

Comparison of gene expression profiles and responses to zinc chloride among inter- and intraspecific hybrids with growth abnormalities in wheat and its relatives

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Abstract Hybrid necrosis is a well-known reproductive isolation mechanism in plant species, and an autoimmune response is generally considered to trigger hybrid necrosis through epistatic interaction between disease resistance-related genes in hybrids. In common wheat, the complementary *Ne1* and *Ne2* genes control hybrid necrosis, defined as type I necrosis. Two other types of hybrid necrosis (type II and type III) have been observed in interspecific hybrids between tetraploid wheat and *Aegilops tauschii*. Another type of hybrid necrosis, defined here as type IV necrosis, has been reported in F₁ hybrids between *Triticum urartu* and some accessions of *Triticum monococcum* ssp. *aegilopoides*. In types I, III and IV, cell death occurs gradually starting in older tissues, whereas type II necrosis symptoms occur only under low temperature. To compare comprehensive gene expression patterns of hybrids showing growth abnormalities, transcriptome analysis of type I and type IV necrosis was performed using a wheat 38k oligo-DNA microarray. Defense-related genes including many WRKY transcription factor genes were dramatically up-regulated in plants showing type I and type IV necrosis, similarly to other known hybrid abnormalities, suggesting an association with an autoimmune response. Reactive oxygen species generation and necrotic cell death were effectively inhibited by ZnCl₂ treatment in types I, III and

IV necrosis, suggesting a significant association of Ca²⁺ influx in upstream signaling of necrotic cell death in wheat hybrid necrosis.

Keywords Calcium influx · Microarray · Postzygotic barrier · Programmed cell death · Reactive oxygen species · Wheat

Introduction

Hybrid incompatibility between two diverging lineages acts as a postzygotic reproductive barrier, and plays significant roles in intraspecific differentiation and plant speciation (Bomblies and Weigel 2007; Rieseberg and Willis 2007). Most types of hybrid incompatibility are caused by epistatic interaction of at least two loci or alleles, a basis of the Dobzhansky–Muller model (Bomblies and Weigel 2007; Presgraves 2010). Hybrid necrosis is a major incompatibility observed in higher plants, and is generally considered to be triggered by an autoimmune response. The causative genes of hybrid necrosis have been isolated in *Arabidopsis*, lettuce and rice, and commonly encode disease resistance-related proteins including NB-LRR-type resistance (R) proteins and R-interacting proteins (Alcázar et al. 2009, 2010; Bomblies et al. 2007; Chae et al. 2014; Jeuken et al. 2009; Yamamoto et al. 2010). In addition, a number of defense-related genes are up-regulated in hybrid plants exhibiting necrotic symptoms in *Arabidopsis* and *Nicotiana* (Bomblies et al. 2007; Masuda et al. 2007).

In intraspecific crosses of common wheat (*Triticum aestivum* L.), hybrid necrosis caused by interaction between dominant alleles of the *Ne1* and *Ne2* loci is frequently observed (Hermsen 1963; Tsunewaki 1960, 1970). The complementary *Ne1* and *Ne2* loci are respectively located

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on chromosome arms 5BL and 2BS, and the induction of necrotic cell death by *Ne1–Ne2* is called type I hybrid necrosis (Chu et al. 2006; Takumi et al. 2013; Tsunewaki 1960, 1992). Type I necrosis functions as a reproductive barrier contributing to intraspecific differentiation mainly between European and American varieties and middle Asian and African varieties in common wheat (Pukhalskiy et al. 2000). Distribution of the *Ne1* and *Ne2* dominant alleles is postulated to be due to selection advantages such as rust fungus resistance (Bomblies and Weigel 2007), although no direct evidence proving a relationship between type I necrosis and disease resistance has been reported. In leaves of wheat showing type I necrosis, superoxide is highly accumulated and antioxidant enzymes show high levels of activity (Dalal and Khanna-Chopra 2001; Khanna-Chopra et al. 1998; Sugie et al. 2007).

Hybrid necrosis has also been reported in interspecific crosses of wheat relatives (Nishikawa 1960; Roy 1955; Sears 1944). In interspecific hybrids showing necrotic symptoms, interplay between the distinct nuclear genomes triggers the incompatible responses, and causal genes are believed to be located in the genome of both parental species. Common wheat is an allohexaploid species (AABBDD genome) that originated through allohexaploidization between cultivated tetraploid wheat *Triticum turgidum* L. (AABB genome) and the wild diploid relative *Aegilops tauschii* Coss. (DD genome) (Matsuoka 2011). In many interspecific crosses reflecting the allopolyploid speciation process of common wheat, abnormal growth phenotypes have been found (Matsuoka et al. 2007; Nishikawa 1960, 1962a, b), and the abnormalities in the triploid hybrids could act as postzygotic hybridization barriers inhibiting the formation of common wheat. Two types of hybrid necrosis, types II and III, are observed in many F₁ triploid plants (ABD genome) of tetraploid wheat and *Ae. tauschii* (Mizuno et al. 2010, 2011; Nishikawa 1960, 1962b). Cell death occurs gradually beginning with older tissues in hybrid lines showing type III necrosis, similarly to type I necrosis, whereas type II necrosis lines show a necrotic phenotype under low temperature conditions (Mizuno et al. 2011; Nishikawa 1960). In addition to necrotic symptoms, low temperature represses mitotic cell division in the shoot apices of hybrid plants displaying type II necrosis (Mizuno et al. 2011). Causal D-genome genes, *Net2* for type II necrosis and *Nec1* for type III necrosis, are respectively assigned to chromosomes 2D and 7D, but chromosome localization of the complementary genes, *Net1* and *Nec2*, on the AB genome is unknown (Mizuno et al. 2010, 2011). A number of defense-related genes are up-regulated in both type II and III necrosis, and some are commonly up-regulated (Mizuno et al. 2010, 2011). Thus, molecular mechanisms involved in expression of the hybrid necrosis phenotypes share signal pathways related

to plant disease responses. Two types of hybrid chlorosis, one with severe and one with mild symptoms, are observed to a limited extent in triploid hybrids and synthetic hexaploid wheat lines (Mizuno et al. 2010; Nakano et al. 2015). Unlike type II and III necrosis and the two types of hybrid chlorosis, severe growth abortion (SGA) is always lethal, because SGA-exhibiting ABD triploids cease development and growth after expansion of the second or third leaves (Mizuno et al. 2010; Nishikawa 1960). In spite of their phenotypic difference from type II and III hybrid necrosis, a number of defense-related genes are highly up-regulated in seedlings of SGA-exhibiting hybrids and leaves of synthetic wheat lines with hybrid chlorosis symptoms (Hatano et al. 2012; Nakano et al. 2015).

Hybrid necrosis is frequently observed in interspecific crosses of two wild diploid *Triticum* species, *Triticum monococcum* subspecies *aegilopoides* (Link) Thell. (A^mA^m genome) and *Triticum urartu* Tumanian ex Gandilyan (AA genome) (Yamagishi 1987). This hybrid necrosis, called here type IV necrosis, is hypothetically triggered by epistatic interaction between dominant alleles of *Ned1* in the nuclear genome of *T. monococcum* ssp. *aegilopoides* and *Ned2* in the *T. urartu* genome (Yamagishi 1987). All examined *T. urartu* accessions contain the dominant allele of *Ned2*, whereas *Ned1*-carrier and non-carrier accessions are mixed in *T. monococcum* ssp. *aegilopoides* (Yamagishi 1987). The necrotic phenotype in type IV necrosis appears to resemble the symptoms in type I and III necrosis. However, no additional information on type IV necrosis has been reported.

Gene expression profiles are not necessarily consistent with each other, but some genes are mutually up- and down-regulated in type II and III necrosis and SGA of ABD triploids (Hatano et al. 2012; Mizuno et al. 2011). Here, we performed transcriptome analyses for type I and IV necrosis to elucidate the mechanisms inducing necrotic symptoms at the transcriptional level and compared gene expression profiles with those in plants showing growth abnormalities in ABD triploid and synthetic wheat to clarify the common features underlying the various types of hybrid necrosis in *Triticum*.

Materials and methods

Plant materials

Three accessions of common wheat were used for analysis of type I necrosis. The common wheat variety S-615 has no dominant alleles for *Ne1* and *Ne2*. The near-isogenic lines (NILs) carrying dominant alleles *Ne1* and *Ne2* are designated *Ne1*-S615 and *Ne2*-S615, respectively (Tsunewaki and Koba 1979). The *Ne1* and *Ne2* alleles originated from

common wheat cultivars Prelude and Kharkov, respectively (Tsunewaki 1960), and these dominant alleles were introduced into the S-615 genetic background through 10 backcrosses.

