, plant invertase/pectin methylesterase inhibitor superfamily proteins and a β-1,4-xylosyltransferase protein as cell wall structure modification enzymes, and a β-amylase as polysaccharide degrading enzyme.

For example, the expression of *SWEET12* homologue significantly decreased in the NJ-treated seeds 48 h after GR24 treatment. A sugar transporter of Arabidopsis, AtSWEET12, localizes to the plasma membrane of the phloem and transports sucrose during apoplastic phloem loading by exporting sucrose from phloem parenchyma cells, and in double mutant plants carrying insertions both in

AtSWEET11 and *1*2 leaf assimilate exudation was reduced leading to increased sugar accumulation in the leaves (Chen *et al*., 2012). The down-regulated expression of *SWEET12* by NJ treatment in *O. minor* seeds may cause the alteration of the sugar metabolism by inhibition of sucrose transport. However, expression level of *AtSWEET12* was low in germinated seeds of Arabidopsis (Chen *et al*., 2012), therefore, the expression profile of *AtSWEET12* homologous gene in seeds of *O. minor* may be a characteristic to the root parasitic weeds and a promising candidate for the control of germination.

In addition, the expressions of contigs encoding some kinases decreased promptly after perception of GR24 in the NJ-treated seeds. The identified kinase showing significantly decreased expression is known as a receptor-like kinase (RLK) or a receptor-like cytosolic kinase (RLCK). The plant RLK was firstly identified in maize (*Zea mays*) (Walker and Zhang, 1990), and subsequently, several RLKs were isolated from Arabidopsis (Chang *et al.*, 1992; Kohorn *et al*., 1992; Walker, 1993). The Arabidopsis genome sequence has revealed a surprisingly extensive array of receptor-like kinase (RLK) genes and it was reveled that Arabidopsis contained more than 400 RLKs and 200 RLCKs (Shiu and Bleecker, 2001). Theses RLKs are known to function in various aspects of development and plant defense (Becraft, 2002; Morris and Walker, 2003), however, germination regulating RLKs or RLCKs were not found to the present. The identified RLKs and RLCKs in the NJ-treated seeds may have function as germination inducers, and further intensive study will promote a better understanding of the germination mechanism and contribute to the advancement of germination control.

Finally, further data analysis focusing on time course of transcriptional changes in early germination process of *O. minor* will provide insight into the molecular mechanism such as regulatory mechanisms regulated by strigolactone-responsive genes and detailed sugar use in the characteristic germination process of root parasitic weeds in the Orobanchaceae, and that characteristic mechanisms are expected as the clues for searching other targets for the control.

Chapter 5 General conclusion

Whereas the chemical control of root parasitic weeds in the Orobanchaceae representing use of herbicides showed effectiveness to some extent, it still has difficulty in their selectivity. Systemic herbicides may be effective for control of root parasitic weeds because the herbicides can translocate from hosts to the parasites through the conductive tissue of the parasites directly connected to those of hosts. Systemic herbicide such as EPSPS, ALS inhibitors were used for *Orobanche* and *Phelipanche* control, and systemic herbicide dicamba and hormonal herbicides 2,4-dichlorophenoxyacetic acid were used for *Striga* control (Aly, 2007). However, these are non-selective herbicides, thus, damage to host plants is concerned unless the hosts are herbicide-resistant plants. Additionally, the herbicide treatment to the root parasitic weeds after the aboveground emergence is too late to prevent yield losses of host plants because most of the damage to the hosts has already occurred underground (Eizenberg *et al*., 2006). Thus, regulation of the root parasitic weeds in underground is essential for the effective control in infested areas. In this thesis, a novel target site by chemical control was searched, and the germination process, key developmental stage in underground, was focused on. It was considered that the control method by inhibition of the germination solves the problems described above. Previous results showing involvement of structurally unknown trisaccharide in germination of *O. minor* seeds and selective inhibitory effect of NJ, a glycosidase inhibitor, on the germination indicated the relationship between the NJ inhibitory effect and the trisaccharide metabolism in germination (Joseph *et al.*, unpublished), and the possibility of the trisaccharide metabolism as a target for the selective

control. Therefore, I aimed to elucidate the role of the trisaccharide in germination of *O. minor*, and to evaluate the effect of NJ on the trisaccharide metabolism.

