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Ectopic expression of ubiquitin-conjugating enzyme gene from wild rice, *OgUBC1*, confers resistance against UV-B radiation and *Botrytis* infection in *Arabidopsis thaliana*

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ABSTRACT

A previously unidentified gene encoding ubiquitin-conjugating enzyme was isolated from leaves of wild rice plant treated with wounding and microbe-associated molecular patterns. The *OgUBC1* gene was composed of 148 amino acids and contained a typical active site and 21 ubiquitin thioester intermediate interaction residues and 4 E3 interaction residues. Both exogenous application of salicylic acid and UV-B irradiation triggered expression of *OgUBC1* in leaves of wild rice. Recombinant OgUBC1 proteins bound to ubiquitins *in vitro*, proposing that the protein might act as E2 enzyme *in planta*. Heterologous expression of the *OgUBC1* in *Arabidopsis thaliana* protected plants from cellular damage caused by an excess of UV-B radiation. A stable expression of chalcone synthase gene was detected in leaves of OgUBC1-expressing Arabidopsis, resulting in producing higher amounts of anthocyanin than those in wild-type Col-0 plants. Additionally, both pathogenesis-related gene1 and 5 were transcribed in the transgenic Arabidopsis in the absence of pathogen infection. The OgUBC1-expressing plants were resistant to the infection of *Botrytis cinerea*. Taken together, we suggested that the *OgUBC1* is involved in ubiquitination process important for cellular response against biotic and abiotic stresses in plants.

1. Introduction

Sunlight is the most important factor for photosynthesis that provides chemical energy for living organisms. They are exposed to ultraviolet (UV), visible and infrared lights emitted from the sun and actively respond to them. Among UV-A (315–400 nm), UV-B (280–315 nm) and UV-C (100–280 nm), UV-A and a part of UV-B only reach the earth's surface and the amount of UV-B is increasing due to diminution of ozone layer. UV-B radiation is known to be a damage agent to DNA, RNA, and proteins [1–3]. It can also regulate developmental processes, including leaf characteristics, and flowering time in plants. [4–6]. As well, an excess of UV-B radiation stimulates an oxidative burst and biosynthesis of stress-related hormones in plants [7]. The cellular responses activated by UV-B radiation are similar to those for plant immune response against pathogen infection, indicating that UV radiation can promote disease resistance [8–10]. In fact, plants pre-exposed to UV radiation showed reduced susceptibility in response to the infection of pathogenic microbes [11–13].

Recently, sensor protein for UV-B, UV RESISTANCE LOCUS 8 (UVR8), whose mutant was hypersensitive to UV-B radiation, was identified in *Arabidopsis thaliana* [14,15]. The UVR8 is a β-propeller protein similar to human guanine nucleotide exchange factor REGULATOR OF CHROMATIN CONDENSATION 1 [14]. In the absence of UV-B radiation, homodimerized-UVR8 is to be inactive state. Whereas the monomerized-UVR8 protein physically interacts with a ring-finger-type ubiquitin ligase COP1 (CONTITUTIVELY PHOTOMORPHOGENEIC 1) in order to activate transcriptional initiation of genes important for UV protection in the presence of UV-B [15–17]. COP1, as a multifunctional repressor of photo-morphogenesis, is involved in an ubiquitin–proteasome system [18,19]. The fact that UVR8 cooperates with COP1 to trigger downstream signaling implies that ubiquitination is a crucial step for tolerance response against UV-B.