To generate interspecific hybrids with type IV necrosis, we used three accessions of *T. monococcum* subspecies *aegilopoides* (Link) Thell. (syn. *T. boeoticum* Boiss.), and five accessions of wild diploid *T. urartu* Tumanian ex Gandilyan. KU-101-3 and KU-8001 were used as *Ned1* carriers, and KU-3620 as a non-carrier in *T. monococcum* ssp. *aegilopoides*. For *T. urartu*, KU-199-5, KU-199-8, KU-199-9, KU-199-11 and KU-199-16 were used and crossed with pollen of *T. monococcum* ssp. *aegilopoides* accessions. Many of the F₁ seeds from *T. urartu* crossed with pollens of *T. monococcum* ssp. *aegilopoides* failed to germinate, as reported previously (Yamagishi 1987; Fricano et al. 2014); some plants that germinated were used for phenotyping and RNA extraction.

For studies of type III necrosis, we used two synthetic hexaploid wheat lines showing an early flowering phenotype. They were produced from two cross combinations between a tetraploid wheat accession, *T. turgidum* ssp. *durum* (Desf.) Husn. cv. Langdon (Ldn), and two *A. tauschii* Coss. accessions, KU-2059 and KU-2828 (Mizuno et al. 2010). The Ldn/KU-2059 line showed normal growth (wild type; WT) and the Ldn/KU-2828 line showed type III necrosis.

Transcriptome analysis

Two F₁ hybrids of common wheat, *Ne2*-S615/S-615 for WT and *Ne2*-S615/*Ne1*-S615 for type I necrosis, and two interspecific hybrids of diploid wheat, KU-3620/KU-199-11 for WT and KU-101-3/KU-199-5 for type IV necrosis, were used for microarray analysis. Total RNA was extracted using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany) from leaves of seedlings grown at normal temperature (23 °C) for 7 days. Necrotic symptoms started to appear on the 1st (lowest) leaves in the 7-week-old seedlings of the type I and type IV necrosis hybrids. A KOMUGI 38k oligonucleotide DNA microarray (Agilent Technologies, Santa Clara, CA) was supplied by the National BioResource Project (NBRP)-Wheat, Japan (<https://www.nbrp.jp>) for analysis. Detailed information on the 38k microarray platform can be found in Kawaura et al. (2008) and the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) website under GPL9805. Cyanine-3 (Cy3) labeled cRNA was prepared from 0.5 µg RNA using the Quick Amp Labeling kit (Agilent Technologies) according to the manufacturer's instructions. Hybridization of Cy3-labeled cRNA to the microarrays and washing were performed using a Gene Expression Hybridization kit and

Gene Expression Wash Pack (Agilent Technologies). Signal intensities were detected by Feature Extraction Software 9.5 (Agilent Technologies). Slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (G2505B) using one color scan setting for 1×44k array slides. Scanned images were analyzed with Feature Extraction Software using default parameters to obtain background-subtracted and spatially detrended processed signal intensities. Two independent experiments were conducted for each sample. All microarray data were deposited as series GSE64342 in the NCBI GEO database (<http://www.ncbi.nlm.nih.gov/geo/>), including supplementary files, GSM1568808 through GSM1568811 for type I necrosis and GSM1568812 through GSM1568815 for type IV necrosis.

Functions of probes and genes were predicted by BLASTx searches (*E* value <1e⁻³) against the NCBI non-redundant protein database. Based on the 20 top BLASTx hits, Gene Ontology (GO) annotation was performed using the Blast2GO algorithm (Conesa et al. 2005). Annotations were refined using the ANNEX function (Myhre et al. 2006) and mapped to plant-specific GO-Slim terms, both available in Blast2GO. GO terms of differentially expressed (fold change ≥3 or ≤1/3) probes were extracted and compared.

For comparison of the microarray data for type I and type IV necrosis with previously reported data for growth abnormalities in ABD triploids and synthetic wheat hexaploids, data sets including GSE19613, GSE24566, GSE33357 and GSE59640 in the NCBI GEO were respectively used for type III necrosis, type II necrosis, SGA and hybrid chlorosis. RNA samples were extracted from leaves and crown tissues of synthetic hexaploids after 6 weeks of incubation at 4 °C for plant lines with a type II necrosis phenotype, or with no low-temperature treatment for other plants (Hatano et al. 2012; Mizuno et al. 2010, 2011; Nakano et al. 2015). For the analyses of type II and type III necrosis and hybrid chlorosis, the RNA samples were extracted from leaves starting to appear the abnormal growth symptoms (Mizuno et al. 2010, 2011; Nakano et al. 2015). In total, 22,018 probes were selected from the 38k microarray based on evaluation of their signal intensity (non-saturated uniform signal greater than background signal), and used for the comparison. A heat map was constructed using the function “heatmap.2” of the R statistical package “gplots” (version 2.11.0) (Warnes 2012). Hierarchical clustering of samples and probes was performed using the “hclust” function of the “stats” package in R (version 2.15.2) with the agglomeration method set to average (R Core Team 2012). The distance between samples and probes was calculated using the function “Dist”, available in the package “amap” (version 0.8.7) (Lucas 2011), based on Pearson's correlation.

Measurement of photosynthetic activity

A portable JUNIOR-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used for measurement of the maximum photochemical quantum yield of photosystem II (F_v/F_m). The first leaves of 1-, 2- and 3-week-old WT and hybrid necrosis plants were incubated in the dark for 1 h. F_v/F_m was measured at ten independent locations on each leaf blade of three F_1 plants according to the instructions in the operating manual for the fluorometer. For type IV necrosis, three F_1 plants for each cross were analyzed, and the first leaves of 4-, 6- and 8-day-old WT and hybrid necrosis plants were used for measurement of photosynthetic activity.

Quantitative reverse transcription (qRT-PCR) analysis

qRT-PCR was performed using RNA isolated from leaves of WT and hybrid necrosis plants. Each plant was grown at 23 °C, and total RNA was extracted using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). First-strand cDNA was synthesized from DNase I-treated RNA samples using ReverTra Ace Reverse Transcriptase (Toyobo, Osaka, Japan) and an oligo(dT)₂₀ primer. Accumulation of transcripts of each gene was detected using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) with THUNDERBIRD SYBR qPCR Mix (Toyobo) and gene-specific primer sets. The gene-specific primer sets for qRT-PCR are listed in Supplemental Table 1. The *Actin* gene was used as an internal control, and relative expression was calculated as $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001), representing the value relative to the transcript levels in leaves of WT (*Ne2-S615/S-615* or *KU-3620/KU-199-16*). Mean values were calculated based on three technical replicates.

Zinc chloride treatment and trypan blue and diaminobenzidine (DAB) staining

Each hybrid plant was grown at 23 °C, watering every day with 0.2 % Hyponex (Hyponex Japan, Osaka, Japan) solution with or without 0.02 mM $ZnCl_2$, which functions as a calcium antagonist. The $ZnCl_2$ -treated plants were used for staining with trypan blue and DAB, measurement of photosynthetic activity and extraction of RNA.

Trypan blue was used for detection of dead cells. Leaf samples were submerged in 0.5 % trypan blue staining solution (Nacalai Tesque). After boiling for 1 min, the samples were incubated at room temperature for 25 min and washed with water containing 2.5 mg/mL chloral hydrate. DAB (Wako, Osaka, Japan) was used for H_2O_2 detection. Leaf samples were infiltrated with 2 mg/mL DAB solution (pH 3.8) for 1 h, and then treated with 99 % ethanol for

chlorophyll removal. The trypan blue- and DAB-stained leaves were observed under bright field illumination under an Olympus BX60 microscope (Olympus, Tokyo, Japan).

Results

Phenotypes of type I and type IV necrosis

Hybrid plants showing type I necrosis were generated by crossing *Ne1-S615* and *Ne2-S615* NILs. All of the F_1 plants clearly showed a hybrid necrosis phenotype of gradual premature death of leaves or plants (Fig. 1a). At the four-leaf stage, F_1 plants with type I necrosis exhibited complete necrotic cell death of the first leaf. Type IV necrosis plants were produced by interspecific crossing between *KU-101-3* and *KU-199-16*, and all F_1 plants exhibited necrotic symptoms at the seedling stage (Fig. 1b). The first leaves showed complete cell death at the two-leaf stage. F_1 plants showing type I or IV necrosis were killed during the vegetative growth phase at normal ambient temperature (23 °C).

According to a previous report (Yamagishi 1987), some wild einkorn wheat accessions show hybrid necrosis in interspecific crosses with *T. urartu* accessions, and others exhibit a WT phenotype without any necrotic symptoms. We confirmed appearance of the necrotic phenotype in some cross combinations between wild einkorn wheat and *T. urartu* (Table 1). *KU-3620* showed a WT phenotype without any necrotic symptoms in all examined crosses with four *T. urartu* accessions, whereas necrotic symptoms were clearly observed in leaves of hybrid plants between two other ssp. *aegilopoides* accessions and *T. urartu* accessions.

