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Programmed cell death (PCD) is an important biological process that occurs during normal plant growth and development or under stress conditions. Toxin3 is a mycotoxin that is produced by the pathogenic fungus *Drechslera gigantea* and can cause severe PCD in *Arabidopsis thaliana*. Jasmonates play a key role in plant growth, development and survival, and the JA pathway is involved in mediating plant defense responses to pathogens. Protein1 and Protein2 in *Arabidopsis thaliana* can participate in plant disease resistance and injury stress responses and in the regulation of plant growth and development through the regulation of the jasmonic acid (JA) signaling pathway.

This study revealed that Protein1 and Protein2, as members of the Arabidopsis ubiquitin ligase family, exhibit significant ubiquitin ligase activity. The analysis of the relationship between the mycotoxin Toxin3 and the expression of *Protein1* and *Protein2*, as well as functional analysis of Protein1 and Protein2 mutants and transgenic lines, showed that both were involved in the physiological process of Toxin3-induced PCD in plants. Subsequent experiments showed that mutations in DPL1, a key factor in the jasmonic acid signaling pathway, resulted in the blocking of the Protein1 and Protein2 response to Toxin3, suggesting that Protein1 and Protein2 might mediate the JA signaling pathway and participate in the process regulating Toxin3-induced PCD in plants.

**1 Different characteristics of Protein1 and Protein2 responses after Toxin3 treatment**

**1.1 The effect of Toxin3 on the transcription levels of *Protein1* and *Protein2***

We studied the effect of Toxin3 on the transcription levels of *Protein1* and *Protein2* by real-time PCR. *Arabidopsis thaliana* leaves were grown for 4 weeks and then treated with 10 mM MgCl2 (Mock) or 10 μM Toxin3 (Toxin3). RNA was extracted from leaves collected at 0 h, 24 h, 48 h, and 72 h of treatment, and the *Protein1* and *Protein2* transcript levels were measured by real-time PCR using *INTER10* as an internal reference. The transcript level at each time point was corrected. The results showed that Toxin3 induced *Protein1* transcription. Conversely, at 72 h, Toxin3 induction exhibited an approximately 15-fold inhibitory effect on *Protein2* transcription, as shown in Figure 2D.

**2 Toxin3-induced PCD requires the synergistic effect of Protein1 and Protein2**

**2.1 Phenotypic analysis of Toxin3-induced PCD in Protein1 and Protein2 overexpression and mutant lines**

To analyze the functions of Protein1 and Protein2 in Toxin3-induced PCD, we hybridized *protein1* and *protein2* single mutants to produce a *protein1 protein2* double mutant. For Col-0, *protein1*, *protein2* and the leaves of *protein1 protein2*, respectively, 10 mM MgCl2 (Mock) or 10 μM Toxin3. After 72 h of treatment, and the leaves were sampled and photographed to determine the occurrence of PCD. The results are shown in Figure 3A. The degree of PCD in the *protein1 protein2* double mutant was significantly reduced compared to that in the wild-type (Col-0).

Additionally, we produced transgenic plants overexpressing *Protein1* and *Protein2* and selected two overexpression lines for each gene (*Protein1ox5*, *Protein1ox6,* *Protein2ox7* and *Protein2ox8*). The leaves of the Col-0 control group were treated with 10 mM MgCl2 (Mock) or 10 μM Toxin3. After 72 h of treatment, the leaves were photographed, and the occurrence of PCD was observed. Compared with the wild-type control Col-0 plants, both Protein1- and Protein2-overexpressing plants had more severe levels of Toxin3-induced PCD, as shown in Figure 3A.

Together, the results of Toxin3 induction of PCD in mutant and overexpression strains indicate that Protein1 and Protein2 both play a positive regulatory role in the process of Toxin3-induced PCD. To verify this conclusion, we conducted two subsequent experiments: quantitative analysis of electrical conductivity and statistical analysis of the seedling susceptibility to PCD and the proportion of PCD changes in seedlings.

