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
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 *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 Marubushi 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) Gellan gum (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 Marubushi 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 RNasy 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 AGT TGA CA AAG 3', reverse 5'-CAA AGA TCC TGT GTG TTG AGA ACC-3', Actin: forward 5'-AGT CCT TTT CCA GCC ATCCA-3', reverse 5'-GTT TGA 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, 1,2-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) Gellan gum (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) Gellan gum (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 5 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 µm 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 µl 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 µm nylon mesh, after which 1.6 ml Otto II buffer (Doležel and Bartos 2005; Otto 1990) containing 2 µg 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).

**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...
PAL and phenolic compounds are related to 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 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...
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 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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References
Amrhein N, Godeke K, Kefeli V (1976) The estimation of relative intracellular phenylalanine ammonia-lyase (PAL)-activities and modulation in vivo and in vitro by competitive inhibitors. Ber Dtsch Bot Ges 99: 5247–5259
Bombles K, Lempe J, Epplle P, Warthmann N, Lanz C, Dangl JL, Weigel D (2007) Autoimmune response as a mechanism for a Dobzhansky–Muller-type incompatibility syndrome in plants. PLoS Biol 5: 1962–1972
Chen C, Chen H, Lin YS, Shen JB, Shan JX, Qi P, Shi M, Zhu MZ, Huang XH, Feng Q, et al. (2014) A two-locus interaction causes interspecific hybrid weakness in rice. Nat Commun 5: 3357
Dempsey DA, Vlot AC, Wildermuth MC, Klessig DF (2011) Salicylic acid biosynthesis and metabolism. Arabidopsis Book 9: e0156
Dolezel J, Bartos J (2005) Plant DNA flow cytometry and estimation of nuclear genome size. Ann Bot 95: 99–110
Fu CY, Wang F, Sun BR, Liu WG, Li JH, Deng RF, Liu DL, Liu ZR, Zhu MS, Liao YL, et al. (2013) Genetic and cytological analysis of a novel type of low temperature-dependent intrasubspecific hybrid weakness in rice. PLoS One 8: e75886
Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol 40: 347–369
Huang J, Gu M, Lai Z, Fan B, Shi K, Zhou YH, Yu JQ, Chen Z (2010) Functional analysis of the Arabidopsis PAL gene family in plant growth, development, and response to environmental stress. Plant Physiol 153: 1526–1538
Ito N, Takabatake R, Seo S, Hiraga S, Mitsuhashi I, Ohashi Y (2002) Induced expression of a temperature-sensitive leucine-rich repeat receptor-like protein kinase gene by hypersensitive cell death and wounding in tobacco plant carrying the N resistance gene. Plant Cell Physiol 43: 266–274
Jiang Q, Chen H, Pan X, Pan Q, Shi Y, Li X, Zhang G, Wang Y, Xie S, Shen S (2008) Proteomic analysis of wheat (Triticum aestivum L.) hybrid necrosis. Plant Sci 175: 394–401
Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. Bioinformatics 23: 1289–1291
Kushalappa AC, Yogendra KN, Karre S (2016) Plant innate immune response: Qualitative and quantitative resistance. CRC Crit Rev Plant Sci 35: 38–55
Lawton MA, Lamb CJ (1987) Transcriptional activation of plant defence genes by fungal elicitor, wounding, and infection. Mol Cell Biol 7: 335–341
Manabe T, Marubashi W, Onozawa Y (1989) Temperature-dependent conditional lethality in interspecific hybrids between Nicotiana suaveolens Lehm. × N. tabacum L. In: Proceedings of the 6th International Congress, SABRAO, pp 459–462
Marubashi M, Kobayashi M (2002) Temperature-dependent apoptosis detected in hybrids between Nicotiana debneyi and N. tabacum expressing lethality. Plant Biotechnol 19: 267–270
Marubashi W, Yamada T, Niwa M (1999) Apoptosis detected in hybrids between Nicotiana glutinosa and N. repanda expressing lethality. Planta 210: 168–171
Masuda Y, Yamada T, Kuboyama T, Marubashi W (2007) Identification and characterization of genes involved in hybrid lethality in hybrid tobacco cells (Nicotiana suaveolens × N. tabacum) using suppression subtractive hybridization. Plant Cell Rep 26: 1595–1604
Mino M, Maekawa K, Ogawa K, Yamagishi H, Inoue M (2002) Cell death processes during expression of hybrid lethality in interspecific F1 hybrid between Nicotiana gossei Domin and Nicotiana tabacum. Plant Physiol 130: 1776–1787
Mizuno N, Hosogi N, Park P, Takumi S (2010) Hypersensitive response-like reaction is associated with hybrid necrosis in interspecific crosses between tetraploid wheat and Aegilops tauschii Coss. PLoS One 5: e11326
Mizuno N, Shitsukawa N, Hosogi N, Park P, Takumi S (2011) Autoimmune response and repression of mitotic cell division occur in inter-specific crosses between tetraploid wheat and Aegilops tauschii Coss. that show low temperature-induced hybrid necrosis. Plant J 68: 114–128
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497
Oka H (1957) Phylogenetic differentiation of the cultivated rice. Jpn J Genet 32: 83–87
Okada M, Yoshida K, Takumi S (2017) Hybrid incompatibilities
in interspecific crosses between tetraploid wheat and its wild diploid relative *Aegilops umbellulata*. *Plant Mol Biol* 95: 625–645