The ubiquitin–proteasome system is the famous protein degradation mechanism in eukaryotic cells. The seriate response requires an ubiquitin-activating enzyme (E1, UBA), an ubiquitin-conjugating enzyme (E2, UBC), and an ubiquitin ligase (E3, Ub

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ligase). The active ubiquitin moiety attached to E2 was transferred to a lysine residue in target protein, which is facilitated by E3. The E3 interacts with both E2 and its protein substrate, which provides substrate specificity [20]. Our knowledge about role of E2s was still relatively limited, compared to that of E3s. One of COP1-interacting proteins, COP10 was an E2 enzyme variant, lacking a cysteine residue important for ubiquitin conjugation, necessary for COP1-mediated protein degradation in Arabidopsis [21,22]. COP10 formed protein complex with other E2s, UV-damaged DNA-binding protein 1A (DDB1A) and de-etiolated 1 (DET1), and the resulting complex had a crucial role in COP1-mediated photo-morphogenesis [23]. Among 37 E2s in Arabidopsis, AtUBC2 was involved in tolerance response to UV stress as well as activation of floral repressor gene [24,25]. AtUBC13 seemed to participate in post-replication DNA repair system [26]. These three observations suggest that some of E2s are key components for light signaling and photo-morphogenesis in plants.

Previously we have successfully identified wound- and microbe-associated molecular patterns (MAMPs)-induced genes from leaves of wild rice and reported the roles of several genes in adaptive responses against biotic and abiotic stresses [27–30]. These studies strongly proposed that wild rice possesses a series of genes capable of conferring either disease resistance or environmental tolerance on crop plants. We identified an *OgUBC1* gene encoding an E2 from wild rice. The *OgUBC1* was strongly expressed in leaves either treated with MAMPs, and salicylic acid (SA), or exposed to UV-B radiation. The recombinant OgUBC1 protein showed a typical activity of E2 *in vitro*. Ectopic expression of the *OgUBC1* triggered transcription of defense- and stress-related genes, and accumulation of anthocyanin in leaves of Arabidopsis. In addition, the OgUBC1-expressing plants were tolerant against UV-B-mediated cell damage and resistant to the infection of *Botrytis cinerea*. The study suggests that the OgUBC1 takes part in the ubiquitin–proteasome system required for cellular responses against biotic and abiotic stresses.

2. Materials and methods

2.1. Gene, plants and pathogen

Wild rice plants (*Oryza grandiglumis*) were grown in the National Crop Experiment Station, Rural Development Administration in Korea, and the fully expanded leaves were used in this study. *A. thaliana* Col-0 grew under environmentally controlled growth chamber (22 ± 1 °C, 16 h light/8 h dark). The OgUBC1-expressing Arabidopsis was generated by floral dipping method and screened on Murashige and Skoog media containing hygromycin (20 mg/L) [31]. To obtain the full-length cDNA, RT-PCR was performed using SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen, USA). Gene specific primers used for amplification were OgUBC1-full-F (5′-ATGGCGTCCAAGAGGATCCTGAAGGAG-3′) and OgUBC1-full-R (5′-TTAAGCCCATGCGTACTTCTGCGT-3′). *B. cinerea* was grown on potato dextrose agar media at 25 °C for 10 days. Four-week-old Arabidopsis were inoculated with *B. cinerea* by 5 μL of spore solution (5×10^5 conidia/mL). The disease susceptibility was evaluated by the size of necrotic lesion and disease index [32].

2.2. Chemical and UV-B treatments

Approximately 2 cm-long leaf segments of wild rice were incubated on solutions containing 20% yeast extract, 100 μM cantharidin (CN), 100 μM endothall (EN), 100 μM jasmonic acid (JA) or 100 μM SA under 16 h light/8 h darkness at 24 °C as described earlier [30]. For UV-B irradiation, 4 week-old Arabidopsis plants were placed under a UV lamp (G40T10E, Sankyo Denki, Japan) that radiates 5 $J/m^{-2}\,s^{-1}$. Different period of illumination was applied to different set of plant materials. Leaf tissues were harvested at the indicated times, and used stored at −80 °C until extraction of total RNA.

