Phenylalanine ammonia-lyase and phenolic compounds are related to hybrid lethality in the cross *Nicotiana suaveolens* × *N. tabacum*

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Abstract Hybrid lethality observed in hybrid seedlings between *Nicotiana suaveolens* and *N. tabacum* is characterized by browning, initially of the hypocotyls and eventually of entire seedlings. We investigated the mechanism underlying this browning of tissues. A phenylalanine ammonia-lyase (PAL) gene codes an enzyme involved in a pathway producing phenolic compounds related to the browning of plant tissues. The expression of PAL rapidly increased with the induction of hybrid lethality. Phenolic compounds were observed to be accumulated in whole parts of hybrid seedlings. Treatment of hybrid seedlings with L-2-aminooxy-3-phenylpropionic acid (AOPP), an inhibitor for PAL, suppressed browning and decreased the phenolic content of hybrid seedlings. Although programmed cell death (PCD) was involved in hybrid lethality, AOPP treatment also suppressed cell death and enhanced the growth of hybrid seedlings. These results indicated that PAL is involved in hybrid lethality, and phenolic compounds could be the cause of hybrid lethality-associated tissue browning.

Key words: AOPP, hybrid lethality, phenolic compounds, phenylalanine ammonia-lyase, programmed cell death.

Introduction

Hybrid lethality, a type of postzygotic isolation in reproductive isolation, is a phenomenon involving the mortality of hybrid plants, and is observed in certain cross combinations in several plant species including *Oryza sativa* (Chen et al. 2014; Fu et al. 2013; Oka 1957; Shiragaki et al. 2019), *Nicotiana* spp. (Tezuka and Marubashi 2004; Tezuka et al. 2007), and *Arabidopsis thaliana* (Bomblies et al. 2007). This phenomenon can prevent breeders from introducing desirable genes into domesticated species through crossbreeding.

In the genus *Nicotiana*, hybrid lethality has been reported in several interspecific crosses between cultivated species such as *Nicotiana tabacum* and wild species including *N. suaveolens* (Marubashi and Kobayashi 2002; Tezuka and Marubashi 2004; Tezuka et al. 2007, 2010; Yamada et al. 1999). Hybrid seedlings of the cross *N. suaveolens* × *N. tabacum* show browning of both hypocotyls and roots as early symptoms; eventually, entire seedlings turn brown at 28°C. The mechanism

underlying lethality involves programmed cell death (PCD) accompanied by chromatin condensation, nuclear fragmentation, and internucleosomal fragmentation of DNA (Tezuka and Marubashi 2004; Yamada et al. 2000). Programmed cell death is also observed in other *Nicotiana* interspecific crosses for which hybrid seedlings show hybrid lethality (Marubashi and Kobayashi 2002; Marubashi et al. 1999). Hybrid lethality as well as PCD are suppressed at high temperatures (32–36°C) (Manabe et al. 1989; Marubashi and Kobayashi 2002; Mino et al. 2002; Tezuka and Marubashi 2004; Yamada et al. 1999).

Several factors involved in PCD during hybrid lethality in the genus *Nicotiana* have been reported; i.e., reactive oxygen species (ROS) (Mino et al. 2002; Yamamoto et al. 2017), NADPH oxidase (Mino et al. 2002), and ethylene (Yamada and Marubashi 2003; Yamada et al. 2001). Suppression subtractive hybridization has indicated that several genes including plant PCD-related genes, ethylene-related genes, and auxin-related genes are involved in hybrid lethality (Masuda et al. 2007). Furthermore, PCD during hybrid lethality includes

Abbreviations: PAL, phenylalanine ammonia-lyase; PCD, programmed cell death; AOPP, L-2-aminooxy-3-phenylpropionic acid.

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autophagic features induced by the accumulation of protein aggregates, including proteasome subunits (Ueno et al. 2016, 2019). Although several factors have been reported to be involved in hybrid lethality (as mentioned above), it is not understood what factor is directly related to the browning of hybrid seedlings, a key characteristic of hybrid lethality.