Otto F (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Darzynkiewicz Z, Crissman HA (eds) *Methods in Cell Biology*. Academic Press, San Diego, pp 105–110

Pellegrini L, Rohfritsch O, Fritig B, Legrand M (1994) Phenylalanine ammonia-lyase in tobacco (Molecular cloning and gene expression during the hyperosmotic reaction to tobacco mosaic virus and the response to a fungal elicitor). *Plant Physiol* 106: 877–886

Shiragaki K, Iizuka T, Ichitani K, Kuboyama T, Morikawa T, Oda M, Tezuka T (2019) *HWA1*- and *HWA2*-mediated hybrid weakness in rice involves cell death, reactive oxygen species accumulation, and disease resistance-related gene upregulation. *Plants* 8: 450

Takamatsu K, Iehisa JC, Nishijima R, Takumi S (2015) Comparison of gene expression profiles and responses to zinc chloride among inter-and intraspecific hybrids with growth abnormalities in wheat and its relatives. *Plant Mol Biol* 88: 487–502

Tezuka T, Kuboyama T, Matsuda T, Marubashi W (2007) Possible involvement of genes on the Q chromosome of *Nicotiana tabacum* in expression of hybrid lethality and programmed cell death during interspecific hybridization to *Nicotiana debneyi*. *Planta* 226: 753–764

Tezuka T, Kuboyama T, Matsuda T, Marubashi W (2010) Seven of eight species in *Nicotiana* section *Suaveolentes* have common factors leading to hybrid lethality in crosses with *Nicotiana tabacum*. *Ann Bot* 106: 267–276

Tezuka T, Marubashi W (2004) Apoptotic cell death observed during the expression of hybrid lethality in interspecific hybrids between *Nicotiana tabacum* and *N. suaveolens*. *Breed Sci* 54: 59–66

Ueno N, Kashiwagi M, Kanekatsu M, Marubashi W, Yamada T (2016) Time course of programmed cell death, which included autophagic features, in hybrid tobacco cells expressing hybrid lethality. *Plant Cell Rep* 35: 2475–2488

Vieira ID, Fatibello-Fulho O (1998) Flow injection spectrophotometric determination of total phenol using a crude extract of sweet potato root (*Ipomoea batatas* (L.) Lam.) as enzymatic source. *Anal Chim Acta* 366: 111–118

Yamada T, Marubashi W (2003) Overproduced ethylene causes programmed cell death leading to temperature-sensitive lethality in hybrid seedlings from the cross *Nicotiana suaveolens*×*N. tabacum*. *Planta* 217: 690–698

Yamada T, Marubashi W, Nakamura T, Niwa M (2001) Possible involvement of auxin-induced ethylene in an apoptotic cell death during temperature-sensitive lethality expressed by hybrid between *Nicotiana glutinosa* and *N. repanda*. *Plant Cell Physiol* 42: 923–930

Yamamoto T, Shomura S, Mino M (2017) Cell physiology of mortality and immortality in a *Nicotiana* interspecific F1 hybrid complies with the quantitative balance between reactive oxygen and nitric oxide. *J Plant Physiol* 210: 72–83

Zhang C, Wang X, Zhang F, Dong L, Wu J, Cheng Q, Qi D, Yan X, Jiang L, Fan S, et al. (2017) Phenylalanine ammonia-lyase2.1 contributes to the soybean response towards *Phytophthora sojae* infection. *Sci Rep* 7: 7242

Zhu Y, Qian W, Hua J (2010) Temperature modulates plant defense responses through NB-LRR proteins. *PLoS Pathog* 6: e1000844

Zoń J, Miziak P (2017) 1-Aminobenzocyclobutene-1-phosphonic acid and related compounds as inhibitors of phenylalanine ammonia-lyase. *Chem Biodivers* 14: e1600488