2.3. Total RNA extraction and mRNA analyses

Total RNA was extracted from the leaves of wild rice and Arabidopsis plants using Concert™ Plant RNA Reagent (Invitrogen). *OgUBC1*-specific probes were used for the Northern blot analysis. As a control, 18S rRNA-specific probes, which was amplified with 18S rRNA-F (5′-CTTGGATGTGGTAGCCGTTT-3′) and 18S rRNA-R (5′-ATGATAACTCGACGGATCGC-3′) were used: [33]. Gene-specific primers for the genes of Arabidopsis were as follows: MEB5.2-F (5′-AATCTCTTCCAATGTTCTTCTTCT-3′), MEB5.2-R (5′-ATGGATGCC TTGACCTCGAGTTTG-3′), CHS-F (5′-CTGGTACATCATGAGACGCTT-GAC-3′), CHS-R (5′-ATGGTGATGGCTGGTGCTTCTTCT-3′), PR1-F (5′-ATGATCACATCATTACTTCATTAG-3′), PR1-R (5′-ATGAATTTTACT GGCTATTCTCGA-3′), PR5-F (5′-CGACATTGTTCTGATCCATGACCT-3′), PR5-R (5′-ATGGCAAATATCTCCAGTATTCAC-3′), PDF1.2-F (5′-ACATAAATTATTTTTATATTATTG-3′) and PDF1.2-R (5′-ACACAACAC ATACATCTATACATT-3′).

2.4. Analysis of relative ion leakage and anthocyanin level in transgenic Arabidopsis

The relative ion leakage analysis followed the method as described earlier [34]. One gram of leaves from 4 week-old Arabidopsis plants, which were exposed to UV-B during 90 min, was floated on 30 mL sterile water for 24 h at room temperature. Following incubation, the conductivity of the bathing solution was measured with a conductivity meter (Model EC-400L, Istek, Korea), and the measured values set to value A. The leaves were further incubated at 95 °C for 20 min to completely disrupt cell membrane. After cooling to room temperature the conductivity of the bathing solution was measured again. The value referred to as value B. For each measurement ion leakage was expressed as percentage ion leakage, i.e. (value A/value B) × 100. Anthocyanin were extracted and quantified as described previously [35].

2.5. Assay for ubiquitin-OgUBC1 thiol ester linkage

Amplified *OgUBC1* cDNA fragment with two gene specific primers, OgUBC1-5 (5′-CACCATGGCGTCCAAGAGGATCCTGAAG-GAG-3′) and OgUBC1-3 (5′-AGCCCATGCGTACTTCTGCGT-3′) was inserted into the pBAD202 vector (Invitrogen) to produce C-terminal His-tag fusion protein. The resulting *pBAD-OgUBC1* plasmid was introduced into *Escherichia coli* LMG194 strain for producing recombinant OgUBC1 protein (rOgUBC1). The soluble rOgUBC1 protein was expressed by adding 0.025% arabinose and used for analyzing its enzymatic activity. Formation of E2 thiol-ubiquitin ester adducts was determined as described by Sullivan and Vierstra [36] Reaction mixtures containing the His-tagged rOgUBC (E2), 0.25 pg of purified yeast E1 (SigmaAldrich, USA), 0.64 pg of ubiquitin, and 1 unit of inorganic pyrophosphatase (USB, USA) in 20 ul of 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM dithioerythritol were incubated at 30 °C for 0.5 min or as indicated. After terminating the reaction, the reactants were directly subjected to 12% SDS–PAGE at 4 °C. Separated proteins were transferred onto a nitrocellulose membrane (GE Healthcare, UK). The diluted anti-His antibody (1:1000) and anti-mouse IgG (1:5000) was used for the Western blot analyses. Detection was carried out using an ECL™ Western Blotting Analysis System (GE Healthcare).

