The Mycoheterotrophic Symbiosis Between Orchids and Mycorrhizal Fungi Possesses Major Components Shared with Mutualistic Plant-Mycorrhizal Symbioses

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Achlorophyllous and early developmental stages of chorophyllous orchids are highly dependent on carbon and other nutrients provided by mycorrhizal fungi, in a nutritional mode termed mycoheterotrophy. Previous findings have implied that some common properties at least partially underlie the mycorrhizal symbioses of mycoheterotrophic orchids and that of autotrophic arbuscular mycorrhizal (AM) plants; however, information about the molecular mechanisms of the relationship between orchids and their mycorrhizal fungi is limited. In this study, we characterized the molecular basis of an orchid-mycorrhizal (OM) symbiosis by analyzing the transcriptome of *Bletilla striata* at an early developmental stage associated with the mycorrhizal fungus *Tulasnella* sp. The essential components required for the establishment of mutual symbioses with AM fungi or rhizobia in most terrestrial plants were identified from the *B. striata* gene set. A cross-species gene complementation analysis showed one of the component genes, calcium and calmodulin-dependent protein kinase gene *CCaMK* in *B. striata*, retains functional characteristics of that in AM plants. The expression analysis revealed the activation of homologs of

AM-related genes during the OM symbiosis. Our results suggest that orchids possess, at least partly, the molecular mechanisms common to AM plants.

Plants form symbioses with a wide range of microorganisms such as mycorrhizal fungi or nitrogen-fixing bacteria to facilitate their acquisition of nutrients (Martin et al. 2017). Arbuscular mycorrhizal (AM) symbiosis is a widespread mutualistic association between land plants and fungi of the subphylum Glomeromycotina (Parniske 2008; Smith and Read 2008; Spatafora et al. 2017), in which the resource exchange basically relies on a reciprocal “fair trade” (Kiers et al. 2011), although there are certainly some exceptions (Imhof et al. 2012). In contrast to this mutual symbiosis, some plants unilaterally depend on mycorrhizal fungi for their nutritional needs, including carbon and other essential nutrients. This parasitic nutritional mode, termed mycoheterotrophy (Leake 1994), occurs in most major groups of land plants, including liverworts, lycophytes, ferns, and angiosperms (Merckx 2012). A wide range of fungi, including species of Glomeromycotina, Ascomycota, and Basidiomycota, is known as the fungal partner for the mycoheterotrophic plants (Merckx 2012).

The family Orchidaceae, which at 25,000 species is one of the largest families of flowering plants (Atwood 1986; Chase et al. 2015), has the most mycoheterotrophic genera of any plant family (Leake 1994; Merckx 2012). Orchids are dependent on carbon, nitrogen, and phosphorus sources provided by mycorrhizal fungi (Cameron et al. 2006, 2007; Kuga et al. 2014), at least during their germination, a characteristic classified as initial mycoheterotrophy (Merckx 2012). Although many initial mycoheterotrophs develop into autotrophic mature plants, some orchids continue to obtain carbon from mycorrhizal fungi throughout their lifecycle (full mycoheterotrophy) or utilize both photosynthesis and mycoheterotrophy at maturity (partial mycoheterotrophy) (Dearnaley et al. 2016; Merckx 2012). Recent studies have provided some evidence that bidirectional transfer of nutrients occurs in adult orchids (Cameron et al. 2006, 2008; Suetsugu et al. 2017), suggesting that the fidelity of fungal partners may enable them to gain long-term benefits (Leake et al. 2008). Among orchid-mycorrhizal (OM) symbioses, the majority of orchids interact with the polyphyletic *Rhizoctonia* fungi that are saprotrophic (Martos et al. 2009; Ogura-Tsujita et al. 2009) or possibly root endophytic fungi

Nucleotide sequence data from the RNA-seq analysis and genome sequencing analysis in this study have been deposited into the DNA Data Bank of Japan Sequence Read Archive under the accession numbers DRA005999 and DRA006009, respectively.

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(Rasmussen and Rasmussen 2007; Selosse and Martos 2014). The initial mycoheterotrophic dependency on *Rhizoctonia* fungi is likely the ancestral state of the Orchidaceae family, and it has been hypothesized that the unique ability of orchids to recruit free-living saprotrophic fungi into novel mycorrhizas may have dramatically expanded their potential habitat and triggered their radiation (Givnish et al. 2015; Merckx 2012; Ogura-Tsujita et al. 2009).

Recent studies have reported that genes related to AM symbiosis such as GRAS-domain transcription factor genes, nodulin-like genes, and the auxin efflux facilitator gene *PIN1* can be activated during the formation of mycoheterotrophic symbioses in the young seedling (protocorm) stage of autotrophic (i.e., initially mycoheterotrophic) and partially mycoheterotrophic orchids (Perotto et al. 2014; Suetsugu et al. 2017; Zhao et al. 2013). Some partially mycoheterotrophic orchids such as *Epipactis* spp. produce fully achlorophyllous variants that can reach almost the same size as their green counterparts due to the ability to obtain carbon from their symbiotic fungi (Selosse and Roy 2009). Because the achlorophyllous variants exhibit an increased dependence on mycorrhizal fungi (Selosse and Roy 2009; Suetsugu et al. 2017), genes upregulated in the mycorrhizal roots of the achlorophyllous variants could be important for the establishment of symbiosis. Our previous research, comparing the root transcriptomes of partially mycoheterotrophic orchid *Epipactis helleborine* and their achlorophyllous mutants, demonstrated that genes required for AM colonization such as genes encoding a glycerol-3-phosphate acyltransferase (*REQUIRED FOR ARBUSCULAR MYCORRHIZA2*) and subtilisin are more highly expressed in the achlorophyllous variants than in green *E. helleborine* individuals (Suetsugu et al. 2017). Moreover, genes related to gibberellin biosynthesis are down-regulated in the achlorophyllous variants, which is notable because gibberellin reduces hyphal colonization and arbuscule formation during AM symbiosis (Takeda et al. 2015). These previous findings imply that the mycorrhizal symbioses of mycoheterotrophic orchids share at least some common properties with AM-forming autotrophic plants (Perotto et al. 2014; Suetsugu et al. 2017); however, our understanding of the molecular basis of the OM symbiosis is limited (Fochi et al. 2017; Perotto et al. 2014; Zhao et al. 2013; Zhao et al. 2014).

In molecular studies of mycorrhizal symbioses, it is well known that a set of common symbiosis genes (CSG) is involved in the signaling pathway for the perception and transduction of diffusible microbial signals during root colonization and nutrient exchange in angiosperms. CSG were initially reported to be required for the establishment of not only rhizobium-legume (RL) symbiosis but also AM symbiosis in leguminous plants (Genre and Russo 2016; Kistner et al. 2005; Stougaard 2001) and, recently, a third symbiosis involving nitrogen-fixing actinorrhizas was also found to require CSG (Genre and Russo 2016). The CSG in the legume *Lotus japonicus* encode the symbiosis receptor-like kinase SYMRK; cation channels CASTOR and POLLUX; nucleoporins NUP85, NUP133, and SEC13 HOMOLOG1 (SEH1) NENA; the calcium and calmodulin-dependent protein kinase CcAMK; and the calcium signal-decoding protein CYCLOPS (Genre and Russo 2016). CSG mutants have a symbiosis-defective phenotype because the early symbiotic signaling pathway is blocked (Groth et al. 2010; Imaizumi-Anraku et al. 2004; Kanamori et al. 2006; Saito et al. 2007; Stracke et al. 2002; Tirichine et al. 2006; Yano et al. 2008). Candidates for orthologs of CSG have been computationally predicted to be present not only in the genomes of a number of dicot and monocot AM plants that form mutualistic symbiosis with AM fungi but also in the charophyte algae, whose ancestral lineage gave rise to the land plants (Delaux et al. 2013, 2014, 2015; Zhu et al. 2006). In contrast, it was reported that non-AM

plants such as members of the families Brassicaceae and Pinaceae generally have a defective or partial set of CSG (Delaux et al. 2014; Garcia et al. 2015). The sequences and functional conservation of CSG in orchids remains unknown.

