Research Paper

A hypersensitive response-like reaction is involved in hybrid weakness in F₁ plants of the cross Capsicum annuum × Capsicum chinense

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Hybrid weakness in Capsicum is characterized by the termination of leaf differentiation after the development of several leaves. F₁ plants in some crosses between Capsicum annuum and Capsicum chinense show weakness; this phenomenon has not been investigated in detail since first reported. In the present study, we characterized morphologically and physiologically hybrid weakness in Capsicum. F₁ plants did not show weaker growth than their parents 20 days after germination (DAG), but at 40 DAG, the hybrid weakness phenotype was evidenced by almost complete arrest of new leaf formation, delayed increase in plant height, and reduced upper internode length. The shoot apical meristem (SAM) of F₁ plants exhibited delayed development and an abnormal structure characterized by a flat shape and the presence of fuzzy cell layers on the surface. These abnormal SAMs of F₁ plants may lead to dwarfism. Dead cells and accumulation of H₂O₂ were visually detected in leaves of F₁ plants, and cell death was considered to be programmed, as it was accompanied by internucleosomal fragmentation of DNA. The expression of immunity marker genes PR1 and PR2 was upregulated in leaves of F₁ plants. These results suggest that a hypersensitive response-like reaction is involved in Capsicum hybrid weakness.

Key Words: reproductive isolation, interspecific cross, programmed cell death, defense response, pepper.

Introduction

Capsicum genus belongs to the Solanaceae family and includes economically important plants, used not only as edible products but also for medicinal and cosmetic purposes. Capsicum species are cultivated all over the world and produced at an average of 40 million tons per year (FAO 2017). These species have attracted attention because they contain capsaicin, a metabolite that has beneficial effects such as in stress relief, pain relief, and fat breakdown (Bach and Yaksh 1995, Kobayashi et al. 1998, Watanabe et al. 1994). Capsicum genus comprises approximately 35 species (Carrizo García et al. 2016) including 5 cultivated species: Capsicum annuum, Capsicum chinense, Capsicum frutescens, Capsicum baccatum, and Capsicum pubescens. C. annuum (pepper) is the most important cultivated species and thus has been bred for long.

Interspecific crosses are effective methods to introduce new desirable traits from related species into commercial cultivars. Today, the main goal of C. annuum breeding programs is to select high-yield cultivars resistant to pests, diseases, and abiotic stresses, and to improve their fruit quality (Ramalho do Rêgo and Monteiro do Rêgo 2016). These useful traits, which C. annuum does not have, are often found in related species. Some examples of traits introduced in pepper are tobacco mosaic virus resistance (Boukema 1980), tomato spotted wilt virus resistance (Moury et al. 2000), and multiple flowering (Subramanya 1983). Therefore, breeders have attempted to conduct interspecific crosses repeatedly. However, reproductive isolation mechanisms often prevent the achievement of successful interspecific hybrids or affect their offspring.

Prezygotic and postzygotic barriers result in reproductive isolation. Among prezygotic isolation mechanisms, fertilization failure because pollen grains cannot germinate on stigma, or pollen tubes cannot reach ovaries or ovules, are common. It has been reported that Capsicum pollen tubes failed to reach the ovary in reciprocal crosses of C. frutescens × C. baccatum, though pollen grains germinated (Martins et al. 2015). Postzygotic isolation mechanisms include abnormal development of the embryo and/or endosperm in F₁ seeds, abnormal growth and/or sterility in F₁ plants, and hybrid breakdown after F₁ generation.
The phenotypes of abnormal growth most frequently studied in F₁ are plant death and plant weakness. These phenomena, called hybrid lethality, hybrid necrosis, and hybrid weakness, have been reported in many plant species including Oryza sativa (Chen et al. 2014, Fu et al. 2013, Oka 1957), Nicotiana spp. (Tezuka and Marubashi 2004, Tezuka et al. 2007), and Arabidopsis thaliana (Blomblys et al. 2007). In most cases, the interaction of two complementary genes causes abnormal plant growth, and at least one gene of a set of the complementary genes previously identified encodes protein related to plant disease response (Blomblys et al. 2007, Chen et al. 2014).