Microarray analysis of type I and type IV necrosis

To comprehensively compare gene expression profiles between WT (*Ne2-S615/S-615*) and type I necrosis plants (*Ne2-S615/Ne1-S615*), we analyzed the transcriptomes using a wheat-specific 38k oligo DNA microarray (Kawaura et al. 2008). After hybridization with the RNA samples, probes showing significant ($P < 0.05$) difference in signal intensity compared to the WT were defined as either up- or down-regulated genes. Of the 37,826 probes on the wheat microarray, 405 (1.1 %) and 1254 (3.3 %) probes were respectively regarded as genes up- and down-regulated in leaves of the type I necrosis plants compared with WT plants (Supplemental Table 2). Based on a BlastX search against the NCBI nr protein database, 1219 differentially expressed probe sequences were assigned to one or more GO annotations. GO analysis showed that the up- and down-regulated genes could be categorized into 17 functional categories (Fig. 1c). Expression of genes related to

Fig. 1 Transcriptome analysis of type I and type IV necrosis. **a** Leaves of hexaploid wheat hybrids with (*lower*) and without (*upper*) type I necrosis symptoms. **b** Leaves of interspecific hybrids of two wild diploid wheat species with (*lower*) and without (*upper*) type IV necrosis symptoms. **c, d** Summary of microarray analyses. Up- and down-regulated genes in type I (**c**) and type IV (**d**) necrosis were categorized into 17 functional categories

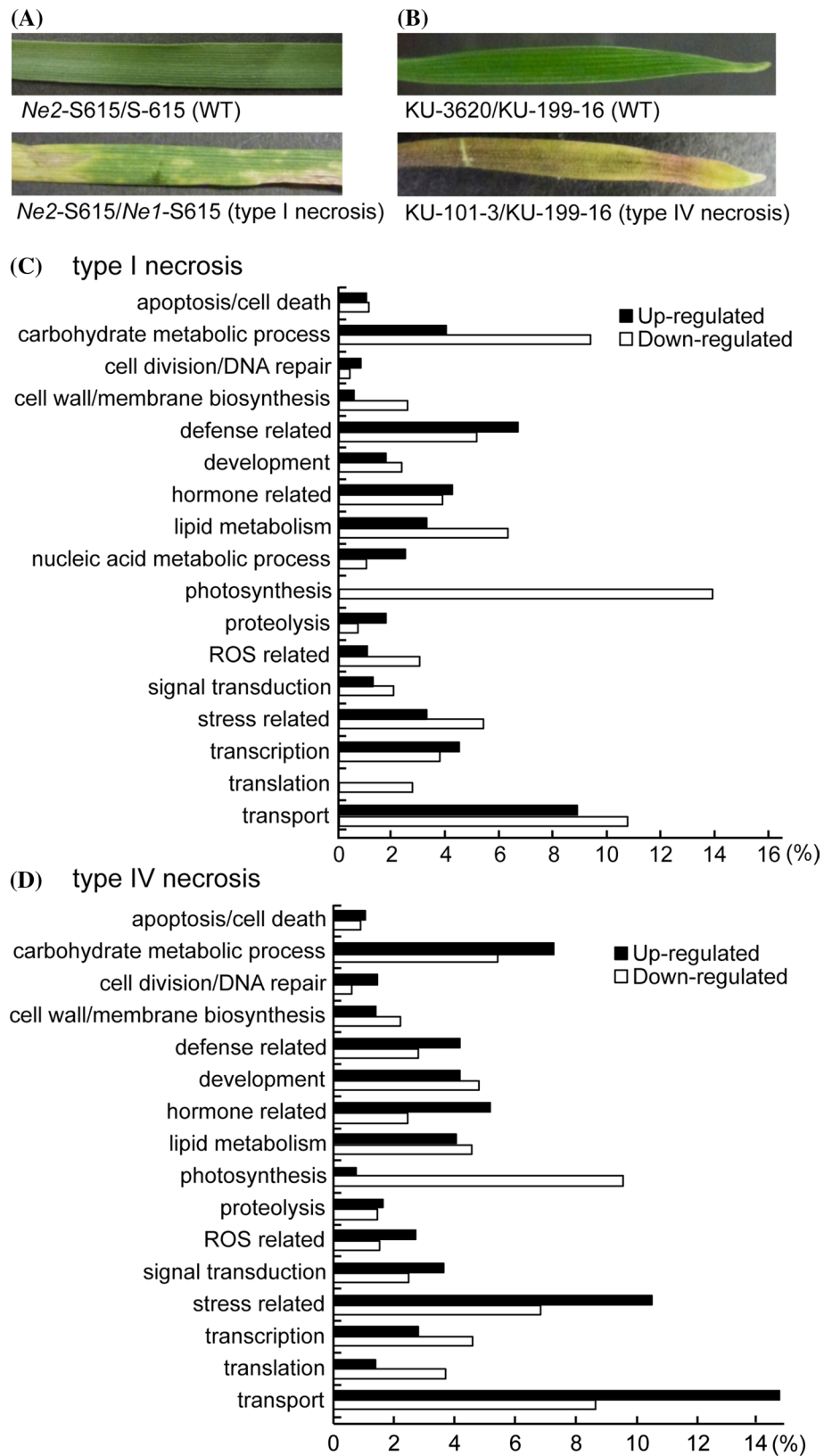


Table 1 Hybrid plant phenotype in interspecific crosses between two wild einkorn wheat species

	<i>T. monococcum</i> ssp. <i>aegilopoides</i>	<i>T. urartu</i>				
		KU-199-5	KU-199-8	KU-199-9	KU-199-11	KU-199-16
KU-101-3		Necrosis	Necrosis	Necrosis	Necrosis	Necrosis
KU-8001		n.d.	Necrosis	n.d.	Necrosis	Necrosis
KU-3620		Wild-type	n.d.	Wild-type	Wild-type	Wild-type

n.d., not determined; KU, Plant Germ-Plasm Institute, Faculty of Agriculture, Kyoto University, Japan

Table 2 Top 12 transcription factor genes identified by microarray analysis as up-regulated in seedling leaves of hybrid plants between *Ne1-S615* and *Ne2-S615*

Accession no.	Protein	<i>E</i> value	Log ₂ ratio
ABF57921	<i>Triticum aestivum</i> MADS-box transcription factor TaAGL2	1.54E ⁻¹⁰¹	6.61
ABF57933	<i>Triticum aestivum</i> MADS-box transcription factor TaAGL31	5.09E ⁻¹⁶⁷	5.02
ABR87001	<i>Hordeum vulgare</i> WRKY transcription factor 3	2.38E ⁻⁷⁹	4.65
DAA05113	<i>Oryza sativa</i> WRKY transcription factor 48	2.20E ⁻³⁵	4.08
CBZ39287	<i>Hordeum vulgare</i> NAC transcription factor	1.35E ⁻¹²⁵	3.21
ABO15542	<i>Triticum aestivum</i> WRKY45 transcription factor	1.02E ⁻⁰⁹	3.08
EU665450	<i>Triticum aestivum</i> WRKY35 transcription factor	2.00E ⁻⁴⁸	2.84
DAA05112	<i>Oryza sativa</i> WRKY transcription factor 47	7.62E ⁻¹⁷	2.45
BAK53496	<i>Triticum aestivum</i> WRKY45-like transcription factor	8.15E ⁻⁰⁶	2.29
ABK55356	<i>Triticum aestivum</i> CBFII-5.2	8.03E ⁻²⁵	2.11
HM027572	<i>Triticum aestivum</i> NAC transcription factor 7	0	2.04
AAK08983	<i>Oryza sativa</i> Myb transcription factor JAMyb	6.83E ⁻⁸⁵	1.96

carbohydrate metabolism, defense and transport were dramatically altered, and many photosynthesis-related genes were down-regulated in leaves of type I necrosis plants.

Similarly, gene expression profiles were compared between WT (KU-3620/KU-199-11) and type IV necrosis plants (KU-101-3/KU-199-5). Of the 37,826 microarray probes, 1323 (3.5 %) and 2805 (7.4 %) probes were respectively regarded as genes up- and down-regulated in leaves of type IV necrosis plants compared with WT plants (Supplemental Table 3). Up- and down-regulated probes were encountered more in type IV necrosis than in type I necrosis. GO annotations of the 2661 differentially expressed probe sequences showed that the up- and down-regulated genes in type IV necrosis could be categorized into 16 functional categories that appeared in type I necrosis (Fig. 1d). The accumulation of transcripts related to carbohydrate metabolic processes, stress and transport was dramatically up-regulated in leaves of type IV necrosis plants. As found in type I necrosis, a number of photosynthesis-related genes were down-regulated in type IV necrosis.

