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GLUCOSE-6-PHOSPHATE DEHYDROGENASE IS REQUIRED FOR HPA1$_{xoo}$ (HARPIN PROTEIN FRAGMENT)-MEDIATED SALT STRESS TOLERANCE IN TRANSGENIC *ARABIDOPSIS THALIANA*

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Abstract

Harpin induces salicylic acid and abscisic acid signaling in plants under biotic and abiotic stress, respectively. Our previous report showed that the effective harpin fragment Hpa1$_{xoo}$ enhanced H_2O_2 production and pathogen resistance in a transgenic *Arabidopsis* mutant. In this study, we examined contents of thiobarbituric acid reactive substance (TBARS), H_2O_2 and glutathione, and glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR) and glutathione peroxidase (GPX) enzyme activity in Hpa1$_{xoo}$-expressing *Arabidopsis* under salt stress. The results revealed increased amounts of TBARS and H_2O_2 in wild-type (WT) compared to mutant plants under salt stress conditions. In contrast, increased levels were observed in the mutant under stress-free conditions. Moreover, a higher reduced glutathione (GSH) content and ratio of GSH/oxidized glutathione (GSSG) was observed in mutant compared to WT plants under bothstress-free and salt stress conditions. In addition, mutant plants exhibited significantly higher G6PDH, GR and GPX activity than WT plants under salt stress. Suppression of G6PDH activity via 6-aminonicotinamide (6-AN, a specific inhibitor of G6PDH) was partly reversed by L-buthionine-sulfoximine (BSO, a specific inhibitor of GSH regeneration) and aggravated by GSH. Combined with previous reports, these findings suggest that the G6PDH enzyme plays a key role in harpin fragment (Hpa1$_{xoo}$)-mediated salt stress tolerance in transgenic *Arabidopsis.*

Key words: *Arabidopsis thaliana*, Harpin protein, Pentose phosphate pathway, G6PDH enzyme, Salinity stress.

Introduction

Plants are prone to various biotic and abiotic stresses within their changing environment (Atkinson & Urwin, 2012; Mittler, 2006). Pathogen infection, insect attack, drought, salinity and extreme temperatures can reduce both crop yield and quality. Environment stress also causes oxidative damage, increasing production of reactive oxygen species (ROS) (Torres & Dangl, 2005), which, in turn, impairs production of macromolecular compounds such as lipids, proteins and nucleic acids (Deng *et al.,* 2016). Maintaining cellular redox homeostasis under both stress-freeand environmental stress conditions is therefore very important (Foyer & Noctor, 2005).Accordingly, plants have evolved highly efficient mechanisms aimed at maintaining cellular redox balance. The thiol pool (e.g., reduced glutathione (GSH)) is indispensable in controlling ROS production under control. GSH uses NADPH as a reductant to reduce its oxidative form (GSSG) via glutathione cycling, while generation of NADPH requires the G6PDH enzyme (Wang *et al*., 2008; Liu *et al.,* 2007).

Salinity seriously impairs growth and reduces crop yield, and despite efforts to improve resistance via traditional breeding, success has been limited (Miklas *et al.,* 2006). This lack of progress is attributable to the complexity of the tolerance trait, which is influenced by coordinated and differential expression of a network of genes. Recently, however, transgenic approaches have successfully enhanced abiotic stress tolerance in agriculturally important crops using only a limited number of target traits (Wang *et al*., 2003).

Erwinia amylovora produces an acidic, heat-stable protein harpin that elicitsa hypersensitive response during plant-pathogen interaction (Wei *et al.,* 1992; He *et al.,* 1994), triggering salicylic acid signaling in plants under pathogen attack (Dong *et al.,* 1999). Recently, an effective fragment (Hpa1$_{Xoo}$) of the harpin protein was screened and introduced into *Arabidopsis*, enhancing H_2O_2 accumulation and biotropic pathogen resistance (Sang *et al.,* 2012). Furthermore, exogenous harpin protein was found to trigger abscisic acid signaling, inducing drought tolerance in *Arabidopsis* (Dong *et al.,* 2005), while antioxidant enzyme was found to be necessary for harpin-mediated drought tolerance in *hrf1*-overexpressing rice (Zhang *et al.*, 2011).