We previously reported a method for inducing the symbiotic germination of *Bletilla striata* (Orchidaceae), which is an initially mycoheterotrophic plant species, with a symbiotic fungus *Tulasnella* sp. strain HR1-1, hereafter referred to as *Tulasnella* (Yamamoto et al. 2017). This method is suitable for quantitative analyses because the *B. striata* seed germinate rapidly and synchronously. Here, we use this method to enable the description of the CSG and the characterization of the molecular basis of OM symbiosis by analyzing transcriptome profiles of *B. striata* symbiotic protocorms. We also explored the functional conservation of the CSG and its concomitant expression in this orchid by performing a cross-species complementation of *CCaMK* and by analyzing the expression of *B. striata* genes identified as orthologs of AM-inducible marker genes in rice (Gutjahr et al. 2008).

RESULTS

RNA sequencing of *B. striata* symbiotic protocorms and genome sequencing of *Tulasnella*.

In orchid mycorrhizae, fungal hyphae colonize protocorm or root cells. The hyphae enter parenchyma cells, branch to form dense hyphal coils called pelotons, and are ultimately degraded. A protocorm infected with mycorrhizal fungi contains dozens of symbiotic cells. Each of these symbiotic cells can be classified into three stages according to the morphological characteristics of their fungal symbionts (Yamamoto et al. 2017). The early stage is characterized by fungal invasion and the formation of pelotons within the cortical cells, the middle stage is characterized by well-developed pelotons with a clear fungal cell wall, and the late stage is characterized by the disruption of the peloton. These symbiotic cells in the protocorm were observed at least 1 week after the symbiotic germination of *B. striata* with *Tulasnella* and most of the symbiotic cells are at the early stage at that time (Yamamoto et al. 2017). After that, the total number of symbiotic cells in a protocorm continue to increase during the first 3 weeks. The symbiotic cells at the middle and late stages were the most frequently observed after 2 and 3 weeks of symbiotic germination, respectively (Yamamoto et al. 2017). Therefore, we collected samples of the *B. striata* protocorms at each of these ages to compare their transcriptomes using RNA sequencing. The RNA sequencing run produced 4.8 to 14.9 million reads per sample. We assembled the total reads into contigs and predicted 77,768 nonredundant open reading frames (Supplementary Table S1).

To classify the reads and nonredundant gene sets into sequences of plant and fungal origin, we sequenced the *Tulasnella* genome using Illumina sequencing technology. The obtained assembly contained 15,642 scaffolds with 65.9 Mb of sequence data (Supplementary Table S2). Using the fungal assembly, we classified the reads from the RNA sequencing of the symbiotic protocorm into paired or unpaired reads, which therefore originated from the fungus or plant, respectively. On average, $87.5 \pm 2.0\%$ of reads were found to have a putative plant origin. The de novo reference assembly of plant-derived sequences was confirmed by subtracting the result of a BLAST search of the assembled genome scaffolds of *Tulasnella* from the nonredundant gene sets. We then extracted 59,143 coding sequences that were determined to be of plant origin.

Identification of a set of putative common symbiotic genes in *B. striata*.

To determine whether *B. striata* possesses a set of CSG, we searched for orthologs of CSG from other plant species in the

(Continued)

Fig. 1. Phylogenetic relationships of common symbiosis genes in *Bletilla striata* and a variety of other plant species. Neighbor-joining trees show the phylogenetic relationships among the protein sequences of **A**, SYMRK; **B**, CASTOR; **C**, POLLUX; **D**, NUP85; **E**, NUP133; **F**, NENA; **G**, CCaMK; and **H**, CYCLOPS. Accession numbers from the National Center for Biotechnology Information Reference Sequence are given for all sequences. Black filled circles represent monocotyledonous species. Arrows indicate the *B. striata* genes. Phylogenetic trees were constructed using MEGA software version 7.

Fig. 1. (Continued from previous page)

de novo reference assembly of *B. striata*-originated genes using BLAST software (McGinnis and Madden 2004). Orthologs of all the CSG from monocot rice (*Oryza sativa*) and dicot *L. japonicus* CSG (the gene ID of *O. sativa* sequences and *L. japonicus* sequences are listed in Supplementary Table S3) were found in *B. striata* (Supplementary Table S4). To examine the evolutionary relationship of the CSG in *B. striata* and other plant species, phylogenetic trees were constructed from alignments of their full-length protein sequences. All of the *B. striata* CSG were distinctly clustered into a group from monocotyledonous plants (Fig. 1; Supplementary Fig. S1). A set of CSG is also conserved in other photosynthetic orchids *Phalaenopsis equestris* and *Dendrobium catenatum* (Fig. 1).

Differential regulation of *B. striata* genes between protocorm ages.

To identify the plant genes involved in OM symbiosis, we compared the transcriptomes of the two transitional periods of symbiotic protocorms: week one versus week two (W1-W2), which mainly represents the change from the early (fungal invasion) to middle (fungal colonization) stages, and week two versus week three (W2-W3), which mainly represents the transition from the middle to late (hyphal degradation) stages. A total of 1,649 and 1,303 genes had significantly different expression levels (fold change ≥ 2.0 or ≤ -2 , false discovery rate [FDR] < 0.05) during the W1-W2 and W2-W3 transitions, respectively (Fig. 2A; Supplementary Tables S5 and S6). Among these differentially expressed genes (DEG), 1,417 and 1,071 were altered specifically during W1-W2 and W2-W3, respectively, whereas only 232 DEG were present in both comparison sets (Fig. 2A).

To characterize these DEG, we performed a gene ontology (GO) enrichment analysis using the BLAST2GO tool (Conesa et al. 2005). After dividing DEG into four groups (W1 $>$ W2 or W1 $<$ W2 groups in W1-W2 transition and W2 $>$ W3 or W2 $<$ W3 groups in W2-W3 transition), the frequency of each GO term was compared between each DEG group and the de novo reference assembly of plant-originated genes. There were 85 significantly overrepresented GO terms, when redundant higher hierarchy terms were removed from the list (FDR < 0.05). These GO terms were clustered into five main groups based on the FDR values (Fig. 2B). Cluster 1 contained 30 terms that were overrepresented in W1. Cluster 2 included 22 terms that had a relatively higher FDR than the others and had no particular pattern. Clusters 3 and 4 included 25 terms that were overrepresented in W2. Cluster 5 contained eight terms that were overrepresented in W3. Among these, only 14 terms (16% in total significant GO terms) were detected more than two comparison sets (Table 1), indicating that the cellular environment of the protocorms is altered drastically throughout the three stages of OM symbiosis. Several overrepresented GO terms, as described in Table 1, particularly within the clusters 3 and 4 that show the group upregulated in W2, were shared with the GO terms that overrepresented in upregulated genes during AM or RL symbiosis (Handa et al. 2015). There were also no significant GO terms functionally classified as being involved in response to biotic and abiotic stimuli or specifically involved in stress response (Table 1). In total, 13 GO terms were overrepresented in common DEG between W1-W2 and W2-W3 (Supplementary Table S7). In particular, these terms included some significantly overrepresented GO terms related to nitrogen metabolism such as “nitrate reductase activity”, “nitric oxide metabolic process”, and “nitric oxide biosynthetic process”.