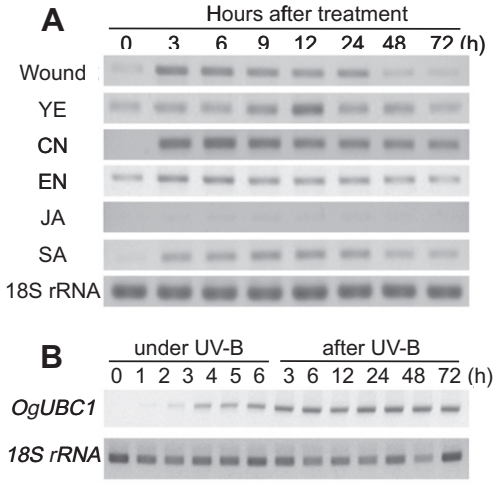

Fig. 1. Transcription of *OgUBC*1 gene in leaves of wild rice. (A) *OgUBC1* mRNA levels in leaves at different time points either after wounding or during soaking on yeast extract (YE), cantharidin (CN), endothall (EN), jasmonic acid (JA) and salicylic acid (SA). (B) Expression of *OgUBC1* by UV-B radiation. Wild rice plants were grown under illumination of UV-B radiation during 6 h (UV treatment) and transferred under natural solar radiation (after UV treatment). Twenty micrograms of total RNA were used for the RNA analyses with *OgUBC1*-specific probes. 18S rRNA-specific probes verified the equivalence of the RNA loaded in the each lane.

3. Results and discussion

3.1. OgUBC1 expression was stimulated by diverse exogenous signals in leaves of wild rice

Previously, we identified an *OgUBC1* gene encoding a putative E2 in leaves of wild rice plants treated with wounding and MAMPs (GenBank accession No. GU936576) [27]. To test which signals affected on expression of *OgUBC1*, we applied different signal molecules to leaves of wild rice and checked *OgUBC1* expression at given time points. Both treatments with wounding and MAMPs effectively induced *OgUBC1* expression (Fig. 1A). It is well documented that mechanical wound stimulated JA-dependent response in plants. However, *OgUBC1* mRNA was not detected in JA-treated leaves at the experimental conditions. Rather, exogenous treatment of SA strongly activated transcription of *OgUBC1* in leaf tissue (Fig. 1A). These analyses revealed that *OgUBC1* expression was somehow controlled by both SA- and MAMP-dependent pathway rather than JA-related events. As mentioned earlier, plant responses activated by SA partly overlapped with those by UV radiation. To test the possibility, we checked *OgUBC1* expression in leaves of wild rice after UV irradiation. The level of *OgUBC1* transcript had continuously increased in leaves of wild rice during UV-B irradiation and still maintained up to 72 h even if plants were transferred to normal light condition after irradiation (Fig. 1B). Radiation of UV triggered SA accumulation in plants [12]. To test if expression of *OgUBC1* affected SA accumulation, we measured the level of SA in leaves of wild-type Col-0 and OgUBC1-expressing Arabidopsis with or without UV-B radiation. However, we did not see any differences in SA level between wild type and OgUBC1-expressing plants (data not shown), which suggested that OgUBC1 might act downstream of SA accumulation stimulated by UV-B radiation.

3.2. The recombinant OgUBC1 protein had an ubiquitin conjugation activity

It is likely that OgUBC1 is a one of components in ubiquitin–proteasome system to resist to certain stressed conditions. To test if OgUBC1 protein successfully accepted an activated ubiquitin by E1, we purified rOgUBC1 protein and analyzed its enzymatic activity *in vitro*. With α-His antibody, we detected ubiquitin-conjugated rOgUBC1 proteins, resulting from co-incubation with ATP and E1. This result strongly proposed that the rOgUBC1 protein had ubiquitin-conjugating activity (Fig. 2A). Ubiquitin-OgUBC1 combination was not observed in ubiquitin conjugating reaction either without ATP or E1. This specificity was further confirmed by adding inorganic pyrophosphatase to the reaction mixture as pyrophosphatase renders the conjugation between E2 and ubiquitin effectively. In addition, the thiol-ester intermediates between rOgUBC1 and ubiquitin was highly accumulated, whereas amount of naïve rOgUBC1 was gradually declined from 0.5 to 30 min (Fig. 2B). Thus we concluded that the OgUBC1 is one of potential E2s in wild rice plants.