In general, ROS production favors biotrophic pathogen resistance (Barna *et al.,* 2012; Torres *et al.,* 2006), but is not beneficial to abiotic stress tolerance in plants (Gill & Tuteja, 2010). Moreover, salicylic acid signaling can antagonize abscisic acid signaling in plants under environmental stress (Mauch-Mani & Mauch, 2005). Nevertheless, harpin protein can induce both biotic and abiotic stress resistance in plants (Dong *et al.,* 1999, 2005). We therefore speculated that different protein regions of harpin possess different functions, similar to the human estrogen receptor (Tora *et al.,* 1989); however, it is also possible that the harpin fragment plays multiple roles. In this study, transgenic *Arabidopsis* expressing the harpin fragment (Hpa1$_{Xoo}$) was used to test this hypothesis, with the aim of understanding whether this harpin fragment induces abiotic tolerance as well as pathogen resistance, and if so, the possible underlying mechanisms.

Materials and Methods

Plant and experimental design: Seeds of *Arabidopsis thaliana* (Col) and Hap1$_{xoo}$ transgenic plants (Sang *et al.,* 2012) were sown in pots containing perlite: vermiculite (1:3) and grown under a 16 h: 8 h light/dark cycle (200

μmol photon $m^{-2}s^{-1}$) in a greenhouse at 22–25°C and 50–70% relative humidity. Plants were cultivated for five weeks with 10 mL 1/10 strength Hoagland solution applied every two days (preliminary experiment, data not shown). For salt stress treatment, NaCl (0, 50, 100 and 150 mM) was applied to *Arabidopsis* seedlings grown in 10% Hoagland solution (aerated with air) at 25°C under a 12h: 12h photoperiod. H_2O_2 (10 mM), GSH (10 mM), L-buthionine-sulfoximine (BSO, 25 mM; a specific inhibitor of GSH biosynthesis; May & Leaver, 1993) and 6-aminonicotinamide (6-AN, 5 mM; specific inhibitor of the pentose phosphate pathway, PPP; Mou *et al.*, 2003) were respectively foliar sprayed onto the seedling (1 ml per seedling, respectively; three times during the first 24 hours) for further investigation under 100-mM NaCl conditions. Third and fourth leaves from the top of salt-stressed plants were harvested and frozen in liquid nitrogen until analysis.

H_2O_2 content and lipid peroxidation assay: H_2O_2 content was determined according to the xylenol orange method of Gay *et al.,* (1999), and thiobarbituric acid reactive substance (TBARS) was used for evaluation of lipid peroxidation according to Heath & Packer (1968) with some modifications (Deng *et al.*, 2016). Briefly, leaves from salt-stressed plants were homogenized in 2.5% trichloroacetic acid (TCA), centrifuged at 4000 ×g at 25°C then 200 μl of the supernatant added to the mixture solution (trichloroacetic and thiobarbituric acids). The solution was heated at 98°C then quickly cooled in ice water and centrifuged again. Absorbance of the supernatant was monitored at 532 and 600 nm to determine the TBARS content using an extinction coefficient of 155 mM^{-1} cm^{-1}.

G6PDH, GPX and GR activity: G6PDH enzyme was extracted according to Esposito *et al.*, (2001) with some modifications. Briefly, 1.0 g of leaves was ground in liquid N_2then 2 ml of extract buffer added to 8 ml of solution (50 mM Hepes-Tris (pH 7.8), 3 mM $MgCl_2$, 1 mM EDTA, 1 mM PVP, and 1 mM dithiothreitol). The homogenate was centrifuged at 12 000 ×g for 15 min at 4°C then 100 uL supernatant added to the dehydrogenase (G6PDH + 6PGDH) and 6-phosphogluconate dehydrogenase (6PGD) assay buffers. The reduction of NADP to NADPH was assayed as the rate of change in absorbance at 340 nm during the first 5 min, and G6PDH activity calculated as the total dehydrogenase activity minus 6PGD activity (Tian *et al.*, 1998).GPX activity was measured according to Athar & Iqbal (1998) at 340 nm during the initial 1 min of the reaction at 25°C. GR activity was determined spectrophotometrically according to Foyer and Halliwell (1976) and expressed as nmol NADPH min^{-1} g^{-1} dry weight.