PHENYLALANINE AMMONIA-LYASE (*PAL*) is a disease resistance-related gene (Dempsey et al. 2011). *PAL* is the first enzyme in the phenylpropanoid pathway and converts L-phenylalanine produced in the shikimate pathway into cinnamic acid. Phenolic compounds related to the browning of plants are produced via cinnamic acid (Dempsey et al. 2011). When plants are infected with a pathogen, *PAL* induces the production of salicylic acid and phytoalexin (Hahlbrock and Scheel 1989; Lawton and Lamb 1987; Pellegrini et al. 1994).

Because disease resistance responses are suggested to be involved in hybrid lethality in *Nicotiana* (Masuda et al. 2007), and because *PAL* induces the production of phenolic compounds related to plant browning, we predicted that *PAL* is involved in hybrid lethality and is directly related to the browning of seedlings during hybrid lethality. Therefore, in this study, we investigated the expression of *PAL* and the effect of a competitive inhibitor of *PAL* on hybrid lethality to determine whether *PAL* and phenolic compounds are related to hybrid lethality in the cross *N. suaveolens* × *N. tabacum*.

Materials and methods

Plant materials

Interspecific hybrid seedlings were obtained from the cross between *Nicotiana suaveolens* Lehm. (♀) and *N. tabacum* L. 'Hicks-2' (♂) (seeds from the Leaf Tobacco Research Center, Japan Tobacco Inc., Oyama, Japan). Although hybrid lethality is observed in the reciprocal hybrid seedlings, it is difficult to obtain hybrid seedlings from the cross between *N. tabacum* (♀) and *N. suaveolens* (♂), because test-tube pollination and ovule culture are necessary to produce the hybrid seedlings (Tezuka and Marubashi 2004). Therefore, only hybrid seedlings from the cross *N. suaveolens* (♀) × *N. tabacum* (♂) were used in the present study. Seeds of the parents were placed in a nursery bed filled with culture soil (Super Mix A; Sakata Seed, Yokohama, Japan) in a constant-temperature room (25°C, 12/12 h light/dark cycle, approximately 80 μmol m⁻² s⁻¹). Two weeks after germination, the seedlings were transplanted to pots (9 cm diameter, 10 cm depth) filled with a 1:2 mixture of vermiculite (Type GS; Nittai, Osaka, Japan) and culture soil (Super Mix A). Four weeks after germination, seedlings were transferred to larger plastic pots (15 cm diameter, 12 cm deep) filled with a 1:2 mixture of vermiculite and culture soil in a greenhouse (natural day length; Osaka Prefecture University, Sakai, Japan), where the temperature was maintained above 15°C. Flowers of *N. suaveolens* were emasculated 1 day before anthesis and

pollinated with fresh *N. tabacum* pollen. Seeds were harvested about a month after pollination and preserved in a desiccator under low-temperature conditions (4°C) until use in our experiments.

Real-time RT-PCR for *PAL* gene

Seeds of both parent species and the resultant hybrid were sown in flat-bottomed test tubes (25 mm diameter, 100 mm length) that contained 10 ml of half strength of Murashige and Skoog medium (1/2 MS medium) (Murashige and Skoog 1962) supplemented with 1% (w/v) sucrose and 0.2% (w/v) Gellangum (pH 5.8) in an incubator at 28°C (24 h light). Immediately after germination, seedlings were transferred to 36°C, a temperature at which hybrid lethality is completely suppressed (Tezuka and Marubashi 2004; Yamada et al. 2000). After being in culture for 20 days, seedlings were transferred to 28°C to induce hybrid lethality. This experimental system, including the transfer of seedlings preliminarily cultured at 36 to 28°C, enabled synchronization of the timing of induced hybrid lethality among seedlings.