**2.2** **Overexpression of Protein1, Protein2 and Toxin3 in mutants** **and a statistical analysis of sensitivity**

To further analyze the positive regulatory relationship of Protein1 and Protein2 in the process of Toxin3 induction of PCD, we performed statistical analysis of the sensitivity of the Protein1 and Protein2 overexpression and mutant lines to Toxin3. First, Col-0, *protein1*, *protein2*, *protein1 protein2* were cultured for one week. *Protein1ox5*, *Protein1ox6*, *Protein2ox7,* and *Protein2ox8* seedlings were transferred to new Murashige and Skoog (MS) medium containing 2 μM Toxin3 and were cultured for 6 days. Then, the lines were divided into hypersensitive, sensitive, and insensitive groups according to their sensitivity to Toxin3. This is shown in Figure 3C. For the plants of each ecotype, we performed the classification and counting of supersensitive, sensitive and insensitive plants, as shown in Figure 3C, and calculated the ratio of each sensitivity category in all plants (n>50) in each ecotype. The statistical results obtained are shown in Figure 3D. Compared with that in Col-0, the proportion of *protein1 protein2* that were insensitive to FB1 was greatly increased, while that of *protein1 protein2* was significantly higher than that of Col-0. In *Protein1ox5*, *Protein1ox6*, *Protein2ox7,* and *Protein2ox8,* the proportion of plants with insensitivity to FB1 was greatly reduced, and the proportion of plants with hypersensitivity was greatly increased. Thus, the statistical analyses of FB1 sensitivity also indicated that Protein1 and Protein2 play a positive regulatory role in the process of Toxin3-induced PCD.

Based on the above experimental results, it can be concluded that Toxin3 can induce the expression of *Protein1* and inhibit the expression of *Protein2* and that Protein1 and Protein2 play a positive regulatory role in Toxin3-induced PCD. The positive regulatory effect is achieved by Protein1 and Protein2 as active ubiquitin E3 ligases. It has been reported that Toxin3-induced cell death is dependent on the SA, ET, and JA signaling pathways. We sought to investigate whether the induction of *Protein1* and inhibition of *Protein2* by Toxin3 and the positive regulatory effects of Protein1 and Protein2 on Toxin3-induced PCD might also be related to the SA, ET, and JA signaling pathways. To this end, we conducted the following experiments.

**3 The transcriptional response of Protein1 and Protein2 to Toxin3 requires a complete JA signaling pathway**

**3.1 Pairs of Toxin3 in JA, SA, and ET signaling pathway mutants** ***Protein1* and *Protein2* and the transcription** **impact of the product**

To investigate whether the induction of *Protein1* and inhibition of *Protein2* by Toxin3are related to the SA, ET, and/or JA signaling pathways, we examined the transcript levels of *Protein1* and *Protein2* after Toxin3 treatment of plants with mutations in genes related to the JA, SA, and ET signaling pathways. To understand the effect of Toxin3 on the transcription products of *Protein1* and *Protein2* in the JA, SA, and ET signaling pathways, we used the following mutant materials purchased or stored in the laboratory: mutants of the JA signaling pathway genes *dpl1-1*, *dpl1-2*, *nvb2-2*, and *nvb2 nvb3 nvb4* triple mutant; mutants of the SA signaling pathway genes *mbkH*, *obf4,* *mqt1-3,* and *tef2*; mutants of the ET signaling pathway genes *igc2-1*, *ihm2-2* and *ihm3-1 inp1-1* (double mutant); JA and SA signaling pathway double mutants *mbkH dpl1-1* and *obf4 dpl1-1*; and triple mutant affecting all three signaling pathways, *mqt1-1 ked1 ihm2-2*. Among the mutant strains, *dpl1-1* was derived from the Col-gl ecotype, while the others were derived from the Col-0 ecotype.

The seedlings of the Col-0, Col-gl, and all the above mutant plants that had been cultured for 5 days were first transferred to the seedlings. The cells were cultured on new MS medium with (Toxin3) or without (MS) 2 μM Toxin3 for another 6 days. The intact seedlings were ground, and RNA was extracted. After reverse transcription of 2 μg of RNA, the transcription levels of *Protein1* and *Protein2* were analyzed by real-time PCR, and *INTER10* was used as the internal reference for the control of the real-time PCR system. The transcription of *Protein1* and *Protein2* was performed using Col-0 MS. The data for other genotypes were corrected at a level of 1. It can be seen from the results in Figure 4A that for *Protein1*, compared with Col-0 or Col-gl, in *dpl1-1*, *dpl1-2*, *mbkH dpl1-1*, *obf4 dpl1-1*, and *mqt1-1 ked1 ihm2-2*, the induction effect of Toxin3 on *Protein1* was greatly weakened and not obvious. Although Toxin3 still induced Protein1 in *nvb2 nvb3 nvb4*, the induction efficiency was greatly weakened. These results suggest that deletion mutations in the receptor of the JA signaling pathway, DPL1-1, can severely impact the effect of Toxin3 on *Protein1*. The effect of Toxin3 on inducing *Protein1* is closely related to the JA pathway. Similarly, for *Protein2*, the inhibitory effect of Toxin3 is also closely related to the JA pathway.