Total glutathione assay: The total content of reduced (GSH) and oxidized glutathione (GSSG) was measured according to Nagalakshmi *et al.,* (2001). GSSG was determined after removal of GSH using 2-vinylpyridine derivatization, and GSH by subtracting GSSG from the total glutathione content (GSH+GSSG).

Data analysis: All data were analyzed using SPSS 13.0 software and means were analyzed using Duncan's multiple range test at $p<0.05$). Three replicates per each treatment were analyzed.

Results

Figure 1 shows the effect of salt stress on TBARS and H_2O_2 contents in transgenic (mutant) and wild-type (WT) *Arabidopsis*. Both TBARS and H_2O_2increased in WT compared to mutant plants under salt stress conditions (Fig. 1). For example, an increase of approximately 27and 19%, respectively, was observed in WT compared to mutant plants under 100 mM NaCl (Fig. 1a-b). In contrast, an increase in TBARS of 9% and $H_2O_2$of 23% was observed in mutant compared to WT plants under stress-free conditions (Fig. 1).

Figure 2 shows the GSH and GSSG contents and redox values (GSH: GSSG) of WT and mutant plants under salt stress conditions. A significant increase in GSH and higher ratio of GSH/GSSG was observed in mutant compared to WT plants under both stress-free and salt stress conditions (Fig. 2a,c). GSH increased by approximately 28, 23, 33 and 94%in the mutant plants under 0, 50, 100 and 150 mM NaCl, respectively (Fig. 2a). In addition, a decrease in GSSG accumulation was observed in mutant plants under 100 and 150 mM NaCl (Fig. 2b).

Figure 3 shows the effect of salt stress on G6PDH, GR and GPX enzyme activities. An initial increase followed by a decrease in all enzyme activities was observed with increasing salt stress, and all three enzymes were significantly enhanced in mutant compared to WT plants under salt-stress conditions (Fig. 3). An increase in G6PDH of approximately 14, 29, 50 and 63% was observed in mutant plants under 0, 50, 100 and 150 mM NaCl, respectively (Fig. 3).

Figure 4 shows the effect of 6-AN on G6PDH enzyme activity, H_2O_2 content and TBARS accumulation in mutant and WT plants under stress-free and 100-mM NaCl conditions. As shown, G6PDH activity significantly decreased with 6-AN treatment (a specific inhibitor of PPP; Mou *et al.*, 2003), especially in WT plants (Fig. 4a). An increase in G6PDH activity of approximately 80% was observed in 6-AN-treated mutant plants under salt stress (Fig. 4a). Moreover, this reduction effect was further enhanced by addition of GSH (Fig. 4a). However, this impairment was partly reversed by BSO application(a specific inhibitor of GSH synthesis, Fig. 4a; May & Leaver, 1993). Compared with G6PDH activity, 6-AN treatment significantly increased H_2O_2 and TBARS accumulation, especially in WT plants (Fig. 4b,c). For example, an increase in H_2O_2 and TBARS of approximately 34 and 26% was observed in 6-AN-treated WT compared to mutant plants under salt stress (Fig. 4b,c). Similarly, GSH and BSO treatment significantly attenuated and aggravated, respectively, the effect of 6-AN on $H_2O_2$and TBARS accumulation in both WT and mutant plants (Fig. 4b,c).

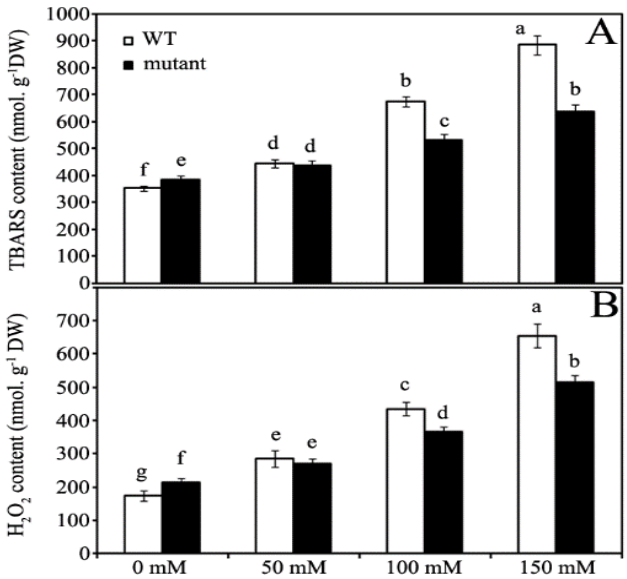

Fig. 1. Oxidative damage under salt stress.
Effects of salt stress (0, 50, 100 and 150 mM) on TBARS accumulation (A) and H_2O_2 content (B) in transgenic and wild-type (WT) plants. Data represent means of three independent experiments. Means followed by the same letter are not significantly different (Duncan's multiple range test, $p<0.05$). Bars indicate ± S.E. (n = 3)