Several transcription factor genes, encoding MADS-box, WRKY, NAC and bHLH family proteins, were highly up-regulated in leaves of F₁ plants showing type I or IV necrosis (Tables 2, 3). Many WRKY-type transcription factor genes, which play important roles in immune responses, senescence, and various other processes in plants (Rushton et al. 2010; Ishihama and Yoshioka 2012), were found among the up-regulated transcription factor

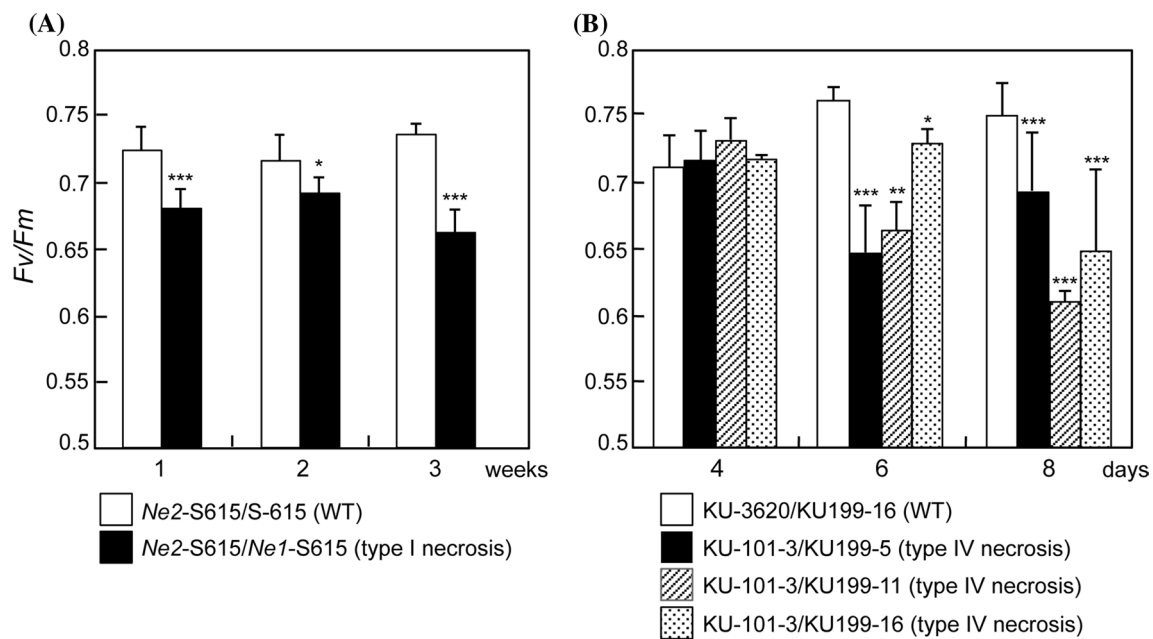
genes in both types of hybrid necrosis. In addition, transcripts of a number of defense-related genes, including chitinase, hypersensitive response protein and sulfur-rich/thionin-like protein genes, accumulated abundantly in F₁ plants showing type I or IV necrosis (Supplemental Tables 4 and 5). Up-regulation of reactive oxygen species (ROS)-related genes, such as curcuminoid synthase-like, oxalate oxidase and germin-like genes, was also observed in type I necrosis. In both types of hybrid necrosis plants, photosynthesis-related genes showed a high rate of down-regulation, including the genes for chlorophyll a/b binding protein and ribulose-1,5-bisphosphate carboxylase (Supplemental Tables 6 and 7).

Photosynthetic activity in type I and type IV necrosis

To examine the effects of down-regulation of photosynthesis-related genes, photosynthetic activities were compared between the WT and hybrid necrotic plants. Photosystem II activity of F₁ plants showing type I necrosis was significantly reduced in the first leaves of 1-, 2- and 3-week-old seedlings compared to WT F₁ plants without any necrotic symptoms (Fig. 2a). A reduction of photosystem II activity was also observed in three cross combinations showing type IV necrosis (Fig. 2b). The *Fv/Fm* values in F₁ plants showing type IV necrosis and grown for 6 and 8 days were significantly different from corresponding values for WT F₁ plants. These observations were consistent with the

Table 3 Top 20 transcription factor genes identified by microarray analysis as up-regulated in seedling leaves of hybrid plants between *T. urartu* KU-199-5 and *T. monococcum* ssp. *aegilopoides* KU-101-3

Accession no.	Protein	<i>E</i> value	Log ₂ ratio
XP_003580130	<i>Brachypodium distachyon</i> nac domain-containing protein	1.69E ⁻⁵⁴	5.59
XP_003563265	<i>Brachypodium distachyon</i> transcription factor bHLH93-like isoform 1	7.26E ⁻²⁴	5.24
BAK02871	<i>Hordeum vulgare</i> MYB transcription factor MYB52	2.51E ⁻¹⁰⁸	4.39
ABO15542	<i>Triticum aestivum</i> WRKY45 transcription factor	1.02E ⁻⁰⁹	3.96
ABR87001	<i>Hordeum vulgare</i> WRKY3 transcription factor	2.38E ⁻⁷⁹	3.89
BAK53496	<i>Triticum aestivum</i> WRKY45-like transcription factor	8.15E ⁻⁰⁶	3.39
ABK55356	<i>Triticum aestivum</i> CBFII-5.2	8.03E ⁻²⁵	2.97
FR821754	<i>Hordeum vulgare</i> NAC transcription factor	6.00E ⁻⁰⁶	2.91
AEV91179	<i>Triticum aestivum</i> MYB-related protein	1.59E ⁻¹⁰¹	2.86
ABU93236	<i>Triticum aestivum</i> R2R3 Myb-like protein	1.16E ⁻⁹⁰	2.76
BAJ99046	<i>Hordeum vulgare</i> transcription factor MYB1r1-like	3.25E ⁻⁵⁵	2.52
ACD80376	<i>Triticum aestivum</i> WRKY22 transcription factor	8.03E ⁻⁶²	2.51
AAU08785	<i>Triticum aestivum</i> NAC domain transcription factor	1.92E ⁻⁸⁶	2.35
AED99724	<i>Lophopyrum elongatum</i> stress-related bZIP transcription factor	1.22E ⁻⁸¹	2.29
ABI83667	<i>Triticum aestivum</i> GSK1 transcription factor 1	1.15E ⁻⁴⁷	2.22
XP_003559600	<i>Brachypodium distachyon</i> transcription factor bHLH25-like	1.08E ⁻⁰⁴	2.06
ABF57921	<i>Triticum aestivum</i> MADS-box transcription factor TaAGL2	1.54E ⁻¹⁰¹	2.05
NP_001068466	<i>Triticum aestivum</i> Myb transcription factor JAMyb	2.41E ⁻¹⁰⁰	2.00
XP_003580559	<i>Brachypodium distachyon</i> transcription factor PIF1-like	2.14E ⁻⁵²	1.65
ACD80387	<i>Triticum aestivum</i> WRKY40 transcription factor	9.31E ⁻⁷⁴	1.63

**Fig. 2** Comparison of chlorophyll fluorescence between wild-type (WT) and necrosis-showing hybrid plants. Means with standard deviations were calculated from data of nine experiments. Student's *t* test was used to test for statistical significance (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) between WT and necrosis-showing hybrid plants.

a Type I necrosis. The first leaves of 1-, 2- and 3-week-old seedlings were incubated in the dark and then used to calculate the ratio of variable to maximum fluorescence (*F_v/F_m*). **b** Type IV necrosis. The first leaves of 4-, 6- and 8-day-old seedlings were incubated in the dark and then used to calculate *F_v/F_m*