Several authors detected cell death in F₁ plants showing abnormal growth, such as Arabidopsis thaliana (Blomblys et al. 2007), Oryza sativa (Chen et al. 2014, Fu et al. 2013), and Triticum spp. × Aegilops spp. (Mizuno et al. 2010, Okada et al. 2017). Moreover, the cell death phenotype in Nicotiana spp. was ascribed to programmed cell death (PCD), as it was accompanied by chromatin condensation, nuclear fragmentation, and internucleosomal fragmentation of DNA (Tezuka and Marubashi 2004, Tezuka et al. 2007). In Oryza sativa, PCD was also documented, and DNA fragmentation took place; however, it was undetermined whether the DNA fragmentation was internucleosomal (Chen et al. 2014). The expression of pathogenesis-related genes was dramatically induced in plants showing hybrid necrosis (Blomblys et al. 2007, Chen et al. 2014, Mizuno et al. 2010).

There are several reports about the abnormal growth of interspecific hybrids in Capsicum. In some reciprocal crosses of C. annuum × C. chinense or C. annuum × C. frutescens, F₁ plants show dwarfism; this phenomenon was named “hybrid dwarfism” (Yazawa et al. 1989, 1990). In this article, however, we called this phenomenon “hybrid weakness” because the term “hybrid dwarfism” would be inappropriate based on the results of the present study. Hybrid weakness is caused by two complementary dominant genes, and C. annuum cultivars having one of these complementary dominant genes are distributed throughout East Asia (Yazawa et al. 1989).

In the cross C. chinense (♀) × C. annuum (♂), F₁ plants show more severe dwarfism than that described as hybrid dwarfism or weakness. This phenotype, named “stunted growth”, is caused by the interaction between the cytoplasm of C. chinense and a nuclear gene of C. annuum (Inai et al. 1993). Besides, in crosses C. frutescens (♀) × C. baccatum (♂) or C. chinense (♀) × C. baccatum (♂), F₁ plants show abnormal growth as if they were affected by a virus; for that reason, this phenomenon is called “virus-like syndrome”. It was reported that virus-like syndrome is caused by the interaction between the cytoplasm of C. chinense or C. frutescens and a nuclear gene of C. baccatum (Pickersgill 1971, Yazawa et al. 1990). Although some classical genetic studies and a few phenotypic observations were published, the detailed phenotypes and molecular mechanisms underlying the F₁ abnormal growth of these three Capsicum crosses, which may be considered postzygotic isolation barriers, have not been elucidated yet.

The aim of this work was to characterize phenotypically and physiologically the hybrid weakness in the F₁ plants of the cross C. annuum × C. chinense in detail. We determined what type of abnormal growth occurred in F₁ plants showing hybrid weakness, considering temporal and spatial variations and focusing on shoot apical meristem (SAM) structure and cell death.

Materials and Methods

Plant materials

We used seeds of C. annuum ‘Shosuke’ (accession JP 123787) supplied by the National Agriculture and Food Research Organization Genebank (Tsukuba, Japan) and of C. chinense PI 159236 supplied by the USDA/ARS Capsicum germplasm collection (Griffin, GA). After seeds of both accessions were germinated on moistened filter papers placed in Petri dishes, all seedlings were transplanted to pots (9 cm diameter, 10 cm depth) filled with culture soil (Sakata Super Mix A, Sakata Seed Co., Tokyo, Japan). The seedlings were cultivated in a constant-temperature room (25°C, 12 h light and 12 h dark, 85 μmol m⁻² s⁻¹). At 30 days after germination (DAG), plants were transferred to bigger pots (21 cm diameter, 15 cm depth) and placed in a greenhouse (natural day length; Osaka Prefecture University, Sakai, Osaka, Japan), where the temperature was maintained above 15°C from October 2016 to February 2017. For additional fertilization, plants were supplied weekly with Otsuka-A prescription (OAT Agrio Co., Ltd., Tokyo, Japan), containing 18.6 mM nitrogen, 5.1 mM phosphorus, 8.6 mM potassium, 8.2 mM calcium, and 0.4 mM magnesium. When plants of both accessions flowered, we carried out interspecific crosses using them as parents, as described in the following section.