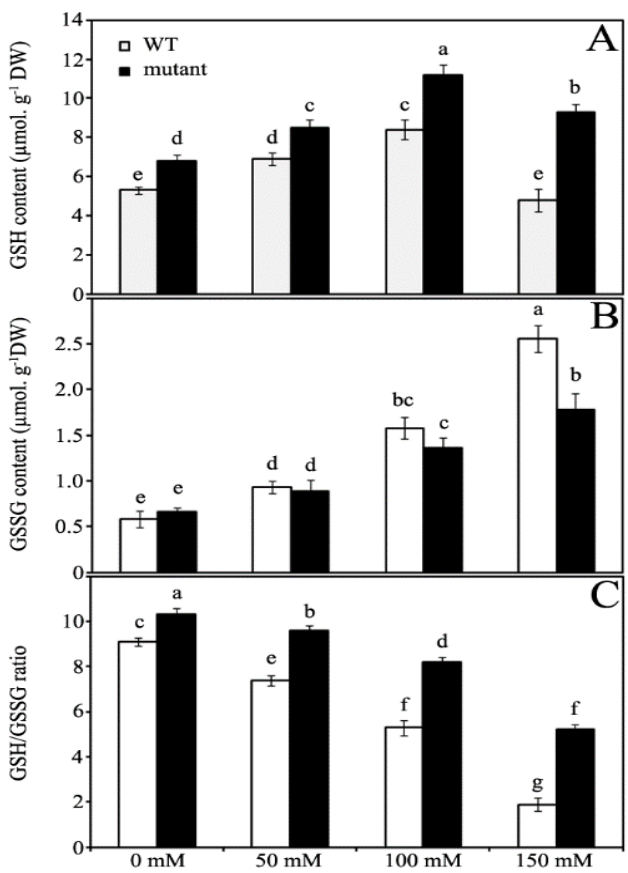

Fig. 2. Glutathione content under salt stress.
Effects of salt stress (0, 50, 100 and 150 mM) on GSH content (A), GSSG content (B) and the GSH/GSSG ratio (C) in transgenic and wild-type (WT) plants. Data represent the means of three independent experiments. Means followed by the same letter are not significantly different (Duncan's multiple range test, $p<0.05$). Bars indicate ± S.E. (n = 3)

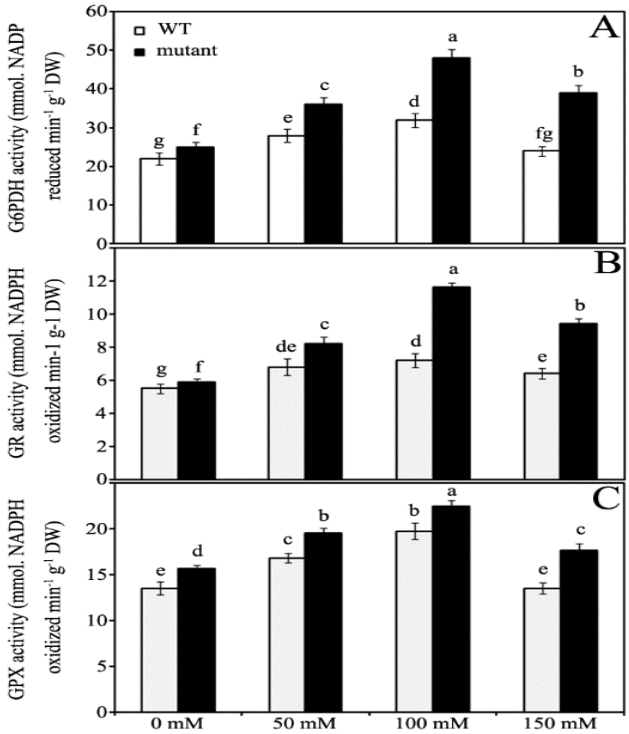

Fig. 3. Enzyme activities under salt stress.
Effects of salt stress (0, 50, 100 and 150 mM) on G6PDH (A), GR (B) and GPX (C) activities in transgenic and wild-type (WT) plants. Data represent the means of three independent experiments. Means followed by the same letter are not significantly different (Duncan's multiple range test, $p<0.05$). Bars indicate ± S.E. (n = 3)