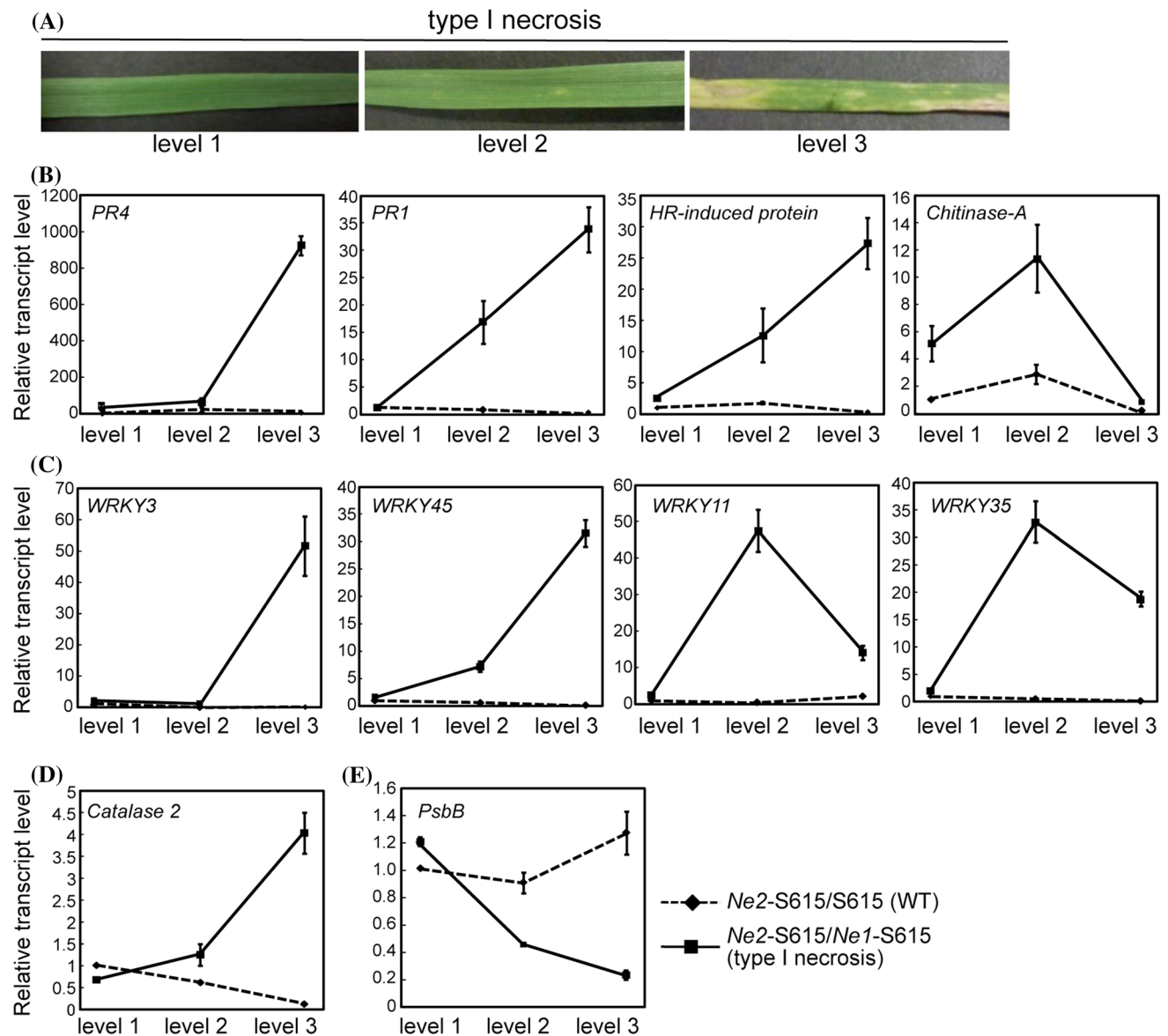


Fig. 3 Comparison of accumulation of transcripts of the indicated genes between WT and necrosis-showing plants in common wheat. **a** Three levels of type I necrosis symptoms on leaves. Total RNA was extracted from leaves showing each level of necrosis. **b** Transcript accumulation patterns of four up-regulated genes related to the defense response from qRT-PCR. The transcript levels are shown as values relative to those in level 1 leaves of the WT line.

Means \pm standard deviations were calculated from data in three experiments. The *Actin* gene was used as an internal control. **c** Transcript accumulation patterns of four up-regulated WRKY transcription factor genes. **d** Transcript accumulation patterns of an up-regulated gene related to ROS alleviation. **e** Transcript accumulation patterns of a down-regulated gene related to photosynthesis

down-regulation of photosynthesis-related genes in plants showing hybrid necrosis.

Expression analysis of defense-related genes in type I and type IV necrosis

To validate the microarray data, qRT-PCR was conducted for 10 genes, nine up- and one down-regulated (Supplemental Table 8). For convenience, the progression of symptoms

was classified into three levels in leaves of type I necrosis-showing seedlings, depending on the observed extent of necrotic symptoms (Fig. 3a). In WT F_1 plants, total RNA was extracted from leaves of seedlings at the same age as in type I necrosis. Transcripts of four defense-related genes were more markedly accumulated at level 2 in F_1 plants showing type I necrosis than in WT F_1 plants (Fig. 3b), indicating that the up-regulation of defense-related genes occurred before the necrotic symptoms appeared in type

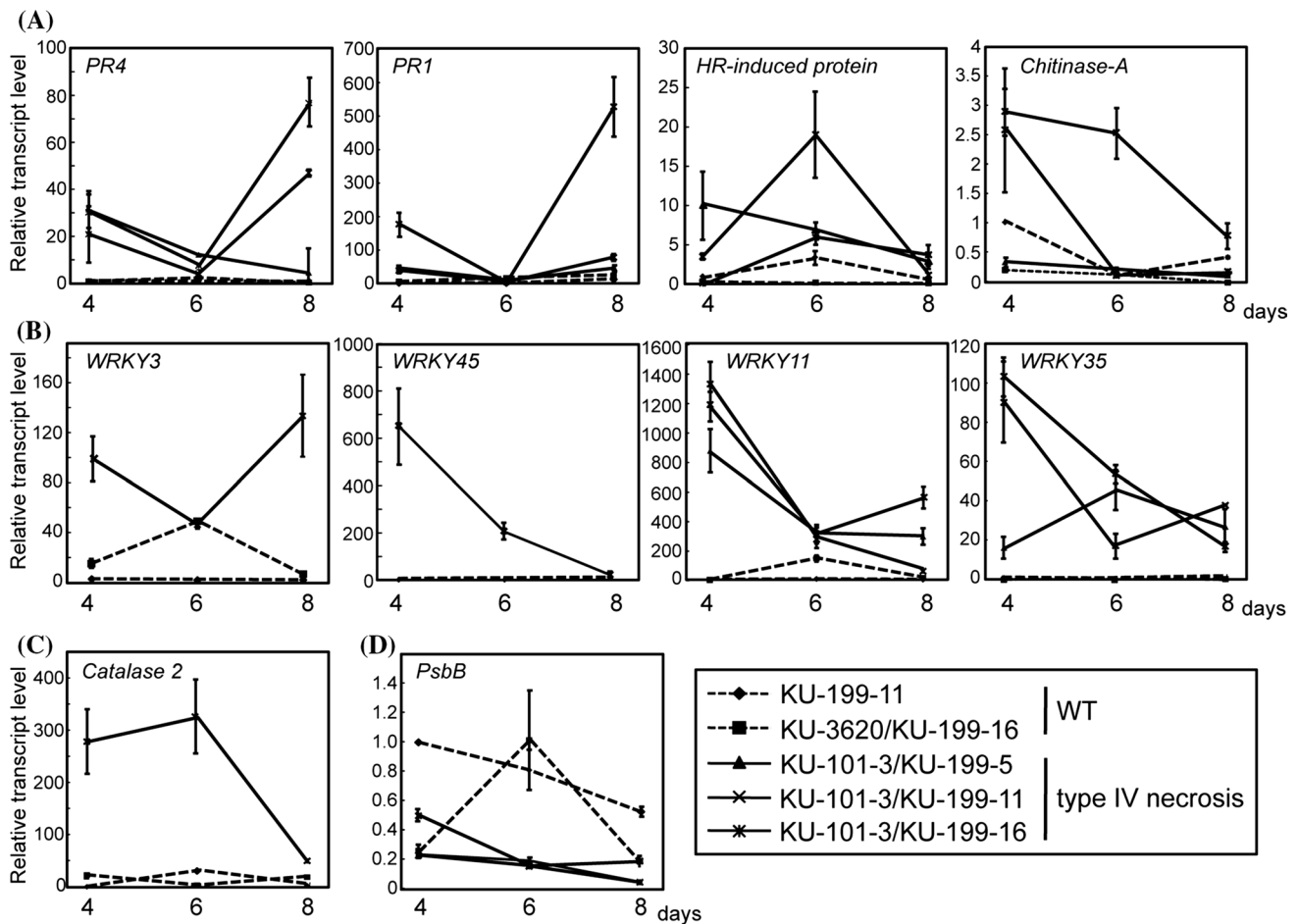


Fig. 4 Comparison of accumulation of transcripts of the indicated genes between WT and necrosis-showing plants in wild diploid wheat. Total RNA was extracted from the first leaves of 4-, 6-, 8-day-old seedlings and used for qRT-PCR. The transcript levels are shown as values relative to those in 4-day-old seedlings of WT (*T. urartu* KU199-11). Means \pm standard deviations were calculated from data in three experiments. The *Actin* gene was used as an internal control.

I necrosis plants. A similarly dramatic up-regulation was also observed at level 2 for three of four WRKY transcription factor genes in type I necrosis plants (Fig. 3c). The *catalase* gene, which functions in ROS alleviation, was highly induced at level 3 in type I necrosis plants (Fig. 3d). Rapid down-regulation of the photosynthesis-related gene *PsbB* was observed at level 2 before necrotic symptoms appeared in type I necrosis plants (Fig. 3e).

qRT-PCR analysis was also performed to compare the expression patterns of the 10 up-regulated genes and one down-regulated gene between two WT lines of wild diploid wheat and three F_1 lines showing type IV necrosis. Total RNA was extracted from the first leaves of 4-, 6- and 8-day-old seedlings. The four defense-related gene transcripts were more abundantly accumulated in type IV necrosis plants than in WT (Fig. 4a). Transcript levels of the four

a Transcript accumulation patterns of four up-regulated genes related to the defense response. b Transcript accumulation patterns of four up-regulated WRKY transcription factor genes. c Transcript accumulation patterns of an up-regulated gene related to ROS alleviation. d Transcript accumulation patterns of a down-regulated gene related to photosynthesis

WRKY transcription factor genes and the ROS-alleviation gene were markedly higher 4 days after sowing in type IV necrosis plants than in WT, and then gradually decreased in the type IV necrosis plants (Fig. 4b, c). Transcript accumulation of *PsbB* was lower in three F_1 lines with type IV necrosis than in WT (Fig. 4d).