Interspecific crosses and cultivation of hybrids

The conventional crossing was carried out as follows: flowers of C. annuum ‘Shosuke’ used as maternal parents were emasculated one day before anthesis and pollinated with pollen of C. chinense PI 159236, used as paternal parents. F₁ seeds were collected from fully ripe fruits. For phenotypic observations, F₁ plants and both parents were cultivated at 25°C in a constant-temperature room, under the same conditions mentioned above. At 100 DAG, the plants were transplanted into pots (21 cm diameter, 15 cm depth), transferred to the greenhouse, and cultivated in the same manner as described in the precedent section. We surveyed the plant height, the number of leaves (excluding those of side branches), and hypocotyl and internode (1st to 5th) lengths every week.

For histological observations and detection of cell death and H₂O₂ accumulation, F₁ plants and both parents were cultivated in pots (9 cm diameter, 10 cm depth) in an incubator (12 h light and 12 h dark, 25°C, 120 μmol m⁻² s⁻¹), in
the same manner as described above. At 28 DAG, some plants were transplanted into pots (15 cm diameter, 12 cm depth) and transferred to the greenhouse, where they grew from October to December 2018. At 100 DAG, these plants were used for RT-PCR assays.

**RAPD analysis**

Total DNA was extracted from individual leaves using a cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980) with minor modifications. Each leaf was ground in a mortar with liquid nitrogen. The ground leaf was mixed with CTAB isolation buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-Cl, pH 8.0) preheated at 60°C, and the mixture was incubated at 60°C for 60 min. The suspension was extracted twice with chloroform/isomyl alcohol (24:1) and centrifuged for 15 min at 500 g. The aqueous phase was transferred to a new tube; nucleic acids were precipitated by the addition of isopropanol (2/3 volume) and centrifuged for 20 min at 1,000 g. The pellet was washed with 70% ethanol and dissolved in 30 μL of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) supplemented with 1 μL of 100 μg ml⁻¹ RNase.

Twenty random 10-mer oligonucleotide primers (Kit A; Operon Technologies, Inc., Alameda, CA, USA) were used. PCR was performed in 10 μL reaction mixture containing 0.2 mM of each dNTP, 1 μL of 10× Standard Buffer, 0.2 μM primer, 0.25 U of Taq polymerase (New England Biolabs Inc., Ipswich, MA), and 0.5 μL of DNA as template. The following PCR conditions were used: initial denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 1 min at 50°C, and 1 min at 72°C, with a final 3 min-extension at 72°C. PCR products were separated by electrophoresis on a 1.5% agarose gel in TBE buffer, stained with ethidium bromide, and photographed under UV light.

**Histological observation**

Shoot apices were excised at 20 and 40 DAG and fixed with FAA (3.7% formaldehyde, 5% acetic acid, 50% absolute ethanol) at room temperature for 1 day. Fixed samples were dehydrated through ethanol-butanol series. Samples were then embedded in paraffin for 3 days at 58°C in an oven. The paraffin block was shaped into a rectangular paraffin block (Olympus BX50, Olympus, Co., Ltd., Tokyo, Japan). The presence of brown-colored areas resulting from DAB oxidation was indicative of H₂O₂ accumulation.

**Detection of internucleosomal fragmentation of DNA**

Total DNA was extracted from individual leaves using a CTAB method as described in RAPD analysis. Aliquots (20 μL) of the extracted DNA were loaded on a 2% agarose (Nippon Gene Co., Ltd., Tokyo, Japan) gel, separated by electrophoresis in Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide, and photographed under UV light.

**RT-PCR assay**

Total RNA was isolated from leaves using TRIzol reagent (Invitrogen Inc., Carlsbad, USA), according to the manufacturer’s protocol and then treated with RNase-free DNase (Promega Co., Madison, USA). First-strand cDNA was synthesized from total RNA (2 μg) using oligo (dT)₁₈ primers and ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). RT-PCR was carried out to analyze the expression of two pathogenesis-related genes, CaPR1 and CaPR2. CaPR1 encodes a basic protein that is inducible by salicylic acid, and CaPR-2 encodes a basic β-1,3-glucanase, hydrolyzing β-1,3-glucans of fungal/oomycete cell walls (van Loon et al. 2006). CaActin was used as the internal control.