Total RNA was isolated from the shoots of seedlings using an ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. RNA purification was performed using a RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA was removed from the RNA solution using a RNase-Free DNase set (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using random hexamers and a SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed in a SmartCycler® II System (Cepheid, Sunnyvale, CA, USA) with Premix Ex Taq™ (TaKaRa, Otsu, Japan). *Actin* was used as an internal control. Primers for qPCR were designed using Primer 3 (Koressaar and Remm 2007). We used the following primers: *PAL*: forward 5'-ACC AGG TGA AGA ATG TGA CAA AGT-3', reverse 5'-CAA AGA TCC TGT TGT TTG AGA ACC-3', *Actin*: forward 5'-AGT CCT CTT CCA GCC ATCCA-3', reverse 5'-GTT GAG CCA CCA CTG AGC AC-3'. Each real-time PCR analysis was performed using three biological replicates.

Treatment of hybrids with AOPP

To investigate the involvement of *PAL* in hybrid lethality, L-2-Aminoxy-3-phenylpropionic Acid (AOPP), a competitive inhibitor of *PAL* (Amrhein et al. 1976; Zoń and Miziak 2017), was used. Hybrid seeds were put in flat-bottomed test tubes (25 mm diameter, 100 mm length) that contained 10 ml of 1/2 MS medium supplemented with 1% (w/v) sucrose and 0.2% (w/v) Gellangum (pH 5.8) in an incubator (16/8 h light/dark cycle, 28°C, approximately 150 μmol m⁻² s⁻¹). One day after germination, hybrid seedlings were transferred to 1/2 MS medium supplemented with 1% (w/v) sucrose, 0.2% (w/v) Gellangum (pH 5.8), with either 0, 0.25 or 0.5 mM AOPP added. Some hybrid seedlings, used as a control, were transferred to 36°C after the subculture was changed to the

fresh 1/2 MS medium without AOPP. The effect of AOPP on hybrid lethality was evaluated by fresh weight, amount of total phenol, and cell death (dead cells, chromatin condensation and nuclear fragmentation) in seedlings. For each of the three biological replicates for fresh weight, we took measurements from a pool of 10 plants.

Measurement of total phenolic compounds

The amount of total phenolic compounds was measured using the Folin–Denis method (Vieira and Fatibello-Fulho 1998). Part of each hybrid seedling (100 mg) was ground in a mortar with liquid nitrogen, and 1 ml of 80% (v/v) methanol was then added. The mixture was transferred to 50 ml tube, and centrifuged for 5 min at 10,000 *g*. The aqueous phase was transferred to a new tube, and methanol was evaporated by incubation at 80°C. The remaining solution (5 ml) and was mixed with 5 ml of 1 N phenol reagent. After 3 min, 5 ml of 10% (w/v) Na₂CO₃ was added. The mixture was incubated at 20°C for 1 h. The absorbance of total phenolic compounds was measured at 700 nm using a spectrophotometer (V-530; Jasco Corp., Hachioji, Japan). The amount of total phenolic compounds was calculated based on the calibration curve made by chlorogenic acid.

Detection of dead cells

Hybrid seedlings were sectioned and treated with an enzyme solution containing 2% (v/v) cellulase Onozuka R-10 (Yakult Pharmaceutical Ind. Co., Tokyo, Japan), 0.2% (v/v) Macerozyme R-10 (Yakult), 0.7 M mannitol and 10 mM CaCl₂, pH 5.6, for 3 h at 30°C. Protoplasts were separated from cellular debris using a 32 μ m nylon mesh. After centrifugation for 5 min at 100 *g*, the supernatant was discarded, and the protoplast pellet was resuspended in 0.7 M mannitol. This cell suspension was dropped onto a glass slide, and stained with 2 μ l Evans blue for 5 min. We observed the protoplasts using a microscope (BX50; Olympus, Tokyo, Japan), and considered protoplasts stained with blue as dead cells. A minimum of 100 protoplasts were observed for each experiment, and each experiment was performed using three biological replicates.