Expression patterns of symbiosis-related genes at each protocorm age.

To further characterize the expression profile of symbiotic germination, we compared our list of DEG with the National

Center for Biotechnology Information nonredundant (nr) database to assign functional annotations. We revealed that the expression of many homologs of the AM-induced genes (Handa et al. 2015) was significantly altered during symbiotic germination (Table 2). In contrast, a few types of genes related to biotic stress such as pathogenesis-related 1-like genes and defense-related WRKY genes changed their expression during W1-W2 or W2-W3 transition. Although it is well known that these genes are typical markers in defense against plant pathogens (Lu et al. 2012; Rivas 2012), no GO terms associated with biotic stress responses were identified, indicating that colonization of *B. striata* protocorms by *Tulasnella* does not trigger a strong defense response. In AM symbiosis, there are many studies of the effects of phytohormones as important regulators of AM development (Gutjahr 2014). It has been reported that gibberellin (GA) reduces hyphal colonization and arbuscule formation during AM symbiosis (Takeda et al. 2015) and auxin is positively correlated with AM colonization (Hause et al. 2007). In phytohormone-related processes resulting from our study, the expression of a GA biosynthesis gene encoding GA 20 oxidase 1-D-like (*GA20ox*) was downregulated in W2 relative to W1 (Table 2). Conversely, a gene encoding a GA-inactivating enzyme, GA 2- β -dioxygenase-like (*GA2ox*), was upregulated in W2 relative to W3 (Table 2). Auxin-responsive genes were upregulated in W2: genes encoding the auxin-responsive protein SAUR32-like and auxin transporter-like protein 3 were highly expressed in W2 relative to W1, and genes encoding the auxin-induced protein 6B-like and auxin-responsive protein SAUR50-like were upregulated in W2 relative to W3. We also identified 17 transporter genes related to nitrogen and sugar transfer showing increased expression during symbiotic germination (Table 2), which corresponds to previous studies on OM symbioses (Fochi et al. 2017; Perotto et al. 2014; Suetsugu et al. 2017; Zhao et al. 2014).

Functional complementation of the *L. japonicus ccamk* mutant using *B. striata CCaMK*.

Although we identified a set of CSG in *B. striata* genome and found some common features of AM symbiosis, the functional conservation of these genes is yet to be elucidated. It was previously reported that one of the CSG, *CCaMK*, is necessary not only for AM and RL symbiosis but also for actinorhizal symbiosis (Svistonoff et al. 2013), which suggests that *CCaMK* could play a key role in the coordination of the symbiotic infection processes. Actually, *CCaMK* is one of the most frequently investigated genes in previous studies that have examined CSG functional conservation (Banba et al. 2008; Chen et al. 2007; Godfroy et al. 2006). Previous studies reported that *O. sativa CCaMK* (*OsCCaMK*) rescued the defective nodulation phenotype of the *ccamk* mutant in *L. japonicus*, resulting in the formation of mature nodules (Banba et al. 2008). Therefore, we examined the potential function of *B. striata CCaMK* (*BsCCaMK*). A homology search showed that *BsCCaMK* is a single-copy gene in *B. striata* as it is in rice, *L. japonicus*, and *Medicago truncatula*, and that it possesses the functional domains required for mutual symbiosis (Takeda et al. 2012) (Supplementary Fig. S2). In addition, the expression of *BsCCaMK* increased during symbiotic germination. We next tested whether *BsCCaMK* possesses the capability of forming both AM and RL symbiosis, instead of an *L. japonicus CCaMK*, by performing a cross-species complementation assay of the *L. japonicus ccamk-3* mutant (Tirichine et al. 2006) with *BsCCaMK*. The cDNA of *BsCCaMK* under the control of the *L. japonicus UBIQUITIN1* (*UBQ1*) promoter (Maekawa et al. 2008) was introduced into *ccamk-3*. Upon inoculating with *Rhizophagus irregularis*, a general species of AM fungi, intracellular symbiosis of AM fungi was fully restored in transgenic *ccamk-3*

roots carrying *BsCCaMK* (*ccamk-3/BsCCaMK*), as well as those transformed with *LjCCaMK* (*ccamk-3/LjCCaMK*) (Fig. 3A). Moreover, in response to DsRed-labeled *Mesorhizobium loti*, a compatible rhizobial bacterium of *Lotus* spp., nodule formation was fully restored on the roots of both *ccamk-3/BsCCaMK* and *ccamk-3/LjCCaMK*, (Fig. 3B). These results demonstrate that *BsCCaMK* conserves the property of forming both AM and RL symbiosis in *L. japonicus*.

Induction of AM symbiosis marker homologs in symbiotic protocorms.

To further explore the presence of molecular systems common to AM plants, we examined the expression of *B. striata* homologs of AM-specific marker genes identified in rice (Gutjahr et al. 2008). We searched for 17 of these rice AM marker genes in the *B. striata* reference sequence using the BLASTX tool, identifying 12 conserved homologs in *B. striata* with sufficiently low E values (E value < 1.0E-05; Supplementary Table S8). Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was conducted for nine randomly selected homolog genes in symbiotic protocorms sampled weekly for 3 weeks, including at the start of the experiment. The expression of eight genes (*BsAM1*, *BsAM2*, *BsAM11*, *BsAM14*, *BsAM20*, *BsAM25*, *BsAM34*, and *BsAM39*) significantly increased during symbiotic germination, whereas the expression of *BsAM18* decreased (Fig. 4). We then refer to these eight genes as OM marker genes.

To examine whether the activation of these OM marker genes occurred in relation to symbiosis, the same expression analysis was performed using protocorms growing on a sucrose-containing medium with *Tulasnella*. Generally, OM relationships are unstable; symbiotic fungi could potentially become pathogenic parasites or fail to colonize the protocorms when the temperature or carbon source is not suitable for symbiosis (Beyrle et al. 1995; Harvais and Hadley 1967). On the sucrose-containing medium, we found serendipitously that *B. striata* protocorms having symbiosis with *Tulasnella* contained significantly lower numbers of symbiotic cells with the destruction of the peloton structure, whereas hyphal growth in axenic culture was unaltered with the additional sucrose (Supplementary Fig. S3). Quantitative RT-PCR was conducted for the aberrant symbiotic protocorms sampled every week from germination to week three. The expression of *BsAM20* and *BsAM39* were significantly lower when compared with those in the normal medium (Fig. 4). *BsAM25* and *BsAM34* transcripts were not detected as germination progressed on the sucrose-containing medium, unlike in the expression on the normal medium (Fig. 4).