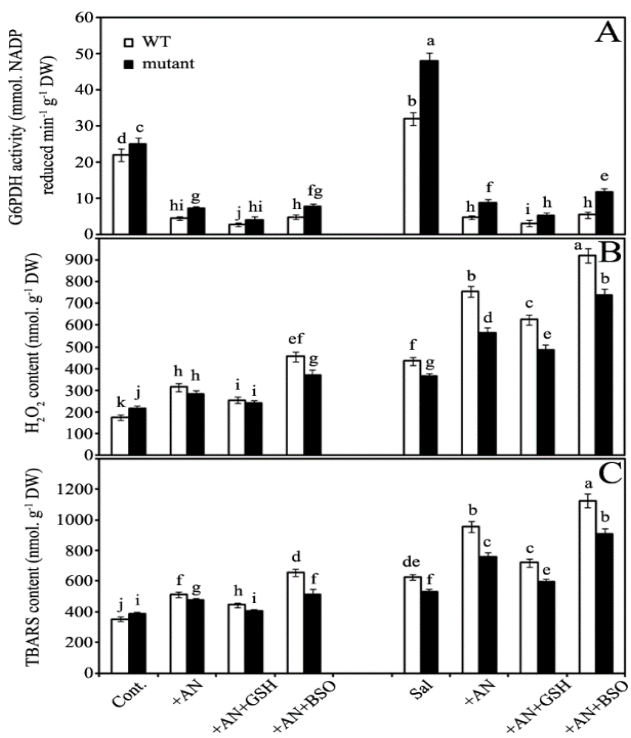

Fig. 4. Effect of 6-AN on enzyme activity, H_2O_2 content and TBARS level.
Effects of 6-AN on G6PDH activity (A), H_2O_2 content (B) and TBARS level (C) in transgenic and wild-type (WT) plants under 100 mM NaCl conditions. Data represent the means of three independent experiments. Means followed by the same letter are not significantly different (Duncan's multiple range test, $p<0.05$). Bars indicate ± S.E. (n = 3)

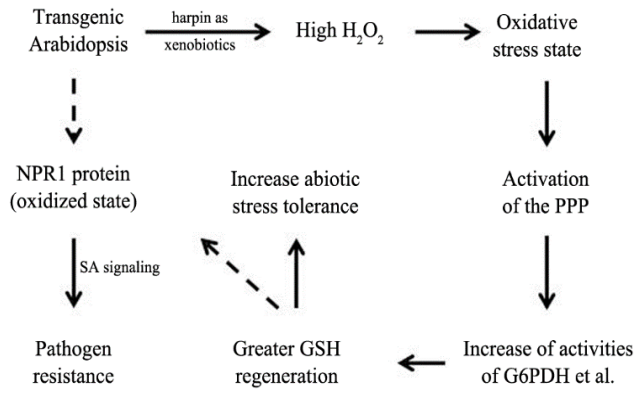

Fig. 5. Hypothetical model.
A "xenobiotic" hypothetical model based on the H_2O_2 priming effect is proposed to illustrate endogenous harpin fragment induction of abiotic and biotic stress resistance. During this process, activation of PPP is required for the regeneration of reduced glutathione. PPP, pentose phosphate pathway; G6PDH, glucose-6-phosphate dehydrogenase

Discussion

This study evaluated the protective effect of harpin fragment ($Hpa1_{xoo}$) in *Arabidopsis* plants under salt stress (Fig. 1a), revealing increased salt tolerance in transgenic plants. While increased accumulation of H_2O_2 was observed in WT compared to transgenic plants under salt stress conditions (Fig. 1b), an increase in H_2O_2 was observed in transgenic but not WT plants under non-stress conditions (Fig. 1b). This is in accordance with our previous findings (Sang *et al.,* 2012).