Comparison of gene expression profiles among wheat hybrids with growth abnormalities

We compared gene expression profiles among the four types (type I to IV) of wheat hybrid necrosis, mild and severe hybrid chlorosis, and SGA in wheat ABD triploid plants based on differences in signal intensity of 22,018 microarray probes relative to WT. These probes selected for the comparison revealed non-saturated uniform signals

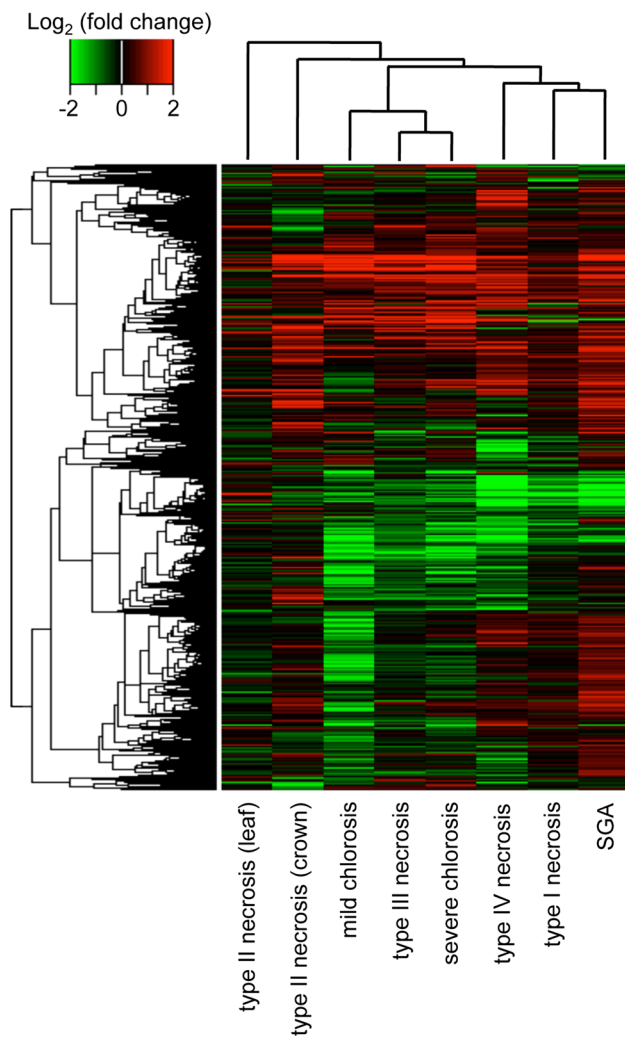


Fig. 5 A heat map of 22,018 probes in eight types of hybrid incompatibility. *Red* and *green* respectively indicate higher and lower expression of sequences complementary to the probes in each incompatible line compared with WT (see *color bar*)

that were greater than the background signal. A heat map showed the relationships between the comprehensive gene expression profiles of the eight growth abnormalities in various wheat hybrids (Fig. 5). Types I and IV necrosis had gene expression profiles that were closely related to SGA. Hybrid chlorosis and type III necrosis in wheat synthetic hexaploids were categorized into the same clade. Type II necrosis was distinct from other hybrid growth abnormalities.

Next, we compared gene expression profiles among three types (type I, III and IV) of wheat hybrid necrosis in which necrotic symptoms appear on leaves of seedlings under normal temperature (23 °C) (Hermsen 1963; Mizuno et al. 2010; Tsunewaki 1960; Yamagishi 1987). Differences in signal intensity of all 37,826 probes relative to WT were compared among leaves showing hybrid necrosis.

Significant positive correlations were detected between the hybrid necrosis plants (Table 4). Moreover, expression profiles were compared for each growth abnormality relative to WT using probes related to defense, carbohydrate metabolism, and photosynthesis. For up-regulated genes, significantly strong positive correlations were observed between the profiles of defense-related genes in type I and type III necrosis and between the profiles of carbohydrate metabolism-related genes in type III and type IV necrosis. For down-regulated genes, significantly strong positive correlations were found in the expression profiles of photosynthesis-related genes between type I and type IV necrosis. However, significant positive correlations were not necessarily detected in the other combinations, and negative correlations were found in some combinations.

Alleviation of necrotic symptoms via zinc chloride treatment

ZnCl₂ functions as a Ca²⁺ antagonist preventing calcium metabolism, and inhibits victorin-induced apoptotic cell death in oat (Hoat et al. 2006). To examine the effect of ZnCl₂ on necrotic cell death in wheat hybrids, we treated seedlings showing the three types of hybrid necrosis with ZnCl₂. ZnCl₂ treatment dramatically suppressed necrotic symptoms on the leaf surface in these types of hybrid necrosis (Fig. 6a). In addition, the numbers of trypan blue-stained cells, which indicate dead cells, in leaves of F₁ seedlings showing type I necrosis and synthetic hexaploid seedlings with type III necrosis, were greatly reduced by the ZnCl₂ treatment (Fig. 6b). This result indicated that ZnCl₂ treatment alleviated necrotic cell death in wheat hybrid necrosis. ROS generation was detected by DAB staining in leaves of type I and type III hybrid necrosis wheat seedlings, and the numbers of brown-colored cells were decreased by ZnCl₂ treatment in both types (Fig. 6b). Thus, ZnCl₂ treatment repressed ROS generation in wheat hybrid necrosis. Effects of ZnCl₂ treatment on photosynthetic activity were also examined for the three types of hybrid necrosis. Time-course studies clearly showed that ZnCl₂ treatment led to significant recovery from the decrease in photosynthetic activity of leaves of hybrid necrosis wheat seedlings (Fig. 6c).

To examine the effects of ZnCl₂ treatment on defense-related gene expression, qRT-PCR was conducted for genes encoding seven defense-related proteins. We chose these defense-related genes which were commonly up-regulated in the hybrid necrosis lines. According to the data from the microarray analyses, transcript accumulation levels of the pathogenesis-related protein 4 (PR4) gene were 4.5-, 30.7- and 8.8-fold increased in type I, type III and type IV necrosis, respectively. For the PR4 gene, two primer sets derived from different cDNAs were used (Supplemental Table 1).

Table 4 Comparison of gene expression profiles among leaves of necrosis-showing hybrid plants with types I, III and IV necrosis

Query	Number of probes	Target expression profile	Correlation coefficient
All genes			
In type IV necrosis versus WT	37,826	Type I necrosis versus WT	0.292**
In type IV necrosis versus WT	37,826	Type III necrosis versus WT ^a	0.313**
In type I necrosis versus WT	37,826	Type III necrosis versus WT ^a	0.188**
Defense-related genes up-regulated			
In type IV necrosis versus WT	87	Type I necrosis versus WT	−0.121
In type IV necrosis versus WT	87	Type III necrosis versus WT ^a	0.172
In type I necrosis versus WT	25	Type IV necrosis versus WT	0.112
In type I necrosis versus WT	25	Type III necrosis versus WT ^a	0.668**
In type III necrosis versus WT ^a	78	Type IV necrosis versus WT	0.03
In type III necrosis versus WT ^a	78	Type I necrosis versus WT	−0.07
Carbohydrate metabolic process related genes up-regulated			
In type IV necrosis versus WT	94	Type I necrosis versus WT	−0.586
In type IV necrosis versus WT	94	Type III necrosis versus WT ^a	0.685**
In type I necrosis versus WT	4	Type IV necrosis versus WT	−0.105
In type I necrosis versus WT	4	Type III necrosis versus WT ^a	0.290
In type III necrosis versus WT ^a	36	Type IV necrosis versus WT	0.658**
In type III necrosis versus WT ^a	36	Type I necrosis versus WT	−0.711
Photosynthesis-related genes down-regulated			
In type IV necrosis versus WT	282	Type I necrosis versus WT	0.664**
In type IV necrosis versus WT	282	Type III necrosis versus WT ^a	−0.177
In type I necrosis versus WT	192	Type IV necrosis versus WT	0.418**
In type I necrosis versus WT	192	Type III necrosis versus WT ^a	−0.793
In type III necrosis versus WT ^a	32	Type IV necrosis versus WT	−0.120
In type III necrosis versus WT ^a	32	Type I necrosis versus WT	−0.728

The Pearson coefficient values were calculated based on the differences in signal intensities of three sets of the categorized probes between the wild-type and abnormal growth hybrids

^a Significant correlations: ** $P < 0.01$

^a Mizuno et al. (2010)

From the microarray data, class I and class III chitinases, RPP13-like protein1-like, and phenylalanine ammonia lyase (PAL) genes were 3.2- to 112.7-times up-regulated in type I and type III necrosis. The WRKY3 transcription factor gene was 25.0- and 14.9-fold activated in type I and type IV necrosis based on the microarray data, respectively. The qRT-PCR analyses showed that the transcripts of most examined genes accumulated more abundantly in leaves of the hybrid necrosis wheat seedlings than in leaves of WT plants (Fig. 7). Significant differences in the transcript levels were also observed in leaves of ZnCl₂-treated seedlings. Transcript accumulation of some examined genes including *PR4* and *class I chitinase* was markedly reduced by ZnCl₂ treatment in type I and type III necrosis plants, whereas no remarkable reduction was observed for *PAL*, *WRKY3* and *RPP13-like protein1-like*. Thus, ZnCl₂ treatment did not necessarily affect transcript accumulation of defense-related genes in these three types of hybrid necrosis plants.