Gutjahr et al. (2008) reported that a few AM marker genes in rice are expressed in arbusculated cells or in cortical cells flanking intercellularly growing hyphae, suggesting the existence of cell-autonomous signaling events operating selectively in certain cell types during colonization by the AM fungus. In symbiotic protocorms, pelotons are limited to cortical cells and are not found in the apical meristem (Shimura and Koda 2005). To confirm the association between the expression of OM marker genes and the localization of the fungus in the protocorms, we compared their expression in the two halves of the protocorm using quantitative RT-PCR. The top half of the protocorm is likely to contain few or no hyphae, whereas numerous pelotons are observed in the bottom half (Supplementary Fig. S4). *Tulasnella* mRNA encoding a translation elongation factor (*TEF*), which was identified from a search against the assembled genome scaffolds of *Tulasnella* using *Piriformospora indica* *TEF* (GenBank accession number AJ249912.1) as a query sequence, was approximately 3.1 times more abundant in the bottom half of the protocorm than the top half. Similarly, the expression levels of the eight OM marker genes (*BsAM1*, *BsAM2*, *BsAM11*, *BsAM14*, *BsAM20*, *BsAM25*, *BsAM34*, and *BsAM39*) were 1.7 to 73.2

times higher in the bottom half than the top. In contrast, *BsAM18*, which was omitted from OM marker genes, had significantly lower expression in the bottom than the top half. These results were congruent with the notion that the expression of the eight OM marker genes is associated with the presence of the fungus, although the top and bottom halves of the protocorm are likely to differ in many other traits as well.

DISCUSSION

CSG are essential components of the symbiosis signaling pathway, required for the establishment of mutual symbioses with both AM fungi and rhizobia. In this study, we performed

Fig. 2. *Bletilla striata* gene transcription during symbiotic germination. **A**, Venn diagram showing the number of differentially expressed genes (DEG) between transitions week one versus week two (W1-W2) and week two versus week three (W2-W3). **B**, Gene ontology (GO) enrichment analysis of DEG in the de novo reference assembly. Heatmaps were generated using the heatmap.2 function in the gplots R package. Hierarchical clustering was performed on the false discovery rate (FDR) values associated with each GO term.

Table 1. Significantly overrepresented gene ontology (GO) terms during the symbiotic germination of *Bletilla striata* inoculated with *Tulasnella* sp. strain HR1-1

Code ^b	Term	False discovery rate ^a			
		W1 > W2	W1 < W2	W2 > W3	W2 < W3
Cluster 1					
Cellular component					
● GO:0000786	Nucleosome	2.03E-12 (16/61)	...	...	...
○ GO:0009522	Photosystem I	1.10E-03 (7/56)	...	...	...
○ GO:0031305	Integral component of mitochondrial inner membrane	4.46E-03 (3/4)	...	...	...
○ GO:0009654	Photosystem II oxygen evolving complex	4.98E-03 (5/31)	...	...	...
○ GO:0005618	Cell wall	1.35E-02 (8/126)	...	...	...
○ GO:0009535	Chloroplast thylakoid membrane	8.16E-03 (8/114)	...	...	...
Molecular function					
● GO:0046982	Protein heterodimerization activity	5.06E-11 (16/79)	...	...	...
○ GO:0004170	dUTP diphosphatase activity	6.77E-06 (4/0)	...	...	...
○ GO:0003677	DNA binding	7.59E-05 (42/1,441)	...	...	...
○ GO:0004612	Phosphoenolpyruvate carboxykinase (ATP) activity	2.55E-04 (3/0)	...	...	...
○ GO:0016984	Ribulose-bisphosphate carboxylase activity	1.74E-03 (3/2)	...	...	...
○ GO:0008234	Cysteine-type peptidase activity	4.03E-03 (7/73)	...	...	...
○ GO:0016702	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	3.73E-03 (6/48)	...	...	...
○ GO:0016168	Chlorophyll binding	3.08E-03 (6/46)	...	...	...
○ GO:0015266	Protein channel activity	1.26E-02 (3/7)	...	...	...
○ GO:0010333	Terpene synthase activity	8.89E-03 (4/18)	...	3.31E-02 (3/19)	...
● GO:0046872	Metal ion binding	2.78E-02 (64/3,463)	...	...	...
○ GO:0008974	Phosphoribulokinase activity	2.43E-02 (2/1)	...	...	...
Biological process					
○ GO:0046081	dUTP catabolic process	2.55E-04 (3/0)	...	...	...
○ GO:0006226	dUMP biosynthetic process	2.55E-04 (3/0)	...	...	...
○ GO:0019253	Reductive pentose-phosphate cycle	7.85E-04 (3/1)	...	...	...
○ GO:0009853	Photorespiration	4.46E-03 (3/4)	...	...	...
● GO:0006334	Nucleosome assembly	1.35E-02 (5/41)	...	...	...
○ GO:0009765	Photosynthesis, light harvesting	1.26E-02 (5/40)	...	...	...
○ GO:0006260	DNA replication	1.07E-02 (7/89)	...	...	...
○ GO:0009647	Skotomorphogenesis	1.01E-02 (2/0)	...	...	...
○ GO:0018298	Protein-chromophore linkage	3.17E-02 (5/53)	...	...	...
○ GO:0005976	Polysaccharide metabolic process	3.17E-02 (11/272)	...	...	...
○ GO:0006094	Gluconeogenesis	2.85E-02 (3/11)	...	...	...
○ GO:0071555	Cell wall organization	2.58E-02 (9/180)	...	...	...
Cluster 2					
Cellular component					
○ GO:0042719	Mitochondrial intermembrane space protein transporter complex	...	2.45E-02 (2/0)	...	...
● GO:0016021	Integral component of membrane	...	2.45E-02 (123/5,487)	...	4.14E-02 (120/5,490)
Molecular function					
○ GO:0003852	2-Isopropylmalate synthase activity	...	...	4.72E-02 (2/4)	...
○ GO:0004601	Peroxidase activity	...	...	4.55E-02 (5/99)	...
○ GO:0016630	Protochlorophyllide reductase activity	3.94E-02 (2/2)	...	...	...
○ GO:0003983	UTP:glucose-1-phosphate uridylyltransferase activity	...	...	...	3.48E-02 (2/0)
○ GO:0033743	Peptide-methionine (R)-S-oxide reductase activity	...	...	...	4.14E-02 (3/6)
○ GO:0009815	1-Aminocyclopropane-1-carboxylate oxidase activity	...	2.45E-02 (2/0)	...	...
○ GO:0010309	Acireductone dioxygenase [iron(II)-requiring] activity	...	2.45E-02 (3/5)	...	...
○ GO:0017153	Sodium:dicarboxylate symporter activity	...	2.45E-02 (2/0)	...	...
○ GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	...	2.53E-02 (20/490)	...	...
○ GO:0008171	O-methyltransferase activity	...	3.22E-02 (6/53)	...	...
○ GO:0016788	Hydrolase activity, acting on ester bonds	...	...	3.74E-02 (14/747)	...
○ GO:0008977	Prephenate dehydrogenase (NAD+) activity	...	...	2.40E-02 (2/2)	...
○ GO:0004665	Prephenate dehydrogenase (NADP+) activity	...	...	2.40E-02 (2/2)	...

(Continued on next page)

^a Numbers in parentheses represent differentially expressed gene counts/reference counts.

^b Black (filled) circles represent the GO terms shared with the terms that were overrepresented in upregulated genes during arbuscular mycorrhizal or rhizobium-legume symbioses reported by Handa et al. (2015).

transcriptome sequencing and de novo assembly of *B. striata*, which is an initially mycoheterotrophic and non-AM plant, under symbiotic conditions, and analyzed the sequence and functional conservation of CSG in *B. striata*.