Detection of chromatin condensation

Protoplasts were isolated and stained using the same method as described above. Protoplasts stained with 0.5% (v/v) 4',6-diamino-2-phenylindol dihydrochloride (DAPI) were observed under a fluorescence microscope (BX50; Olympus, Tokyo, Japan) with U excitation (330–385 nm). We counted the number of protoplasts showing chromatin condensation, a feature of PCD (Supplementary Figure S1) (Tezuka and Marubashi 2004; Yamada et al. 2000). More than 100 protoplasts were observed in each experiment, and each experiment was performed using three biological replicates.

Detection of nuclear fragmentation by flow cytometry

Nuclear fragmentation, a feature of PCD, was detected by flow

cytometry (Tezuka and Marubashi 2004; Yamada et al. 2000). The hybrid seedlings were chopped in ice-cold Otto I buffer (Doležel and Bartos 2005; Otto 1990). The resulting extract was filtered through a 30 μ m nylon mesh, after which 1.6 ml Otto II buffer (Doležel and Bartos 2005; Otto 1990) containing 2 μ g ml⁻¹ DAPI was added. For each sample, the DNA content of at least 10,000 nuclei was analyzed with a CyFlow Space flow cytometer (Partec GmbH, Munster, Germany) (Supplementary Figure S2). Based on the histograms obtained from flow cytometry, the nuclear fragmentation percentage was calculated by the formula [(Area of typical peak/Area of total count) × 100] provided by the WinMDI version 2.9 software for flow cytometric analysis (Scripps Research Institute, La Jolla, CA, USA).

Statistical analysis

Data were analyzed with the SPSS program (version 22) (IBM Corp., Armonk, USA). Tukey HSD tests were used to compare fresh weight, amount of total phenol, and percentages of dead cells, chromatin condensation, and nuclear fragmentation among seedlings.

Results

PAL gene expression during hybrid lethality

When hybrid seedlings were transferred from 36 to 28°C, stems turned slightly brown at 5 h after transfer, and then turned obviously brown from 12 h after transfer. Leaves began to turn yellow at 3 days after transfer and the entire seedlings turned brown at 10–15 days after transfer (Figure 1). No such symptoms were observed in any parental seedlings.

We surveyed *PAL* gene expression during hybrid lethality in order to determine whether *PAL* is involved

Figure 1. Time course changes of visible lethal symptoms in the hybrid seedlings of the cross *N. suaveolens* × *N. tabacum* after transfer from 36 to 28°C. After being cultivated at 36°C for 20 days after germination (A), the seedling exposed to 28°C and cultured for 1 (B), 3 (C), 5 (D), and 12 (E) hours, and 1 (F), 3 (G), 5 (H), 10 (I), and 15 (J) days.

Figure 2. Time course changes of relative transcript level for *PAL* gene in the hybrid seedlings of the cross *N. suaveolens* × *N. tabacum*. Analyses were performed by real-time RT-PCR with RNA extracted from the shoots of seedlings transferred from 36 to 28°C and cultured for 0, 1, 3, 5 and 12 h, and 1, 3, 5, 10 and 15 days. Data were normalized to expression of *Actin*. Values and error bars indicate mean ± SE values ($n=3$). Each sample consisted of a mixture of three individuals.

Figure 3. Appearances of hybrid seedlings of the cross *N. suaveolens* × *N. tabacum* treated with AOPP at 28°C. A hybrid seedling without AOPP treatment at 5 days after germination (DAG) (A) and 14 DAG (D). A hybrid seedling treated with 0.25 mM AOPP at 5 DAG (B) and 14 DAG (E). A hybrid seedling treated with 0.5 mM AOPP at 5 DAG (C) and 14 DAG (F). (G) Overall appearances of hybrid seedlings treated with 0 mM (left), 0.25 mM (center) and 0.5 mM (right) AOPP at 14 DAG. Scale bars indicate 5 mm.