Discussion

Recent studies have suggested that autoimmune responses trigger hybrid necrosis in higher plants, and are accompanied by up-regulation of a number of defense-related genes (Alcázar et al. 2009, 2010; Bomblies et al. 2007; Chae et al. 2014; Jeuken et al. 2009; Yamamoto et al. 2010). Several types of hybrid necrosis have been reported in inter- and intraspecific crosses of wheat and its relatives, and previous studies suggest that all known types of wheat hybrid necrosis are genetically programmed through interaction of two complementary genes (Hermsen 1963; Mizuno et al. 2010; Nishikawa 1962b; Tsunewaki 1960; Yamagishi 1987). The up-regulation of defense-related genes is observed before necrotic symptoms appear in leaves of hybrid plants exhibiting type II and type III necrosis, and necrotic cell death is accompanied by ROS generation and reduction in photosynthetic activity (Mizuno et al. 2010, 2011). Similar

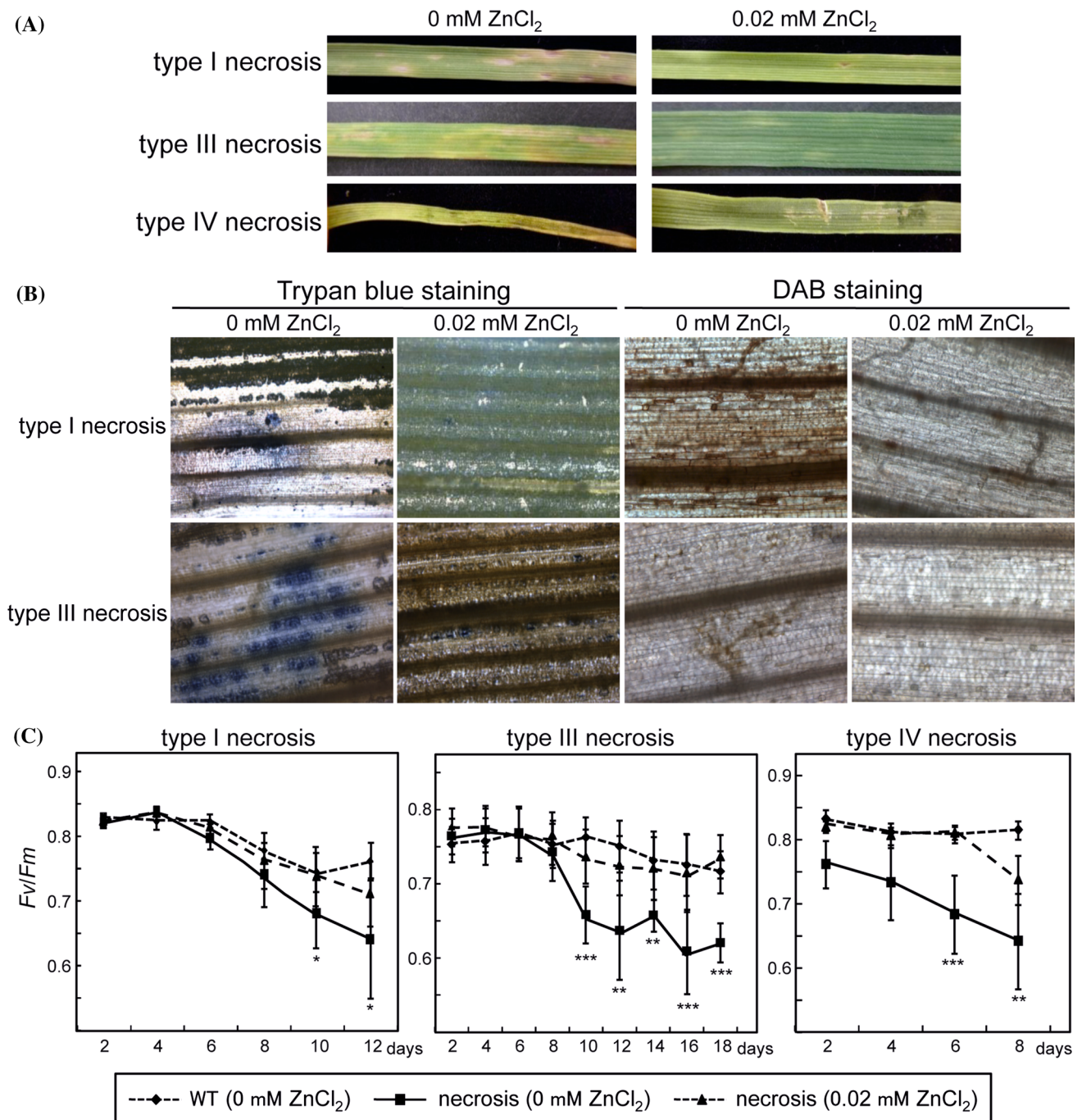


Fig. 6 Effects of ZnCl₂ treatment on necrotic cell death in three types of hybrid necrosis. **a** Leaf phenotype of WT and necrosis-showing plants after 10 days of ZnCl₂ treatment. **b** Trypan blue- and DAB-stained leaves in type I and type III necrosis. **c** Comparison of chlorophyll fluorescence among WT, necrosis-showing plants without ZnCl₂ treatment and necrosis-showing plants after ZnCl₂ treat-

ment. The first leaves were incubated in the dark and then used to calculate the ratio of variable to maximum fluorescence (F_v/F_m). Means \pm standard deviations were calculated from data in nine experiments. Student's *t* test was used to test for statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) between the WT and necrosis-showing plants

alterations in gene expression patterns, photosynthetic activity and ROS generation were found prior to appearance of necrotic symptoms in leaves of hybrid plant seedlings exhibiting type I and type IV necrosis (Figs. 1, 2, 3,

4, 6b). These characteristics indicate that type I and type IV necrosis share at least some similar mechanisms inducing necrotic cell death with type II and type III necrosis. In fact, significant positive correlations were observed