Many orchids have recruited the polyphyletic *Rhizoctonia* fungi in the evolution of mycorrhizal associations (Martos et al. 2009; Ogura-Tsujita et al. 2009; Rasmussen and Rasmussen 2007; Selsosse and Martos 2014). Many of these fungi have a

Table 1. (Continued from previous page)

Code ^b	Term	False discovery rate ^a			
		W1 > W2	W1 < W2	W2 > W3	W2 < W3
Biological process					
○ GO:0006571	Tyrosine biosynthetic process	...	...	4.72E-02 (2/4)	...
○ GO:0031408	Oxylipin biosynthetic process	4.66E-02 (3/14)	...	...	...
○ GO:0045454	Cell redox homeostasis	...	4.46E-02 (8/106)	...	...
○ GO:0006011	UDP-glucose metabolic process	...	...	...	3.48E-02 (2/0)
○ GO:0045039	Protein import into mitochondrial inner membrane	...	2.45E-02 (2/0)	...	...
○ GO:0015711	Organic anion transport	...	3.22E-02 (9/127)	...	...
○ GO:0042128	Nitrate assimilation	...	...	3.48E-02 (2/3)	...
Cluster 3					
Cellular component					
○ GO:0009505	Plant-type cell wall	...	...	1.01E-02 (4/32)	...
● GO:0031225	Anchored component of membrane	...	...	1.61E-02 (4/38)	...
○ GO:0005576	Extracellular region	2.70E-02 (12/307)	...	1.70E-03 (11/308)	...
Molecular function					
○ GO:0016620	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	...	...	9.72E-03 (5/63)	...
○ GO:0045548	Phenylalanine ammonia-lyase activity	...	...	7.24E-03 (3/9)	...
○ GO:0003866	3-Phosphoshikimate 1-carboxyvinyltransferase activity	...	...	2.98E-04 (3/1)	...
○ GO:0016705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	...	...	4.24E-05 (13/294)	...
● GO:0005506	Iron ion binding	...	...	3.30E-05 (13/286)	...
● GO:0004497	Monoxygenase activity	2.78E-02 (10/223)	...	2.85E-06 (13/220)	...
○ GO:0043546	Molybdopterine cofactor binding	...	2.45E-02 (2/0)	6.00E-03 (2/0)	...
○ GO:0050464	Nitrate reductase (NADPH) activity	...	2.45E-02 (2/0)	6.00E-03 (2/0)	...
○ GO:0043531	ADP binding	...	1.41E-02 (5/25)	6.00E-03 (4/26)	...
● GO:0020037	Heme binding	2.73E-02 (12/308)	2.45E-02 (15/305)	2.58E-10 (20/300)	...
Biological process					
○ GO:0009800	Cinnamic acid biosynthetic process	...	...	7.24E-03 (3/9)	...
○ GO:0006559	L-phenylalanine catabolic process	...	...	1.28E-02 (3/12)	...
○ GO:0016049	Cell growth	...	...	1.21E-02 (4/34)	...
○ GO:0010215	Cellulose microfibril organization	...	...	5.21E-04 (4/11)	...
○ GO:0009423	Chorismate biosynthetic process	...	...	1.81E-03 (3/4)	...
○ GO:0042744	Hydrogen peroxide catabolic process	2.06E-02 (5/46)	...	3.56E-03 (5/46)	...
○ GO:0006809	Nitric oxide biosynthetic process	...	2.45E-02 (2/0)	6.00E-03 (2/0)	...
Cluster 4					
Cellular component					
○ GO:0005744	Mitochondrial inner membrane presequence translocase complex	1.26E-02 (3/7)	3.81E-03 (4/6)	...	...
Molecular function					
○ GO:0015035	Protein disulfide oxidoreductase activity	...	5.80E-03 (8/66)	...	...
Biological process					
○ GO:0006662	Glycerol ether metabolic process	...	1.17E-03 (7/32)	...	...
○ GO:0030150	Protein import into mitochondrial matrix	3.94E-02 (3/13)	8.16E-05 (6/10)	...	...
● GO:0055114	Oxidation-reduction process	2.31E-03 (45/1,911)	1.46E-03 (59/1,897)	4.05E-08 (41/1,915)	...
Cluster 5					
Cellular component					
○ GO:0005634	Nucleus	...	...	...	1.13E-04 (69/2,278)
Molecular function					
○ GO:0043565	Sequence-specific DNA binding	...	...	...	3.68E-05 (19/268)
○ GO:0051087	Chaperone binding	...	...	...	2.27E-07 (10/33)
○ GO:0001406	Glycerophosphodiester transmembrane transporter activity	...	...	...	2.70E-03 (3/1)
○ GO:0005509	Calcium ion binding	...	...	...	1.10E-02 (16/310)
○ GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding	...	...	...	5.82E-03 (8/71)
Biological process					
○ GO:0001407	Glycerophosphodiester transmembrane transport	...	...	...	2.70E-03 (3/1)
○ GO:0006355	Regulation of transcription, DNA-templated	...	3.81E-03 (38/1,064)	...	1.96E-17 (67/1035)

free-living, saprotrophic stage but may also have an endophytic stage without apparent infection symptoms (Selosse et al. 2009; van der Heijden et al. 2015; Weiß et al. 2016). It has been suggested that there are two trajectories for the recruiting of the mycorrhizal fungi: the mycorrhizal association evolved directly from soil saprotrophic ancestors or indirectly through endophytic ancestors (Selosse et al. 2018). Recruiting these fungi as symbiotic partners may induce the need to cope with problems such as the fungi secreting a variety of cell-wall-degrading enzymes that could induce plant immunity (Floudas et al. 2012; Juge 2006). Actually, hydrolytic enzymes involved in cell wall degradation abound in an orchid mycorrhizal fungus, *Tulasnella calospora* (Lahrmann et al. 2015), which shows the highest identity with the *Tulasnella* strain used in this study. However, although *B. striata* can form mycorrhizal associations with saprotrophic fungi (Masuhara and Katsuya 1989; Yamamoto et al. 2017), which have the potential to elicit strong defense responses in plants, our transcriptome data provided no

evidence of strong defense activation at the GO level in *B. striata* during symbiotic germination. This is similar to the results of other transcriptome studies of orchids (Perotto et al. 2014; Suetsugu et al. 2017). On the fungal side, it is possible that OM fungi can produce molecules that suppress plant defense responses, such as the secreted protein 7 in *R. irregularis* and *Laccaria bicolor* (Kloppholz et al. 2011; Plett et al. 2014). On the plant side, these findings suggest that *B. striata* recruits a signaling pathway to exploit saprotrophic fungi without inducing a significant stress response. In this study, we revealed that *B. striata* possesses all of the CSG previously identified in other plant species. One of the genes, *BsCCaMK*, had highly conserved functional domains of *CCaMK* and complemented the function of *LjCCaMK*. Moreover, *B. striata* possesses 12 genes that are highly similar to the AM marker genes in rice (Gutjahr et al. 2008). In all, 8 of 12 homolog genes (*BsAMI*, *BsAM2*, *BsAM11*, *BsAM14*, *BsAM20*, *BsAM25*, *BsAM34*, and *BsAM39*) were significantly induced during mycoheterotrophic

Table 2. M-induced, phytohormone-related, and transporter genes that are differentially expressed in *Bletilla striata* protocorms associated with *Tulasnella* sp. strain HR1-1