Plants have evolved a wide range of mechanisms to cope with biotic and abiotic stresses, while emerging evidence suggests that hormone as well as ROS signaling pathways play key roles in the crosstalk between biotic and abiotic stress signaling (Fujita *et al.,* 2006). In general, increased levels of H_2O_2 favor plant tolerance of biotic but not abiotic stressors (Torres *et al.,* 2006; Gill & Tuteja, 2010). Interestingly, treatment with compounds such as β-aminobutyric acid (BABA, Zimmerli *et al.,* 2000; 2008) and harpin protein (Dong *et al.,* 1999; 2005) were found to enhance both pathogen resistance and abiotic stress tolerance in plants. Moreover, harpin protein produced by bacteria, as well as xenobiotic BABA, were found to induce abscisic acid signaling in plants under abiotic stress (Dong *et al.* 2005; Zimmerli *et al.* 2008). Furthermore, our recent study showed that the harpin fragment $Hpa1_{xoo}$, like BABA, induces ROS production in plants (Dubreuil-Maurizi *et al.,* 2010; Sang *et al.,* 2012). Data also suggest the existence of reactive intermediates during xenobiotic degradation in plant cells (Sandermann, 1988), while antioxidant enzymes such as glutathione reductase were also found to be necessary for xenobiotic degradation (Malan *et al.,* 1990). Thus, the harpin-induced increase in H_2O_2, which can be considered xenobiotic in plants), is also thought to be involved in the degradation process (Fig. 1b; Sang *et al.,* 2011).

The glutathione system was previously found to act as a stress marker in plants during adaptation to environmental change (Tausz *et al.,* 2004). Thus, in $Hpa1_{xoo}$-expressing*Arabidopsis*, this xenobiotic-like protein is likely to be continuously generated and subsequently degraded. During this process, slight oxidative stress induced by increased $H_2O_2$is expected, resulting in "over-compensation" during long-term responses. As a result, GSH production and the GSH/GSSG ratio increase in stress-adapted plants due to activation of the defense system (Tausz *et al.,* 2004). We therefore compared the glutathione content and activity of associated enzymes (e.g. G6DPH, GR and GPX) in WT and harpin-expressing *Arabidopsis* plants under both stress-free and salt stress conditions (Figs. 2 and 3). The results revealed increased GSH and a higher GSH/GSSG ratio in transgenic plants (Fig. 2). Furthermore, glutathione biosynthesis-associated enzymes (e.g. G6PDH and GR) exhibited increased activity in transgenic *Arabidopsis* compared to the WT, especially under salt stress conditions (Fig. 3). Moreover, 6-AN, a specific inhibitor of PPP (Mou *et al.,* 2003), was subsequently used to investigate the possible roles of G6PDH enzyme during $Hpa1_{xoo}$-mediated salt stress in transgenic *Arabidopsis* (Fig. 4), revealing its seemingly indispensable role (Fig. 4). This further suggests that the harpin fragment ($Hpa1_{xoo}$) acts as a xenobiotic, inducing slight oxidative stress and activating the pentose phosphate pathway (Palmer, 1999 Juhnke *et al.,* 1996), which is closely associated with GSH regeneration. In general, PPP is thought to play a key role in plant protection against abiotic stress (Wang *et al.,* 2008; Liu *et al.,* 2007). Thus, increased abiotic stress tolerance is expected in this transgenic *Arabidopsis* (Fig 1a; Dong *et al.,* 2005; Zhang *et al.,* 2011).The harpin-, or harpin-fragment-, induced glutathione generation and higher GSH/GSSG ratio observed in this study also suggest enhanced biotrophic pathogen resistance. It is well-known that oxidized NPR1, which is required for the salicylic acid signaling pathway, is only reduced by GSH, entering the nucleus and regulating subsequent plant systemic-acquired resistance (Mou *et al.,* 2003). Figure 5 proposes a hypothetical xenobiotic model based on H_2O_2 priming to illustrate harpin-mediated abiotic and biotic stress resistance in plants.

To summarize, similar to harpin protein, the harpin fragment $Hpa1_{xoo}$was found to induce abiotic stress tolerance in plants. G6PDH enzyme was found to be the key enzyme; however, details of the underlying mechanism require further clarification. For example, it remains unknown whether peroxisome is required for harpin fragment degradation in transgenic plants. Overall, this study provides novel insight into harpin-mediated biotic and abiotic stress resistance from a xenobiotic and redox system perspective.

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