3.3. OgUBC1 conferred UV-B tolerance in transgenic Arabidopsis

To test if the OgUBC1 had any roles in tolerance response against an excess of UV-B radiation, both 4 week-old wild type Col-0 and OgUBC1-overexpressing Arabidopsis were exposed to UV-B radiation for 4 h. Degree of damage was measured 10 days after UV-B illumination (Fig. 3A). Transgenic plants showed less severe symptoms than wild type in response to UV-B radiation. For quantifying the differences between wild type and OgUBC1-expressing plants, the severity of damage was divided into 3 levels (Fig. 3B upper panel). In wild type, nearly 40% of plants were severely impaired by UV-B irradiation, belonging to damage level 3. On the other hand, UV-B irradiation could not result in severe symptoms in OgUBC1-expressing plants tested in this study. Only two lines of transgenic plants (L4 and L9) showed partly severe symptoms (level 3), 7% and 9%, respectively. Most of transgenic plants, however, exhibited either weak or moderate symptoms (Fig. 3B). The result indicated that heterologous expression of *OgUBC1* gave a certain level of protective function to Arabidopsis against an excess of UV-B radiation. To test whether or not the

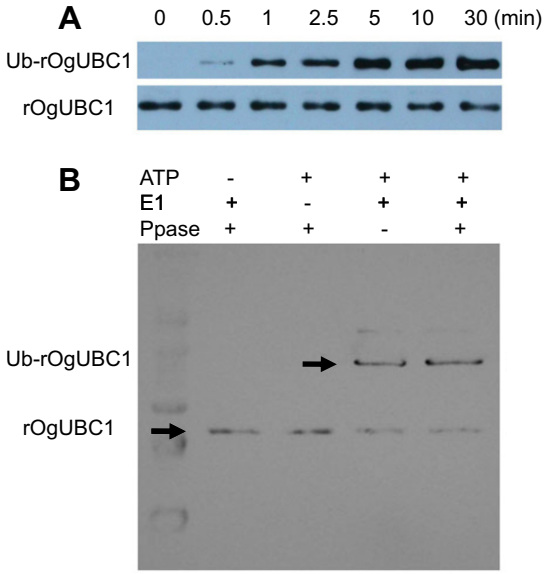

Fig. 2. Enzymatic activity of recombinant OgUBC1 protein. (A) The rOgUBC1 proteins bound to the ubiquitin. The rOgUBC1 protein coupled with 6× His was incubated with E1, and ubiquitin at 30 °C during 30 min, and then the reaction mixture were subjected to an anti-His Western blotting. The upper and lower panels indicated conjugated and un-conjugated rOgUBC1 proteins with ubiquitin, respectively. (B) Inorganic pyrophosphatase (PPase) was necessary for effective interaction between rOgUBC1 and ubiquitin.