Annotation ^a	Sequence ID	Fold change ^b	FDR ^c
W1-W2			
Ammonium transporter 3 member 1-like	DN38547_c0_g2_i1::g.160833::m.160833	1.19	4.40E-02
Ankyrin repeat domain-containing 65-like	DN19080_c0_g1_i1::g.64529::m.64529	1.57	3.19E-03
Ankyrin repeat-containing At3g 12360-like	DN24832_c0_g1_i1::g.8235::m.8235	1.03	3.86E-02
Blue copper -like	DN149578_c0_g1_i1::g.6945::m.6945	-1.69	3.97E-04
Chitinase 4-like	DN50382_c0_g1_i2::g.195442::m.195442	-1.07	2.68E-02
GDSL esterase lipase At1 g28570-like	DN102229_c0_g2_i1::g.7228::m.7228	-2.08	4.86E-03
GDSL esterase lipase At1g74460-like	DN59482_c0_g5_i1::g.120683::m.120683	-2.41	5.64E-10
GDSL esterase lipase At2g23540-like	DN43229_c0_g1_i1::g.140659::m.140659	2.20	6.23E-04
GDSL esterase lipase At3g48460	DN43208_c0_g1_i1::g.140713::m.140713	-1.21	9.76E-03
GDSL esterase lipase At5g33370-like	DN151092_c0_g1_i1::g.1602::m.1602	1.20	2.84E-03
GDSL esterase lipase At5g55050-like	DN42230_c0_g1_i1::g.154911::m.154911	5.45	9.67E-04
GDSL esterase lipase At5g55050-like	DN42230_c0_g1_i2::g.154912::m.154912	5.63	2.66E-04
GDSL esterase lipase At5g55050-like	DN42230_c0_g1_i3::g.154913::m.154913	4.87	1.07E-02
GDSL esterase lipase EXL3-like	DN60397_c1_g6_i1::g.14051::m.14051	2.31	2.83E-06
GDSL esterase lipase EXL3-like	DN134368_c0_g1_i1::g.52::m.52	-1.31	3.86E-02
Germin 3-8	DN45919_c0_g1_i1::g.205096::m.205096	-1.40	8.40E-03
Germin 9-3	DN48078_c0_g2_i1::g.60695::m.60695	-1.32	1.69E-02
Glutathione S-transferase	DN52467_c0_g1_i1::g.80511::m.80511	1.86	3.77E-02
Glycerol-3-phosphate acyltransferase 1-like	DN12938_c0_g1_i1::g.205235::m.205235	-1.05	1.89E-02
Glycerol-3-phosphate acyltransferase 1-like	DN60213_c7_g1_i1::g.179685::m.179685	-1.05	5.34E-04
Late embryogenesis abundant 2-like	DN9192_c0_g2_i1::g.240541::m.240541	-3.31	9.34E-32
Scarecrow 28	DN48117_c0_g1_i1::g.86946::m.86946	-1.27	1.73E-03
Subtilisin-like protease	DN1702_c0_g1_i1::g.35886::m.35886	-1.75	1.54E-05
Subtilisin-like protease	DN47520_c0_g1_i1::g.113202::m.113202	7.83	1.01E-14
Subtilisin-like protease	DN47520_c0_g1_i2::g.113207::m.113207	5.10	4.99E-02
Subtilisin-like protease	DN47520_c0_g1_i3::g.113208::m.113208	5.56	4.53E-17
Subtilisin-like protease	DN53912_c0_g1_i1::g.26558::m.26558	5.73	2.71E-11
Subtilisin-like protease	DN64239_c0_g1_i1::g.131610::m.131610	5.97	2.29E-02
Subtilisin-like protease	DN110003_c0_g1_i1::g.466::m.466	5.91	1.89E-03
Subtilisin-like protease	DN158370_c0_g1_i1::g.2192::m.2192	5.24	2.05E-02
Triacylglycerol lipase 2-like	DN38290_c0_g1_i1::g.63807::m.63807	1.43	2.96E-07
Gibberellin 20 oxidase 1-D-like	DN57018_c0_g1_i1::g.134402::m.134402	-1.23	2.61E-02
Gibberellin 20 oxidase 1-D-like	DN57018_c0_g2_i1::g.134405::m.134405	-1.26	4.54E-02
Gibberellin-regulated 1-like	DN44665_c0_g1_i1::g.59936::m.59936	2.38	3.83E-06
Gibberellin-regulated 1-like	DN148681_c0_g1_i1::g.2046::m.2046	2.75	2.72E-07
Gibberellin-regulated 9-like	DN34062_c0_g1_i1::g.191199::m.191199	2.10	1.43E-03
Auxin-responsive SAUR32-like	DN10126_c0_g1_i1::g.126638::m.126638	1.31	4.75E-04
Auxin transporter 3	DN50840_c0_g2_i1::g.61019::m.61019	1.13	1.61E-03
Ammonium transporter 3 member 1-like	DN38547_c0_g2_i1::g.160833::m.160833	1.19	4.40E-02
Oligopeptide transporter 1-	DN62436_c5_g1_i2::g.193429::m.193429	1.39	2.33E-04
Oligopeptide transporter 1-like	DN62707_c2_g1_i20::g.48777::m.48777	1.59	4.21E-03
Oligopeptide transporter 1-like	DN62707_c2_g1_i6::g.48758::m.48758	1.10	3.21E-03
Lysine histidine transporter-like 8	DN23033_c0_g1_i1::g.60538::m.60538	2.16	2.63E-03
Lysine histidine transporter-like 8	DN23033_c0_g2_i1::g.60539::m.60539	2.08	1.87E-02

(Continued on next page)

^a Functional annotations were assigned based on National Center for Biotechnology Information nonredundant database.

^b Values were represented as log₂ ratio; W2/W1 for week one versus week two (W1-W2) and W3/W2 for week two versus week three (W2-W3).

^c False discovery rate.

symbiosis with saprotrophic fungi. It is noted that the expression of the *O. sativa* *AM11* was dependent on a common symbiosis signaling pathway because the gene was not expressed in rice CSG mutants (Gutjahr et al. 2008). Taken together, these results indicate that not only the sequences of the major components such as CSG but also the expression patterns of genes related to the signaling pathway of mutualistic AM symbiosis are conserved in *B. striata*.

Based on a BLAST search of a sequence database of other orchids, a set of CSG is conserved in photosynthetic (i.e., initial mycoheterotrophic) orchids other than *B. striata*, indicating that CSG are conserved in a wide range of orchids. The presence of CSG in orchids is functionally and evolutionarily interesting because non-AM plants such as members of the Brassicaceae and Pinaceae families, which associate with the root-colonizing basidiomycete *P. indica* and ectomycorrhizal fungi, respectively, generally have a defective or partial set of CSG (Delaux et al. 2014; Garcia et al. 2015). These findings suggest that orchids have evolved differently than these non-AM plants, implying that there may be different types of endophytisms.

Perotto et al. (2014) reported that orchid cells harbor viable fungal structure pelotons and interact with OM fungi without strong defense activation in protocorms that is similar to AM symbiosis, whereas the OM fungi do not seem to be rewarded from the plant at the protocorm stage. This similarity is supported not only by ultrastructural observations (Hadley et al. 1971; Peterson and Massicotte 2004; Peterson et al. 1996; Uetake and Peterson 1998; Williamson and Hadley 1969) but also by the molecular-based evidence in this study. Given that certain

features of the common symbiosis signaling pathway may pre-date the evolution of the first terrestrial plants that harbored AM-like associations (Delaux et al. 2015; Field et al. 2015; Martin et al. 2017), it is possible to speculate that orchids recruit a genetic system of AM symbiosis to establish mycoheterotrophic symbiosis. To address this speculation, future research will be needed to prove the necessity of the common symbiosis signaling pathway for establishment of mycoheterotrophic symbioses.

MATERIALS AND METHODS

Plant materials and fungal strain.