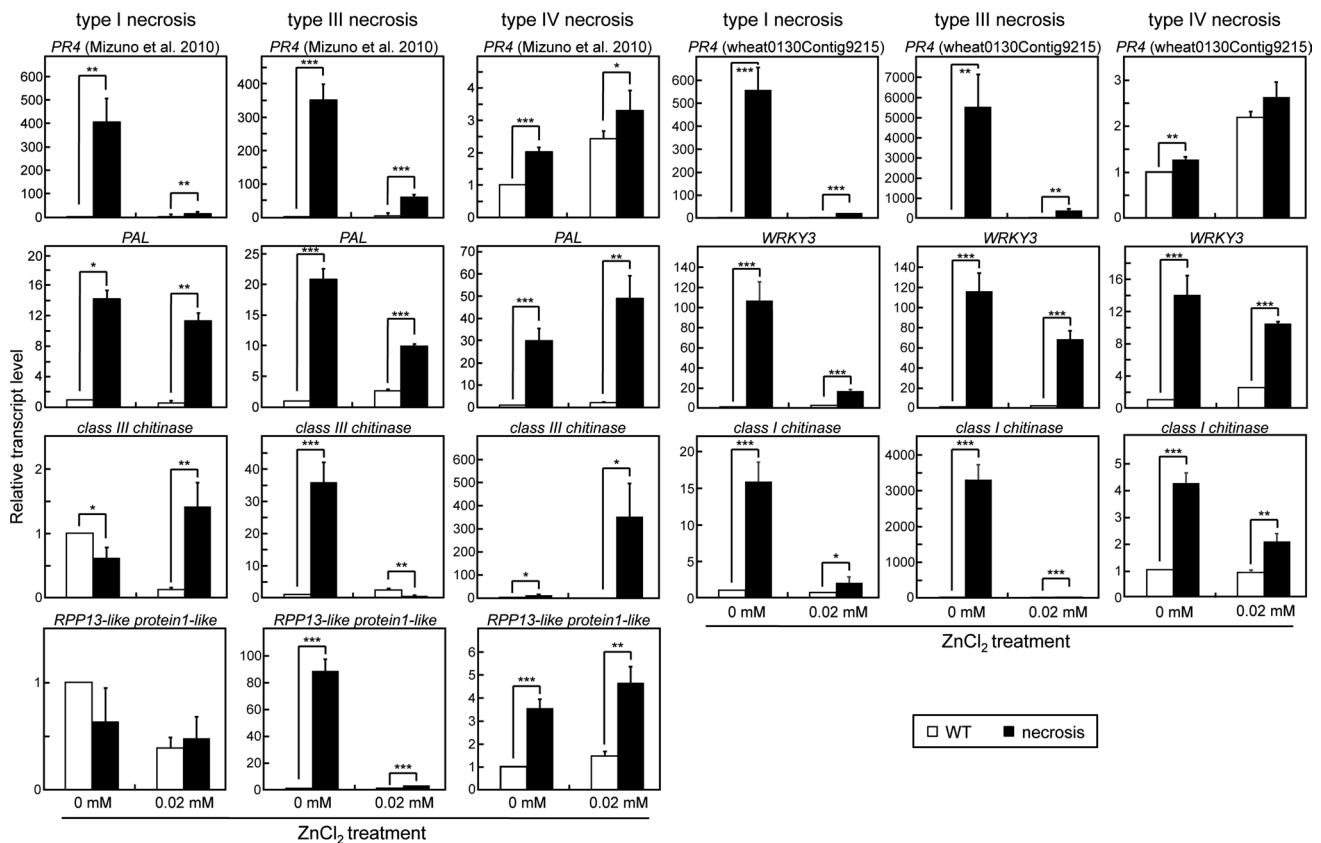


Fig. 7 Effects of ZnCl₂ treatment on transcript accumulation of two defense-related genes in three types of hybrid necrosis. Total RNA was extracted from the first leaves of plants after 10 days of ZnCl₂ treatment and used for qRT-PCR. The transcript levels are shown as values relative to those in leaves of WT without ZnCl₂ treatment.

in the gene expression profiles among plants showing the three types of hybrid necrosis (Table 4). Hypersensitive response (HR)-induced cell death typically occurs as a plant defense response to various plant pathogens (Mur et al. 2008). The programmed cell death in type III necrosis could be regarded as HR-like cell death, and the autoimmune response triggered by interaction of the causal genes (*Nec1* and *Nec2*) appears to result in this type of necrosis, as reported for hybrid necrosis in other plant species (Mizuno et al. 2010). Thus, our observations strongly suggest that an autoimmune response might be triggered by epistatic interactions between *Ne1* and *Ne2* in type I necrosis of common wheat and between *Ned1* and *Ned2* in type IV necrosis of wild diploid wheat.

An oxidative burst occurs in the early stage of HR to pathogen infection (Wojtaszek 1997). ROS generation appears prior to cell death in type III necrosis, and the generated ROS may function as initiators of cell death in type III necrosis, as they do in HR (Mizuno et al. 2010). The HR-like reaction is assumed to occur in type I and type IV necrosis, as observed in type III necrosis, and thus ROS

Means \pm standard deviations were calculated from data in three experiments. The *Actin* gene was used as an internal control. Student's *t* test was used to test for statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) between WT and necrosis-showing lines

would play important roles in initiation and/or progression of necrotic cell death in type I and type IV necrosis. In addition, Ca²⁺-dependent processes mediate various types of programmed cell death including both apoptotic and non-apoptotic cell death (Orrenius et al. 2003), and a Ca²⁺ signal plays important roles in the signal transduction pathways leading to innate immune-associated cell death in plants (Ma and Berkowitz 2007). Ca²⁺ influx into plant cells is associated with NADPH oxidase-dependent ROS generation (Bindschedler et al. 2001; Davies et al. 2006; Song et al. 2006). Inhibition of the cytosolic Ca²⁺ elevation leads to ROS generation and delays plant HR to an avirulent pathogen (Ma and Berkowitz 2007). In fact, ZnCl₂, as a Ca²⁺ antagonist, strongly suppresses victorin-induced cell death, an example of plant defense-associated host cell apoptosis, in oat (Hoat et al. 2006). Our present study showed that ZnCl₂ treatment effectively alleviated ROS generation, reduction in photosynthetic activity, and necrotic cell death in three types of wheat hybrid necrosis (Fig. 6). These results indicated that inhibition of Ca²⁺ signaling repressed necrotic symptoms through

reduction in the generated ROS in plants showing wheat hybrid necrosis. Taking these observations together suggests that epistatic interactions between causative genes of wheat hybrid necrosis might result in Ca^{2+} influx and that the Ca^{2+} influx might play a role in upstream signaling of necrotic cell death.

On the other hand, no clear reduction in gene expression was observed in two plant defense-related genes following ZnCl_2 treatment in three types of wheat hybrid necrosis (Fig. 7). These results indicated that defense-related gene activation might be independent of ROS generation in wheat hybrid necrosis. In general, Ca^{2+} influx is considered to act as a mediator for induction of a set of defense-related genes (Ma and Berkowitz 2007). At least in wheat hybrid necrosis, however, epistatic interaction of the causative genes appears to activate defense-related genes without a Ca^{2+} influx. Thus, defense-related gene activation might proceed in parallel with ROS generation-mediated cell death in wheat hybrid necrosis. To clarify the regulation of defense-related genes via causative gene interaction in hybrid necrosis, much earlier responses in necrotic cells of wheat hybrids should be analyzed in further studies.

Comprehensive gene expression profiles in type I and IV necrosis were positively correlated with that observed in type III necrosis (Table 4). In addition, phenotypic and transcriptional responses to ZnCl_2 treatment resembled each other among the three types of hybrid necrosis (Figs. 6, 7). Comparison of the comprehensive expression profiles with those associated with other growth incompatibilities showed that profiles of type III necrosis and type IV necrosis were similar to those of hybrid chlorosis in synthetic hexaploid wheat and SGA in ABD triploid wheat hybrids, but the profile of type II necrosis was distinct from those observed for other growth incompatibilities (Fig. 5). Necrotic cell death requires low-temperature conditions, and normal growth temperatures such as 23 °C cannot induce any necrotic symptoms in plants showing type II necrosis (Mizuno et al. 2011; Takumi and Mizuno 2011). Low-temperature treatment activates a number of wheat cold-responsive genes, resulting in differences in gene expression profiles for plants showing type II necrosis from profiles seen for other growth incompatibilities. Weakly positive correlations of gene expression profiles were observed even between leaves of plants showing type III necrosis and low temperature-treated leaves and crown tissues in type II necrosis (Mizuno et al. 2011). Thus, hybrid growth incompatibilities, in spite of their phenotypic variation, share at least in part similar alterations in gene expression profiles such as activation of defense- and carbohydrate metabolism-related genes and down-regulation of photosynthesis-related genes in inter- and intra-specific crosses of wheat and its relatives.

In wild diploid wheat, hybrid necrosis was observed in many interspecific cross combinations (Table 1). Epistatic interaction between *Ned1* in *T. monococcum* ssp. *aegilopoides* and *Ned2* in *T. urartu* was previously assumed to trigger necrotic cell death (Yamagishi 1987). All five of the *T. urartu* accessions examined in the present study were considered to carry the dominant allele of *Ned2*, whereas one ssp. *aegilopoides* accession KU-3620 contained no dominant *Ned1* allele. This result agreed with a previous report (Yamagishi 1987). The dominant *Ned2* allele would be fixed in the *T. urartu* population, while distribution of the dominant *Ned1* allele appears to be limited in the ssp. *aegilopoides* population. Thus, post-zygotic reproductive isolation via the *Ned1*–*Ned2* interaction is incomplete between the two wild diploid wheat species. However, other types of reproductive isolation additionally underlie the two wild diploid wheat species (Fricano et al. 2014; Johnson and Dhaliwal 1976; Yamagishi 1987). For example, when ssp. *aegilopoides* accessions are used as pollen parents, F_1 hybrid seeds with *T. urartu* accessions can be obtained, but most of them fail to germinate (Johnson and Dhaliwal 1976; Yamagishi 1987). The failure of germination has been attributed to nuclear-cytoplasm incompatibility or abnormal endosperm development in the hybrid seeds (Dhaliwal 1977; Gill and Waines 1978; Johnson and Dhaliwal 1976). The nuclear genomes of the two wild diploid wheat species are clearly differentiated, as reported by several research groups (Brandolini et al. 2006; Castagna et al. 1994, 1997; Mizumoto et al. 2002). Nuclear genome differentiation might be at least partly caused by reproductive isolation between the two wild diploid wheat species. To clarify the contribution of type IV necrosis to the post-zygotic reproductive isolation between the two wild diploid wheat species, intraspecific distribution of the dominant *Ned1* allele in the ssp. *aegilopoides* population should be elucidated in further study. Moreover, the cytoplasmic effects of *T. urartu* on viable seed development should also be studied to understand more comprehensively the reproductive isolation of wild diploid wheat.

To overcome reproductive barriers among the related plant species, the causal genes for the genetic incompatibilities should be identified and molecular cloning of them should be required. However, large efforts should be needed for the identification and isolation of the reproductive barrier-causative genes. Therefore, the alleviation effects of the ZnCl_2 application on the expression of necrotic symptoms suggest an alternative strategy to overcome at least some of the reproductive barriers. If the ZnCl_2 -applied suppression of necrotic symptoms effectively continued until the grain maturation stage of the hybrid plants, gene flow over hybrid necrosis could be realized. The effects of long-term ZnCl_2 application on development of the hybrid necrosis plants should be tried in the future studies.

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