Primers for RT-PCR (Table 1) were designed based on the genes identified in *C. annuum* using the software Primer-BLAST design tool (Ye et al. 2012). PCR was performed in 10 μL reaction mixture containing 0.2 mM of each dNTP, 10 μM of each forward and reverse primer, 0.25 U of Taq polymerase (New England Biolabs Inc., Ipswich, MA), and 1 μL of cDNA as template. The following PCR conditions were used: initial denaturation at 94°C

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene accession</th>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
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<td>CaPR1</td>
<td>XM_016683907</td>
<td>CaPR1-F</td>
<td>GTTGTGCTAGGGGCTGGTGTT</td>
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<td>CaPR1-R</td>
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<td>XM_016707754</td>
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<td>CaPR2-R</td>
<td>TGAAGGTTGGCAGCAATGT</td>
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<tr>
<td>CaActin</td>
<td>XM_016714317</td>
<td>CaActin-F</td>
<td>TCCAGACAGATGTGGATATCGAAG</td>
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<tr>
<td></td>
<td></td>
<td>CaActin-R</td>
<td>TGGGAATTCAGACAAAGC</td>
</tr>
</tbody>
</table>

**Cell death detection by trypan blue staining**

Trypan blue staining was performed, according to Zhang et al. (2004). Detached leaves were stained by boiling for 8 min in alcoholic lactophenol (ethanol-lactophenol 1:1 v/v) containing 0.1 mg ml⁻¹ of trypan blue and cleared in 70% chloral hydrate solution overnight, then reserved in 70% glycerol. Leaves were observed using a light microscope (Olympus BX50). Trypan blue stains selectively the dead cells due to their increased membrane permeability.

**Detection of H₂O₂ accumulation**

H₂O₂, a well-known reactive oxygen species (ROS), was visualized using diaminobenzidine (DAB), according to the method described by Chandru et al. (2003). Leaves were dipped in DAB solution for 24 h and transferred to boiling 96% ethanol until bleaching. Leaves were then observed using a light microscope (Olympus BX50). The presence of brown-colored areas resulting from DAB oxidation was indicative of H₂O₂ accumulation.
for 1 min, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C, with a final 3 min-extension at 72°C. PCR products were separated by electrophoresis on a 2.0% agarose gel in TBE buffer, stained with ethidium bromide, and photographed under UV light.

Statistical analysis

Data were analyzed with the SPSS program (version 22) (IBM Corp., Armonk, USA). In order to detect significant differences in plant height, leaf number, and internode length, we compared the values obtained in F$_1$ plants with those representing mid-parent values (i.e., the average of ‘Shosuke’ and PI 159236), using two-tailed Student’s t-test.

Results

Morphological appearance of hybrid weakness

From 20 flowers of C. annuum ‘Shosuke’ that were pollinated with pollen from C. chinense PI 159236, we got 6 fruits containing seeds and a total of 35 seeds. The germination percentage of these seeds was 80% (20 of 25 seeds germinated). RAPD analysis was conducted with 20 random primers on hybrid plants from the cross C. annuum × C. chinense for confirmation of hybridity. Two primers (OPA-06 and 19) gave RAPD patterns showing clear polymorphisms between the parents, while other primers could not give clear band patterns or polymorphisms. The plants obtained from the cross had all bands characteristics of both parents (Supplemental Fig. 1). Thus, the plants obtained from the cross were confirmed to be true hybrids.