**4 The positive regulatory effect of Protein1 and Protein2 on Toxin3-induced PCD requires a complete JA pathway**

To determine whether the JA signaling pathway plays an important role in the function of Protein1 and Protein2 in PCD, we examined *protein1 protein2* double mutants, *Protein1ox5* and *Protein2ox7* plants overexpressing *Protein1* and *Protein2*, respectively, and *Protein1* and *Protein2* double mutants or the overexpression plants were crossed with JA pathway *dpl1* or *nvb2* single mutants to obtain the mutants *protein1 protein2 dpl1-2*, *protein1 protein2 nvb2-2*, *Protein1ox5 dpl1-2*, *Protein2ox7 dpl1-2*, *Protein1ox5 nvb2-2,* and *Protein2ox7 nvb2-2*. The materials were preserved in the laboratory to study Toxin3-induced PCD. The leaves of plants with the above genotypes were treated with 10 μM Toxin3. For each genotype, four leaf discs of the same size were sampled from each genotype with a hole punch and were immersed in 10 mM of solution. The MgCl2 conductivity was measured at 2 h, 4 h, 16 h, 24 h, 28 h, 40 h, 48 h, and 72 h after sampling, and the results are shown in Figure 5A. Figure 5A shows that, consistent with the findings in a previous report on the positive regulation of Toxin3-induced PCD by JA, after Toxin3 treatment, the ion permeability was lower in *dpl1-2* than in Col-0, indicating that the degree of PCD in *dpl1-2* was weaker than that in Col-0. The ion permeability of *dpl1-2* was also lower than that of *protein1 protein2*, indicating that the inhibition of PCD in *dpl1-2* was stronger than that in *protein1 protein2*, but the conductivity of *protein1 protein2 dpl1-2* was similar to that in *dpl1-2*. These results indicate that although the increase in Protein1 and Protein2 expression levels can promote Toxin3-induced ion penetration, this promotion effect disappears in lines derived from *dpl1-2*. This finding indicates that DPL1 plays a decisive role in the positive regulation of PCD by Protein1 and Protein2. the conductivity increased faster in *nvb2-2* than in Col-0 over time, indicating the negative regulatory effect of NVB2 in the process of Toxin3-induced PCD. This is similar to the role of NVB2 in the defense response of pathogens. In addition, compared with the wild-type, the reduced ion permeability in *protein1 protein2* and the increased ion permeability in the overexpression plants were both promoted by hybridization with *nvb2-2*, indicating that NVB2 was also involved in the process of the positive regulatory role of Protein1 and Protein2 in Toxin3-induced PCD.

Toxin3 induces the expression of JA response genes, such as *PLANT DEFENSIN1.2* (*PDF1.2*) and *PATHOGENESIS-RELATED5* (*PR5*). As shown in Figure 5B, in *protein1 protein2*, the degree of Toxin3 induction of *PDF1.2* and *PR5* was attenuated, while in *Protein1ox5* and *Protein2ox7*, the expression levels of *PDF1.2* and *PR5* were increased compared with Col-0, indicating that Protein1 and Protein2 can promote the expression of JA marker genes induced by Toxin3. In JA-insensitive *dpl1-2*, *protein1 protein2 dpl1-2*, *Protein1ox5 dpl1-2*, and *Protein2ox7 dpl1-2*, the expression levels of *PDF1.2* and *PR5* induced by Toxin3 were very similar to those of *protein1 protein2*. The downregulation of Protein1 and Protein2 indicated that the role of Protein1 and Protein2 in *PDF1.2*, and *PR5* responses to Toxin3 were dependent on DPL1. Compared with Col-0, *nvb2-2* had increased *PDF1.2* and *PR5* expression levels, further indicating that NVB2 plays a negative regulatory role in the Toxin3-induced JA pathway. In *protein1 protein2*, the expression of *PDF1.2* and *PR5* was inhibited, but in *protein1 protein2 nvb2-2,* not only did this inhibitory effect disappear but the expression level was even higher than that in Col-0. *Protein1ox5 nvb2-2.* *Protein2ox7 nvb2-2* was further increased, suggesting that NVB2 might play a regulatory role in these two Toxin3-responsive genes downstream of Protein1 and Protein2.

Taken together, these results indicate that the complete JA signaling pathway is very important for the positive regulatory roles of Protein1 and Protein2 in Toxin3-induced PCD.