In Chapter 2, the unknown trisaccharide was identified as planteose. The amount of planteose in *O. minor* seeds decreased during germination and the amounts of glucose and fructose, constituent monosaccharide of planteose, increased inversely. This planteose metabolism was conserved in seeds of weedy broomrapes, *O. crenata* and *P. aegyptiaca*, and a witchweed, *S. hermonthica*, suggesting that root parasitic weeds in the Orobanchaceae have common sugar metabolism during germination.

In Chapter 3, inhibitory effect of NJ and other glycosidase inhibitors structurally similar to NJ on germination was studied in detail. NJ showed strong reduction of germination rate of *O. minor* seeds in a dose-dependent manner, however, a compound exhibiting the same effect as NJ was not found among tested inhibitors. The inhibitory effect of NJ on germination was associated with change of sugar metabolism. NJ inhibited the germination by altering sucrose degradation in the planteose metabolic pathway, which caused by suppression of the activities of INVs. Therefor, the importance of planteose metabolism in seed germination of *O. minor* was shown. It was considered that NJ inhibits activities of INVs indirectly because the activities were not inhibited by NJ *in vitro*.

Moreover, inhibitory activity of NJ on germination against other root parasitic weeds in the Orobanchaceae and non-parasitic plants containing planteose was studied. NJ did not inhibited germination other than *O. minor*, but NJ inhibited the radicle or root elongations in some plants. The order of sensitivity of tested plants to

NJ was the reverse order of evolutionary process of parasitism, which suggested that sugar metabolism or a regulatory mechanism of sugar metabolism targeted by NJ is changed according to acquisition of parasitism. Considering the strong inhibitory effect of NJ on germination of *O. minor* and the small effect on non-parasitic plants, the inhibitory site targeted by NJ is a promising target for selective control. Additionally, this target could be applied to other weedy root parasitic weeds, especially broomrapes, because of the similarity of their sugar metabolism during germination, which may be inhibited by NJ treatment. Therefore, chemical control of the planteose metabolism could provide new, specific methods to control the root parasitic weeds.

In Chapter 4, *de novo* assembly of transcriptome in *O. minor* seeds and differential gene expression analysis in the NJ-treated seeds were conducted in order to understand the molecular mechanisms associated with the inhibition of germination by NJ, and to find out other targets that can control the germination. NJ altered the expression of genes encoding sugar transporters, cell wall structure modification enzymes, and polysaccharide degrading enzymes. It was considered that the down-regulated *SWEET12* homologous in the NJ-treated seeds was related to the alteration of sugar compositions caused by NJ treatment because the present result of sugar profile in the NJ-treated *O. minor* seeds was corresponding to the previous result showing the accumulated sucrose level in leaves of the double mutant *atsweet11, 12* in Arabidopsis (Chen *et al*., 2012). Moreover, the result showing that *SWEET12* is expressed in seeds of *O. minor* but low in seeds of

Arabidopsis indicates the importance of this sucrose transporter in seeds of the root parasitic weeds, and a possibility of SWEET12 as a candidate target for the control.

This is the first study showing planteose metabolism in root parasitic weeds in the Orobanchaceae, and selective inhibition of germination of *O. minor* by a chemical targeting the metabolism in the germination process. It is difficult to use NJ itself as a selective herbicide for the root parasitic weeds because NJ is unstable in aqueous solution and its action spectrum may be wide. To develop the selective herbicide, it is necessary to clarify the site of action of NJ, and to establish a simple experimental system which can screen the compounds inhibiting the target site(s) with similar activity to NJ. The actual target site of NJ associated with sucrose degradation in the planteose metabolism is still unknown, but further elucidation of the mechanism of disrupting effect of NJ on sugar metabolism as a possible target for selective chemical control is next challenge to open the way for a new technology in the root parasitic weed control.

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