in hybrid lethality. Although the transcript levels of the *PAL* gene tended to increase in samples from both parents and hybrid seedlings, the levels decreased after reaching a peak at 3 h after transfer in plants of both parents. Meanwhile, the levels in hybrid seedlings were higher than those in both parents at 1 h after transfer

Figure 4. Amount of total phenol of hybrid seedlings of the cross *N. suaveolens* × *N. tabacum* treated with AOPP at 28°C. Values and error bars indicate mean ± SE values ($n=3$). Different letters in graphs indicates significant differences among AOPP treatments (Turkey HSD test, $p<0.05$).

(Figure 2). The level in hybrid seedlings increased further at 3 h and the levels remained elevated at least until 15 days after transfer (Figure 2).

Accumulation of phenolic compounds during hybrid lethality

During hybrid lethality, hypocotyls of hybrid seedlings at 28°C began to turn brown at 4 days after germination (DAG). Whole seedlings turned brown at 14 DAG, although emerging true leaves and root tips sometimes did not show browning (Figure 3). We surveyed the amount of total phenolic compounds in parts of hybrid seedlings, since *PAL* is involved in production of phenolic compounds. In hybrid seedlings cultured at 36°C, no lethal symptoms appeared and there was no change in the amount of total phenol during the culture period (Figure 4). The amount of total phenolic compounds in hybrid seedlings at 28°C was higher than that of hybrid seedlings at 36°C throughout 4 to 14 DAG (Figure 4).

Effect of AOPP on lethal symptoms and phenolic accumulation

Hybrid seedlings cultured at 28°C were treated with the *PAL* inhibitor AOPP. While hypocotyls of hybrid seedlings not treated with AOPP turned obviously brown at 5 DAG (Figure 3A), those of hybrid seedlings treated with AOPP did not (Figure 3B, C). Hypocotyls of hybrid seedlings treated with AOPP began to turn brown at 7 DAG (data not shown). In hybrid seedlings treated with 0.25 mM AOPP, cotyledons faded but true leaves were enlarged compared with hybrid seedlings without AOPP at 14 DAG (Figure 3D, E). Hybrid seedlings treated with 0.5 mM AOPP had green true leaves, and the number of true leaves were higher than in hybrid seedlings treated with 0.25 mM AOPP (Figure 3E, F). Browning of roots

Figure 5. Fresh weight of hybrid seedlings of the cross *N. suaveolens* × *N. tabacum* treated with AOPP at 28°C. Values and error bars indicate mean ± SE values, though error bars are very small and hide under symbols ($n=3$). Different letters in graphs indicates significant differences among AOPP treatments (Turkey HSD test, $p<0.05$).

was suppressed in hybrid seedlings treated with AOPP (Figure 3G). The fresh weights of hybrid seedlings treated with 0.25 and 0.5 mM AOPP were higher than that with no AOPP treatment at both 9 and 14 DAG (Figure 5).

Total phenolic compounds in hybrid seedlings treated with 0.25 and 0.5 mM AOPP at 28°C was higher than that without AOPP at 36°C, but significantly lower than that without AOPP at 28°C (Figure 4).

Effect of AOPP on cell death

We further investigated whether AOPP treatment suppressed cell death during hybrid lethality. Three indicators of cell death, the percentage of dead cells, chromatin condensation, and nuclear fragmentation, were evaluated (Figure 6). At both 9 and 14 DAG, the percentages of dead cells in hybrid seedlings treated with AOPP were lower than that without AOPP treatment, and the dead cell percentage of hybrid seedlings treated with 0.5 mM AOPP was lowest (Figure 6A). The percentage of chromatin condensation increased at 9 DAG regardless of AOPP treatments, and the percentage in hybrid seedlings treated with 0.25 mM AOPP tended to be lower than those without AOPP and with 0.5 mM AOPP at both 9 and 14 DAG (Figure 6B). The percentage of nuclear fragmentation in hybrid seedlings treated with 0.25 mM AOPP didn't increase from 4 to 14 DAG and was lowest among all treatments, whereas the percentages without AOPP and with 0.5 mM AOPP increased (Figure 6C).