Seed of *B. striata* ‘Murasakishikibu’ and its symbiotic fungus, *Tulasnella* sp. strain HR1-1, were used in all experimental work. The origins of these plant and fungal lines have been described in detail previously (Yamamoto et al. 2017). Collected seed were stored at 4°C until they were used. Fungal colonies were cultivated on potato dextrose agar (PDA; Kyokuto, Tokyo) medium at 25°C until required for the symbiotic germination experiments. Seed of *Lotus japonicus* ‘Gifu B-129’ or *ccamk-3* mutant were scarified, sterilized with sodium hypochlorite (1% effective chloride), and soaked in sterilized water overnight. The seed were germinated on moist filter paper and grown in a growth chamber (25°C, cycle of 16 h of light and 8 h of darkness).

Symbiotic germination.

Symbiotic germination procedures were performed according to the method of Yamamoto et al. (2017), with slight

Table 2. (Continued from previous page)

Annotation ^a	Sequence ID	Fold change ^b	FDR ^c
Lysine histidine transporter 1-like	DN54457_c0_g5_i4::g.183467::m.183467	2.24	3.47E-02
Lysine histidine transporter 1-like	DN54457_c0_g5_i8::g.183471::m.183471	2.27	2.94E-02
Sugar transporter ERD6-like 16	DN59467_c6_g7_i18::g.119952::m.119952	1.27	6.52E-03
Bidirectional sugar transporter SWEET16-like	DN39307_c0_g1_i1::g.79589::m.79589	-1.39	9.08E-04
Bidirectional sugar transporter SWEET16	DN39307_c0_g1_i2::g.79591::m.79591	-2.14	1.30E-02
Bidirectional sugar transporter SWEET14-like	DN48582_c0_g1_i1::g.28310::m.28310	1.67	2.72E-07
Bidirectional sugar transporter SWEET14-like	DN48582_c0_g1_i2::g.28312::m.28312	1.85	1.94E-08
Bidirectional sugar transporter SWEET2a-like isoform X1	DN53300_c0_g3_i10::g.155771::m.155771	1.32	2.37E-04
Bidirectional sugar transporter SWEET2a-like	DN53300_c0_g3_i3::g.155766::m.155766	1.28	2.02E-03
Bidirectional sugar transporter SWEET2a-like	DN53300_c0_g3_i4::g.155767::m.155767	1.08	3.15E-03
Bidirectional sugar transporter SWEET6b-like	DN57552_c4_g1_i1::g.104408::m.104408	2.73	1.21E-10
W2-W3			
Abscisic acid 8-hydroxylase 1-like	DN56235_c1_g2_i3::g.241237::m.241237	1.76	4.76E-02
Abscisic acid 8-hydroxylase 1-like	DN56235_c1_g2_i1::g.241235::m.241235	1.79	4.23E-02
Di tri peptide transporter 2	DN114729_c0_g1_i1::g.1994::m.1994	7.83	2.51E-02
Germin 9-3	DN48078_c0_g2_i1::g.60695::m.60695	1.42	5.90E-03
Late embryogenesis abundant At1g64065-like	DN52833_c2_g2_i1::g.252823::m.252823	2.50	6.78E-06
Late embryogenesis abundant At1g64065-like	DN52833_c2_g2_i1::g.252824::m.252824	2.65	2.55E-05
Probable aquaporin NIP-type	DN43429_c0_g2_i1::g.24704::m.24704	-1.93	8.91E-03
Scarecrow 3	DN47026_c0_g2_i1::g.173443::m.173443	1.17	1.36E-03
Scarecrow 3	DN47026_c0_g1_i1::g.173441::m.173441	1.38	1.78E-04
Scarecrow 9	DN61342_c2_g2_i1::g.74268::m.74268	1.07	6.86E-03
Scarecrow 9	DN61342_c2_g1_i1::g.74264::m.74264	1.08	3.89E-03
Scarecrow 14	DN61342_c2_g3_i1::g.74271::m.74271	1.64	5.04E-06
Scarecrow 14	DN61342_c2_g3_i2::g.74274::m.74274	1.69	5.66E-07
Scarecrow 32	DN50312_c0_g2_i3::g.195381::m.195381	1.08	4.18E-02
Scarecrow 32	DN50312_c0_g2_i3::g.195386::m.195386	1.66	4.87E-02
Subtilisin-like protease	DN1702_c0_g1_i1::g.35886::m.35886	-2.03	1.91E-02
Subtilisin-like protease	DN35677_c0_g1_i1::g.181621::m.181621	-3.63	4.96E-02
Subtilisin-like protease	DN154153_c0_g1_i1::g.3731::m.3731	-5.97	2.09E-02
Vacuolar calcium ion transporter	DN55490_c0_g1_i3::g.216679::m.216679	2.46	1.05E-03
Gibberellin 2-β-dioxygenase-like	DN133591_c0_g1_i1::g.8098::m.8098	-2.75	2.79E-02
Auxin-induced 6B-like	DN6072_c0_g1_i1::g.185033::m.185033	-2.23	6.74E-03
Auxin-responsive SAUR50-like	DN138767_c0_g1_i1::g.226::m.226	-3.50	1.92E-02
OPT oligopeptide transporter	DN60623_c0_g2_i3::g.158828::m.158828	1.94	2.68E-02
OPT oligopeptide transporter	DN60623_c0_g2_i5::g.158835::m.158835	2.06	3.14E-02
Bidirectional sugar transporter SWEET16-like	DN39307_c0_g1_i1::g.79589::m.79589	-1.54	1.58E-02

modifications as follows. Plates containing 20 ml of oatmeal agar medium (Difco, Franklin, NJ, U.S.A.), either with or without sucrose at 30 g/liter, were inoculated with a culture of the symbiotic fungus and precultured for a week at 25°C before surface-sterilized seed were placed into the plates for symbiotic germination. The germination experiments were conducted at 25°C in the dark, and protocorms were collected every week for 3 weeks and stored at –80°C for RNA extraction or in a formalin-acetic acid-alcohol (5% formaldehyde, 5% acetic acid, and 45% ethanol) solution at 4°C for fluorescent staining.

RNA preparation for transcriptome analysis.

Total RNA was extracted from 80 mg of *B. striata* protocorms using an RNeasy Plant Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's protocol. The RNA was treated with RNase-free DNase I to remove residual genomic DNA and cleaned using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The quality and quantity of the purified RNA was confirmed by measuring its absorbance at

260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and by electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.). Three biological replicates were prepared for the transcriptome analysis.

RNA sequencing and data analysis.

RNA sequencing libraries were constructed from 1.0 µg of total RNA using a TruSeq RNA Sample Prep Kit v2 and a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, U.S.A.) following the manufacturer's instructions. Following purification, the mRNA fragmentation, cDNA synthesis, and quality and quantity checks were performed according to the method described by Suetsugu et al. (2017). Multiplex sequencing of paired-end or single-end reads was performed on an Illumina HiSeq 1500 instrument at the NIBB Core Research Facilities (Okazaki, Japan), followed by raw data processing, base calling, and quality control using the manufacturer's standard pipeline with RTA, OLB, and CASAVA. Datasets of the

Fig. 3. Complementation of the *Lotus japonicus* *ccamk-3* mutant with *Bletilla striata* CCaMK. Transgenic hairy roots carrying an empty vector (EV), *LjCCaMK*, or *BsCCaMK* were inoculated with **A**, *Rhizophagus intraradices* or **B**, *Mesorhizobium loti* expressing DsRed. All roots were observed after 4 weeks of growth under axenic conditions. GFP = green fluorescent protein. Scale bars: 200 µm (A) and 20 mm (B).

short reads were registered in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (accession number DRA005999).

Construction and de novo assembly of RNA-seq data.