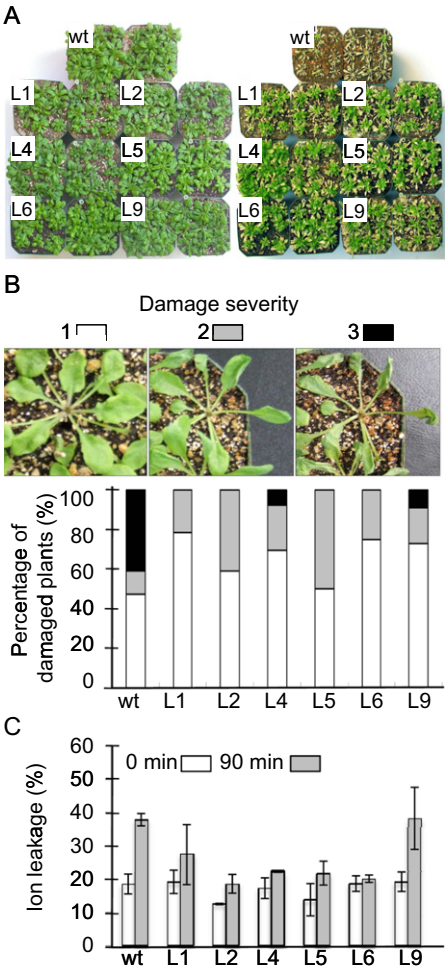

Fig. 3. OgUBC1-expressing Arabidopsis plants were less sensitive to an excess of UV-B radiation. (A) UV-B irradiated both wild type and six different transgenic lines expressing *OgUBC1* during 4 h. Photos were taken 10 days after UV-B irradiation. (B) Six different transgenic plants showed either weak or moderate symptoms, compared to wild-type plants. Representative plants damaged by UV-B irradiation are shown in the upper panel. Damage index 1, <30% (white), index 2, <60% (gray), and index 3, >60% (black). (C) Ectopic expression of *OgUBC1* prevented cell disruption caused by UV-B irradiation. Plants were exposed to UV-B illumination and leaf disks were taken at 90 min in order to check the level of ion leakage.

OgUBC1 prevented cell disruption by UV-B irradiation, ion leakage was measured in leaves of wild type and transgenic plants exposed to UV-B radiation (Fig. 3C). In the absence of UV-B radiation, similar degrees of ion leakage were observed both in wild type and all of transgenic plants. In the treatment of 90 min of UV-B irradiation, the value of wild-type plants reached 36.6%, whereas the value went up to 19∼31% in transgenic plants. Even though some variations were detected among OgUBC1-expressing plants, UV-B radiation led to more severe breakage in cell membrane of wild type than those in OgUBC1-expressing plants. This result suggested that OgUBC1 might act as a positive regulator for the protective mechanisms in plants exposed to UV-B radiation. An interesting thing is that overexpression of Arabidopsis DDB1A (UV-DAMAGED DNA BINDING PROTEIN 1A) conferred UV-tolerance on plants, whereas Arabidopsis ddb2 mutants were hypersensitive to UV radiation [37,38]. Both DDB1A and DDB2 associated with CULLIN4 (CUL4) to form E3 complex that is responsible for DNA repair upon UV radiation in Arabidopsis, indicating that the ubiquitination of certain substrates is one of protective cellular responses to UV radiation [39]. Thus we concluded that the OgUBC1 could play a part in the ubiquitin–proteasome system to coupling with unidentified E3s for UV protection in wild rice plants. The further studies are necessary for identifying E3s and their target proteins important for UV-B tolerance.

3.4. OgUBC1 induced expression of defense-related genes and accumulation of anthocyanin in leaves of Arabidopsis

The stress responsiveness of OgUBC1-expressing plants proposed a possibility that the expression of *OgUBC1* brought some physiological changes in Arabidopsis. First of all, *PR1* and *PR5*, which are representative genes of SA-dependent response, were