Discussion

Hybrid lethality in the cross *N. suaveolens* × *N. tabacum* is characterized by browning of the hypocotyls; this is the first visible sign of lethality, although the whole seedling eventually turns brown. However, the mechanism causing to the browning has been unclear to date. In the

Figure 6. Evaluation of cell death in hybrid seedlings of the cross *N. suaveolens* × *N. tabacum* treated with AOPP at 28°C. (A) Percentages of dead cells evaluated by Evans blue staining. (B) Percentages of chromatin condensation evaluated by microscopic observation. (C) Percentages of nuclear fragmentation evaluated by flow cytometry. Values and error bars indicate mean ± SE values ($n=3$). Different letters in graphs indicates significant differences among AOPP treatments (Turkey HSD test, $p<0.05$).

present study, we determined that gene expression of *PAL* increased in hybrid seedlings immediately after transfer from 36 to 28°C (Figure 2). The amount of total phenolic compounds increased in hybrid seedlings at 28°C but not at 36°C (Figure 4). Furthermore, a *PAL* inhibitor, AOPP, suppressed browning and decreased the phenolic compound content of hybrid seedlings (Figures 3, 4). Programmed cell death during hybrid lethality was also suppressed (Figure 6), and hybrid seedling growth was enhanced by AOPP (Figures 4, 5). Considering these results, it is suggested that early up-regulation of *PAL* and a subsequent increase in phenolic compound content is the cause of browning in hybrid seedlings.

Several studies have reported that *PAL* is involved in hybrid lethality among inter- and intraspecific hybrids in wheat and its relatives (Jiang et al. 2008; Mizuno et al. 2010, 2011; Takamatsu et al. 2015). These studies have reported upregulation of *PAL* expression (Mizuno et al. 2010, 2011; Takamatsu et al. 2015) and accumulation of *PAL* protein (Jiang et al. 2008). Hybrid lethality in wheat and its relatives shows a hypersensitive response-

like reaction; i.e., generation of ROS, cell death, and an upregulation of defense-related genes (Mizuno et al. 2010, 2011; Okada et al. 2017; Takamatsu et al. 2015). Considering the common points between hybrid lethality in the genus *Nicotiana* (Masuda et al. 2007; Mino et al. 2002; Tezuka and Marubashi 2004; Yamada et al. 2000) and that in wheat and its relatives, a hypersensitive response-like reaction is likely to be involved in the upregulation of *PAL* during hybrid lethality in the genus *Nicotiana*.

There is a possibility that *PAL* induces hybrid lethality via a hypersensitive response-like reaction. In hypersensitive responses to a pathogen attack, the expression of *PAL* increases, and salicylic acid is produced via the *PAL* pathway (Huang et al. 2010; Kushalappa et al. 2016; Zhang et al. 2017). Salicylic acid is a signal hormone for plant defense against pathogens and induces a defense response including hypersensitive cell death (Dempsey et al. 2011). In the cross *N. suaveolens* × *N. tabacum*, disease resistance-related genes were identified to be involved in hybrid lethality (Masuda et al. 2007). Additionally, both hybrid lethality and hypersensitive responses are suppressed at high temperatures (Ito et al. 2002; Tezuka and Marubashi 2004; Yamada et al. 2000; Zhu et al. 2010). Therefore, we suggest that AOPP suppresses hybrid lethality via the suppression of the hypersensitive response-like reaction involving *PAL*.

In conclusion, our results suggest that *PAL* plays an important role in hybrid lethality in the cross *N. suaveolens* × *N. tabacum*. The current study has uncovered part of the mechanistic cause of hybrid lethality and may lead to the development of a new method for reducing hybrid lethality.

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