We analyzed the phenotype of hybrid weakness using five F$_1$ plants in comparison with both parents (each n = 5), all of them grown at 25°C. At 20 DAG, no difference was observed in plant height and number of leaves between F$_1$ plants and their parents (Figs. 1A, 1B, 2A, 2B). However, the 2nd internode in F$_1$ plants was significantly longer as compared with the mid-parent value (Fig. 2C). The height of F$_1$ plants was similar to that of C. chinense, although the total internode length excluding hypocotyl of F$_1$ plants was longer than that of both parents. During the following days, while the plant height and the number of leaves of both parents continued to increase, height of F$_1$ plants increased moderately, and the number of leaves almost did not increase due to stop of new leaf development; thus, significant differences were found at 40 DAG (Figs. 1C, 1D, 2A,

![Fig. 1](image-url)
Shoot apex died in one of five F₁ plants (Fig. 1K), and the 5th internode of F₁ plants was significantly shorter than those of both parents at 40, 50, and 60 DAG. In other words, the 5th internode kept on growing in both parents, but not in F₁ plants (Fig. 2C). At 60 DAG, the existing leaves in F₁ plants stiffened, from the lower leaves to the upper ones, and all the leaves of F₁ plants had acquired a hard appearance by 100 DAG; however, the leaves of both parents never became hard. At 270 DAG, the entire leaves of F₁ plants or their margins turned brown (Fig. 1G, 1H), whereas both parents showed normal growth (Fig. 1E, 1I). Abnormal leaves instead of side branches developed from axillary buds in F₁ plants (Fig. 1H, 1K), whereas both parents developed normal side branches (Fig. 1F, 1J). Although both parents had already flowered, F₁ plants did not flower even at 270 DAG.

Abnormal structure of SAM was observed in F₁ plants

The characters of hybrid weakness observed in F₁ of C. annuum × C. chinense cross were the following: cessation of leaf emergence, reduction in plant height, and, sometimes, death of shoot apices. These characters suggested that SAMs of F₁ plants could be abnormal. To prove this, we performed histological observations of SAMs in F₁ plants and their parents. At 20 DAG, SAMs of parents appeared as dome-like structures, composed of an epidermal layer (L1) and a sub-epidermal layer (L2) (Fig. 3A, 3C). On the contrary, SAMs of F₁ plants looked like flat structures, and fuzzy cell layers were observed (Fig. 3B). At 40 DAG, though parents had floral meristems, F₁ plants did not have any floral meristem, and their SAMs remained as abnormal structures (Fig. 3D–3F).

Programmed cell death occurred in the leaves of F₁ plants

The trypan blue assay was used to evidence cell death. We detected blue spots in non-hard, normal leaves of F₁ plants showing hybrid weakness at 50 DAG, whereas the leaves of their parents remained nearly unstained (Fig. 4). Cell death is often induced by the increased production of ROS (Vellosillo et al. 2010, Wang et al. 2018). We detected H₂O₂ in the base of non-hard, normal leaves in F₁ plants at 50 DAG (Fig. 5, middle panel, left), but not in other parts of F₁ leaves (Fig. 5, middle panel, center and right) or in the parents’ leaves (Fig. 5, upper and lower panels). Cell death and H₂O₂ accumulation were confirmed to be reproducibly detected only in leaves of F₁ plants with three biological replicates.

We investigated whether internucleosomal fragmentation...
of DNA (DNA laddering), which is one of the hallmarks of PCD, was present in the leaves of F1 plants. We first investigated leaves of both parents and non-hard, normal leaves of F1 plants at 60 DAG, but any ladder pattern was detected after 20 trials. Then, we investigated leaves of both parents and hard leaves of F1 plants at 100 DAG along with normal leaves of F1 plants at 60 DAG. A distinctive ladder pattern was detected for DNA extracted from hard leaves (Fig. 6). The characteristic sizes of the DNA fragments forming the ladder (integer multiples of around 200 bp) indicated internucleosomal fragmentation of DNA.

**Expression of pathogenesis-related genes was increased in F1 leaves**

We hypothesized that cell death in hard leaves of F1 plants was induced by a pathogen response-like reaction; so, we investigated the gene expression of CaPR1 and CaPR2, which are immunity marker genes associated with the plant defense hormone salicylic acid (Uknes et al. 1992). The expression of CaPR1 and CaPR2 was upregulated in hard leaves of F1 plants, while this response was not detected in any of the parents (Fig. 7).

**Discussion**

After showing a normal initial growth (approximately until 20 DAG), F1 plants in the cross of C. annuum × C. chinense grew less than their parents, resulting in lower plant height, fewer leaves, and a shorter 5th internode. Additionally, abnormal structures in SAMs of F1 plants (Fig. 3) are likely to be related to these phenotypes of hybrid weakness.