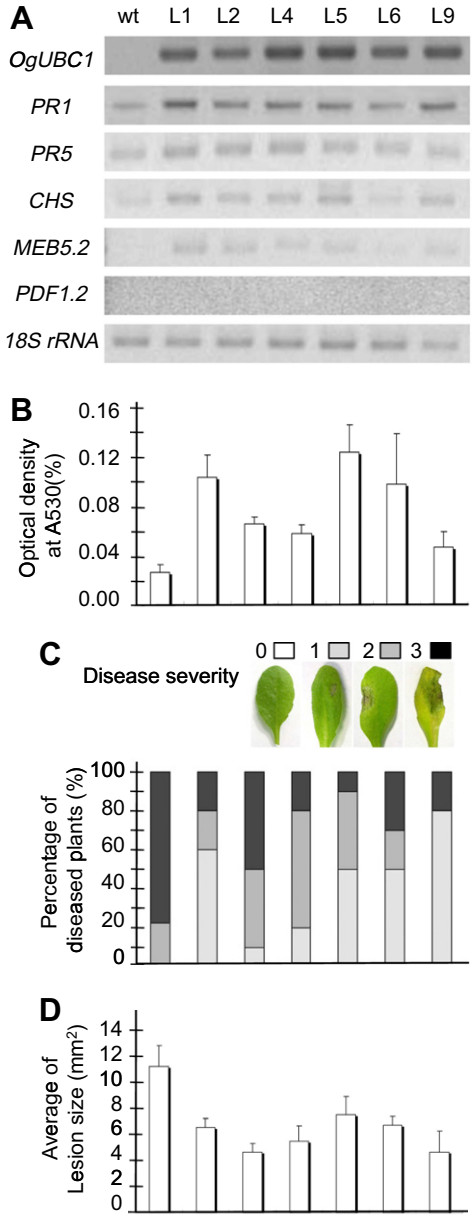

Fig. 4. Enhanced defense responses in OgUBC1-expressing Arabidopsis. (A) Expression of defense-related genes in leaves of wild type and OgUBC1-expressing plants in the absence of any stresses. (B) An increase of anthocyanin content in OgUBC1-expressing plants, compared to those in wild type. Anthocyanin levels in Arabidopsis leaves (0.1 g) were determined by measuring the optical density at 530 nm (OD_{530}). (C) Heterologous *OgUBC1* expression conferred disease resistance on Arabidopsis against infection of *Botrytis cinerea* (5×10^5 conidia/mL). Disease severity was scored in infected leaves 6 days after inoculation (0, no necrosis; 1, mild necrosis; 2, half necrosis; 3, severe necrosis symptom, upper panel). (D) Lesion developments by *B. cinerea* were restricted in OgUBC1-expressing plants.

robustly induced upon overexpression of *OgUBC1* in transgenic Arabidopsis (Fig. 4A). However, expression of PDF1.2, an inducible gene by JA-dependent signaling, was hardly detected in OgUBC1-expressing Arabidopsis. As we mentioned earlier, *OgUBC1* mRNA were strongly expressed in leaves of wild rice treated with SA, but not JA (Fig. 2A). The observations demonstrated that not only expression of *OgUBC1* was dependent on SA-related signaling, but OgUBC1 also regulated expression of SA-induced genes through an undefined mechanism(s). It was known that genes for the pigment accumulation were differentially expressed in plants exposed by UV-B radiation [40]. mRNA of chalcone synthase gene (*CHS*) was slightly induced in leaves of OgUBC1-overexpressing plants, compared to wild-type plants (Fig. 4A). Since CHS catalyzes first step in flavonoid biosynthesis, we expected that the tolerance of OgUBC1-expressing Arabidopsis might be the outcome of enhanced accumulation of the protective antioxidant pigment against UV-B radiation. To test the prospect, we measured endogenous anthocyanin levels in leaves of wild type and OgUBC1-expressing plants (Fig. 4B). Even if levels of anthocyanin varied in independent lines, 2–4-fold higher amounts of anthocyanin were accumulated in transgenic Arabidopsis, compared to those in wild-type plants. These results suggested that OgUBC1 enhanced accumulation of antioxidant pigment via regulating expression of CHS gene, which leads to UV protection in plants.

3.5. OgUBC1-expressing Arabidopsis were resistant against B. cinerea infection

In order to test the role of OgUBC1 against pathogen infection, OgUBC1-expressing Arabidopsis plants were inoculated with *B. cinerea*. Eighty percent of wild-type plants were scored as 3 and the remained 20% as 2 (Fig. 4C). Even if the disease susceptibilities were varied among 6 independent transgenic lines, most of them were resistant against the *Botrytis* infection, compared to wild type. Lesion diameter in all of transgenic lines was smaller (4.7–7.5 mm^2) than those in wild type (11.2 mm^2) (Fig. 4D). Thus we proposed that ectopic expression of *OgUBC1* was enough to confer disease resistance on Arabidopsis against *Botrytis* infection. In conclusion, we suggested that SA- and UV-B-induced *OgUBC1* is a potential genetic source for developing genetically modified plants exhibiting resistance response against biotic and abiotic stresses.

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