Prior to assembly, the reads were trimmed to remove low-quality ends (< QV30), and adapter sequences (parameter: -o 7) using the cutadapt program. Reads shorter than 50 bp were removed. De novo assembly of the trimmed reads was conducted using Trinity, ver. 2.0.6 (Grabherr et al. 2011), with default settings. The coding sequences were predicted using TransDecoder

with default settings (Haas et al. 2013). To reduce the sequence redundancy of the assembled transcript sequences, similar contigs were grouped using CD-HIT (parameter: -c sequence identity threshold = 0.98) (Li and Godzik 2006). The nucleotide sequences of the assembly presented in this study are listed in Supplementary Dataset S1.

Fungal genome sequencing and de novo assembly.

Genomic DNA was extracted from *Tulasnella* grown on the PDA medium using a DNeasy Plant Mini Kit (Qiagen) and

Fig. 4. Expression of *Bletilla striata* marker genes during symbiotic germination with *Tulasnella* sp. strain HR1-1. Quantitative reverse-transcription polymerase chain reaction was performed with total RNA isolated at different time points (week zero to week three of germination) and different conditions (-Suc = symbiotic germination on normal oatmeal agar medium and +Suc, symbiotic germination on oatmeal agar medium containing sucrose at 30 g/liter). Relative expression values were determined using the relative standard curve method. Error bars indicate standard error from three biological replicates. ND = not detected. Different lowercase letters indicate significant differences between groups ($P < 0.05$, Tukey-Kramer test).

purified using Genomic DNA Clean & Concentrator (Zymo Research, Irvine, CA, U.S.A.) in accordance with the manufacturer's protocol. Size-selected genomic DNA libraries were prepared using the Pippin Prep system (Sage Science, Beverly, MA, U.S.A.) for paired-end libraries with a mean insert size of 350 or 800 bp and the Blue Pippin system (Sage Science) or electroelution were used for mate-pair libraries with large insert sizes (>4 kbp). The quality and quantity of the DNA library were confirmed with electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies), and with quantitative RT-PCR using an ABI 7500 Real-Time PCR system (Thermo Fisher Scientific). Multiple DNA libraries were sequenced using an Illumina MiSeq by PE150 v2 or PE300 v3 platform (Illumina). Datasets of the short reads were registered in the DDBJ Sequence Read Archive (accession number DRA006009). To remove contaminating reads, the sequence reads, including adapter sequences, were extracted from all mate-pair reads. After adapter trimming, de novo assembly, scaffolding, and gap closing were performed using Platanus, ver. 1.2.1 (Kajitani et al. 2014) with default parameters.

Differential expression analysis.

Data from three biological replicates were used in this analysis. To classify the single-end reads into sequences of plant and fungal origin, the reads were first mapped to the fungal genome using Bowtie2 (Langmead and Salzberg 2012). The unpaired reads classified as above were mapped to the de novo reference assembly of *B. striata* using Bowtie2 and the transcript abundance was estimated using eXpress, ver. 1.5.1 (Roberts and Pachter 2013). Differences in library size were corrected using the trimmed mean of M value normalization method, and EdgeR (Robinson et al. 2010) was used to identify DEG with a fold change ≥ 2.0 or ≤ -2.0 and FDR < 0.05.

Gene annotation and GO analysis.

DEG were annotated using BLAST searches against the nr database. GO enrichment analysis of DEG was performed using BLAST2GO (Conesa et al. 2005) with one-sided Fisher's exact test (FDR < 0.05). The enrichment of DEG was calculated by comparing the de novo reference assembly of plant-derived sequences.

Plasmid construction.

The promoter region of *LjUBQ10*, which is expressed constitutively in *L. japonicus* (Guether et al. 2009), was amplified by PCR from the *L. japonicus* TAC clone LjT06D07 using Prime STAR HS DNA Polymerase (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's protocol. The gene-specific primers used are shown in Supplementary Table S9. The amplified DNA was cloned into pDONR/Zeo (Thermo Fisher Scientific), then transferred into the Gateway-compatible binary vector pKGWFS7 (Karimi et al. 2002). This construct was used as a negative control for the complementation analysis. The *BsCCaMK* cDNA was obtained by performing an RT-PCR on the total RNA of the *B. striata* protocorms using the Prime Script II first-strand cDNA synthesis kit (Takara Bio Inc.) following the manufacturer's protocol. *BsCCaMK* was amplified using Prime STAR HS DNA polymerase (Takara Bio Inc.) and gene-specific primers; then, the amplified cDNA was cloned into pENTR/D-TOPO (Thermo Fisher Scientific). The *BsCCaMK* entry clone and the *LjCCaMK* entry clone (Yano et al. 2008) were transferred into the Gateway-compatible binary vector pUB-GW-GFP carrying the reporter green fluorescent protein (GFP) gene driven by 35S promoter (Maekawa et al. 2008).

Complementation analysis.

The empty vector plasmid or the pUB-GW-GFP vectors carrying the *BsCCaMK* or *LjCCaMK* constructs were transformed

into the *ccamk-3* mutant of *L. japonicus* (Tirichine et al. 2006) using hairy-root transformation mediated by *Agrobacterium rhizogenes* AR1193, as previously described (Maeda et al. 2006). Plants with GFP-positive hairy roots were selected and transplanted into pots of sterilized river sand and vermiculite (2:1 [vol/vol]) supplied with 1/10-strength Hoagland solution containing 0.1 mM KNO₃ for the AM fungi infection assay or B&D medium containing 0.5 mM KNO₃ for the nodulation assay. Seven days after transplantation, *R. irregularis* DAOM 197198 (Premier Tech, Quebec, QC, Canada) or *M. loti* MAFF 303099 constitutively expressing DsRed (Maekawa et al. 2009) were used to inoculate plants for the AM fungal infection and nodulation assays, respectively. The plants were grown for 4 weeks in a growth cabinet under axenic conditions (25°C, cycle of 16 h of light and 8 h of darkness). The colonization phenotypes of the *R. irregularis*-inoculated roots were observed under a SZX16 stereomicroscope (Olympus, Tokyo) and an BX53 optical microscope (Olympus) after diaminobenzidine staining (Kobae and Ohtomo 2016). The nodulation phenotypes of *M. loti*-inoculated roots were examined under a Multi Viewer System VB-S20 fluorescence microscope (Keyence, Osaka, Japan).

Gene expression analysis.

Protocorms were harvested and stored at -80°C until they were used for RNA extraction. Total RNA was extracted from 5 mg of *B. striata* protocorms or 50 hand-sectioned protocorms for each condition using RNAiso Plus (Takara Bio Inc.), following the manufacturer's protocol. First-strand cDNA synthesis was performed using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) in accordance with the manufacturer's protocol. Quantitative RT-PCR assays were carried out using the THUNDERBIRD SYBR qPCR Mix (Toyobo) on a CFX connect real-time detection system (Bio-Rad Laboratories, Hercules, CA) using the following program: 95°C for 10 min; 25 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. Fold changes were calculated using the expression of a housekeeping gene, *UBIQUITIN5*, as the internal control. Three technical replicates and three biological replicates were performed for each sample, and their threshold cycle values were averaged.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

cutadapt program: <https://cutadapt.readthedocs.io/en/stable/>
 LegumeBase, NBRP *Lotus/Glycine*:
<https://www.legumebase.brc.miyazaki-u.ac.jp/about.jsp>
 MEGA software version 7: <https://www.megasoftware.net/>
 R package: <https://cran.r-project.org/web/packages/gplots/index.html>