Yazawa et al. (1989) reported that cell division in SAMs could be low for certain interspecific Capsicum crosses; however, no detailed description about the cell layers composing SAMs was provided. In general, the surface of a
SAM comprises a layer structure named tunica, which is composed of L1 and L2 layers. Surface cells of SAMs appear aligned because L1 and L2 cells divide by anticlinal division. The cells located under the tunica form the corpus. We detected a disordered disposition of cells in L1 and L2 layers and the flat structure of the SAM in F1 plants (Fig. 3). A disordered disposition of L1 and L2 cells and a flat structure was also found in shoot meristems of the Arabidopsis mutant wuschell (wus) (Laux et al. 1996). Generally, the WUS gene controls the size of the stem cell in SAM (Laux et al. 1996). Additionally, SAMs in fasciata 1 (fas1) mutant of Arabidopsis showed ectopic expression of WUS, which resulted in the disorder of L1 and L2 layers, as well as in a flat structure (Kaya et al. 2001). Therefore, some abnormality of the gene WUS may be involved in Capsicum hybrid weakness.

In two plant genera, PCD has been detected and linked to abnormal growth of F1 plants. Hallmarks of PCD such as chromatin condensation, nuclear fragmentation, and DNA internucleosomal fragmentation were detected in the hybrid lethality phenotype resulting from interspecific crosses of Nicotiana spp. (Marubashi et al. 1999, Tezuka and Marubashi 2004, Tezuka et al. 2007). Likewise, DNA fragmentation was detected by TUNEL assays in hybrid rice showing weakness (Chen et al. 2014), although it was unclear whether the fragmentation was internucleosomal.

In this study, we demonstrated that PCD, accompanied by H2O2 generation and DNA internucleosomal fragmentation, is involved in Capsicum hybrid weakness. These results suggest that PCD is universally involved in the abnormal growth of F1 plants, even if the phenotypes of abnormal growth are different. Cell death was also observed in F1 plants displaying abnormal growth in other genera such as Arabidopsis (Bombilies et al. 2007) and Triticum spp. × Aegilops spp. crosses (Mizuno et al. 2010, Okada et al. 2017). Nevertheless, further studies focused on the phenomenon of cell death using various plant species will be needed to clarify universal involvement of PCD in the abnormal growth of F1 plants.

Hypersensitive response (HR) is known as a plant defense reaction against pathogens. Our results suggest that PCD observed in the hybrid weakness resulting from the cross C. annuum × C. chinense is induced by an HR-like reaction. It has been reported that the HR is induced when resistance (R) gene products recognize specific effector molecules secreted by pathogens (Coll et al. 2011). Subsequently, MAPK cascades lead to increases in calcium levels, and calcium accumulation triggers salicylic acid and ROS production (Coll et al. 2011, Wang et al. 2018). Salicylic acid activates the expression of defense genes, such as PR genes, and H2O2 accumulation leads to the hardening of leaves and PCD (Vellosillo et al. 2010, Wang et al. 2018). Plants exhibiting HR sometimes show dwarfism (de Quadros et al. 2019, Huang et al. 2018). Additionally, there are some reports indicating that HR-like reaction was involved in the abnormal growth of F1 plants (Mizuno et al. 2010, Okada et al. 2017). In the present study, dwarfism, accumulation of H2O2, expression of PR genes, hardening of leaves, and PCD were detected in Capsicum hybrid weakness. Hence, we propose that an HR-like reaction would be involved in Capsicum hybrid weakness.

In conclusion, the morphological characters of Capsicum hybrid weakness are shorter height, fewer number of leaves, shorter 5th internode in dwarf F1 plants, and SAMs with abnormal structure. Moreover, hybrid weakness in the F1 plants was associated with an HR-like reaction, which involved PCD, H2O2 generation, and PR gene expression.

Author Contribution Statement

All authors conceived and designed this research. KS conducted the experiments, analyzed the data obtained, and wrote the manuscript. All authors read and approved the manuscript.

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HR-like reaction is involved in hybrid weakness in *Capsicum*


