Mechanisms of different cultivars of *Cucurbita pepo* in resistance to *Podosphaera xanthii* infection through improvement of antioxidative defense system and gene expression

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Research article

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Abstract

Background: Powdery mildew is one of the world's most destructive diseases of cucurbit and the major cause of losses in its production worldwide. A number of strategies have been developed and applied to discover some suitable and alternative safe methods to manage the powdery mildew disease occurrence, but little information is regarding to screen of resistant pumpkins (*Cucurbita pepo* L.) germplasm and explore the mechanisms of their preventing the disease occurrence at physiological, biochemical, and molecular levels. Therefore, we evaluated and determined the ability and mechanisms of two commercial pumpkin cultivars in resistance to *Podosphaera xanthii* infection.

Results: Compared with mock-inoculated seedlings, small and sparse spots were observed on the cultivar of Sixing F₁ leaves at the 13th day after inoculation with *P. xanthii*, whereas a large number of disease spots or a layer of white powdery mildew were observed on the surface of Jin₁₂ F₁ leaves. Increased the inoculation time (7, 9, 11 and 13 days) significantly and continuously increased the disease incidence and index of pumpkin seedlings after inoculation. The disease incidence and index of Jin₁₂ F₁ were significantly higher than the cultivar of Sixing F₁. At Day 13, the disease incidence and index of Jin₁₂ F₁ were 80.0% and 72.6, whereas the cultivar of Sixing F₁ was 22.3% and 17.7, respectively. The contents of H₂O₂, MDA, lignin and total phenolics in the leaves of Sixing F₁ and Jin₁₂ F₁ were markedly accelerated after inoculation with *P. xanthii*. However, the cultivar of Sixing F₁ exhibited less reactive oxygen species (ROS) accumulation, a lower rate of lipid peroxidation and higher level of lignin and total phenolics contents after inoculation, whereas the cultivar of Jin₁₂ F₁ exhibited higher level of ROS accumulation and rates of lipid peroxidation, and lower level of lignin and total phenolics contents. Higher activity and transcript level of antioxidant enzymes and gene of PAL expression were observed on different tissues of both cultivars after inoculated with *P. xanthii*. Compared with pumpkin seedlings that were inoculated with sterile water but not *P. xanthii*, the level of PAL activity and gene expression in leaves, petioles and stems of Sixing F₁ and Jin₁₂ F₁ were upregulated and increased significantly at different time points after inoculation. The enhancement expression of PAL activity and gene in different tissues of Sixing F₁ was significantly higher than Jin₁₂ F₁, and higher in leaves, lower in petioles and stems.

Conclusions: Our results indicate that the cultivar of Sixing F₁ exhibited the highest ability in resistance to *P. xanthii* infection in comparison to Jin₁₂ F₁, and one novel possible mechanism is related to the cultivars in resistance to *P. xanthii* infection by activating and enhancing the antioxidative defense system and gene expression to prevent the pathogens infection.

Background

Pumpkin (*Cucurbita pepo* L.) is one of the most important vegetable crops for human nutrition all over the world [1]. However, powdery mildew is a common and major disease of pumpkin that affects the seedlings and mature plants growth [2], and also is one of the limiting factors that causing severely economic losses in pumpkin by shortening the ripening and harvest intervals, reducing photosynthesis

and yields, and decreasing fruit quality in field and greenhouse [3, 4]. Normally, the pumpkin yield losses due to the disease of powdery mildew by 30 to 50% [5]. In addition, *Podosphaera xanthii* has been considered as one of the most important pathogens that causing the disease of cucurbit powdery mildew and reducing the production worldwide [6]. Thus, suitable control measurements and alternative safe methods, such as effective and friendly environment control methods are needed to overcome these problems.

In past few years, some strategies have been adapted to manage the disease of powdery mildew in agriculture, including the use of chemical, biological fungicides and breeding the resistance varieties [7]. Similar study has reported that the application of fungicides is the most efficient method to control powdery mildew in Iran and elsewhere [8]. However, chemical fungicide that is not healthy and safe due to its hazardous effects on human, animals, plants, and beneficial organisms, as well as developing pathogen resistance [9]. Although biological control agents have been applied to control of powdery mildew, their efficacy mainly depends on climatic conditions [10]. Therefore, screening of resistant pumpkin germplasm will be the best way for developing new cultivars to prevent the disease of powdery mildew occurrence. The resistance to powdery mildew was first observed in cucumber (*Cucumis sativus* L. cv. Puerto Rico 37) [11], thereafter, a large number of resistant materials were found in local cucurbit varieties from South and East Asia [12], but little is known about the specific knowledge for discovering and developing new resistant varieties of pumpkin to prevent powdery mildew occurrence in China, and the mechanisms of pumpkin in resistance to *P. xanthii* infection is a very complex phenomenon and the nature remains unresolved.

A number of studies have been demonstrated that plants can develop appropriate defense mechanisms to recognize and resist inevitable pathogen attacks, i.e. plants defend themselves against fungal infection through the activation of complex defense responses [13]. One of the earliest these responses is the rapid generation of reactive oxygen species (ROS), which includes superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) [14]. Meanwhile, phenylpropanoid pathway is another most important secondary metabolism pathways and defense responses in higher plants [15, 16]. Phenylalanine ammonia-lyase (PAL) is the first enzyme in the phenylpropanoid pathway, participating in the formation of a series of structural and defensive lignin and phenolic compounds [17–20]. Furthermore, *PAL* gene has been widely studied in participating in plant growth, development and defense systems [21–23], such as the upregulated expression of *PAL* gene in plants that can help plant to develop resistance to phytopathogens infection [24–26]. However, to our knowledge, there is little published information regarding the mechanisms of different cultivars of *C. pepo* in resistance to *P. xanthii* infection at physiological, biochemical, and molecular levels.

In view of the above background, the aims of our present study were to (i) evaluate the ability and effectiveness of two commercial pumpkin cultivars in resistance to *P. xanthii* infection, and (ii) determine the defense responses pathway in two commercial pumpkin cultivars after inoculated with the pathogen of *P. xanthii* at different time points, and (iii) explore the possible mechanisms involved in two different pumpkin cultivars in response to *P. xanthii* infection at physiological, biochemical, and molecular levels.

Results

Symptoms of *Cucurbita pepo* after inoculation with *Podosphaera xanthii*

Compared with mock-inoculated Jin₁₂ F₁ (Fig. 1C) and Sixing F₁ seedlings (Fig. 1D), small and sparse spots were observed on the cultivar of Sixing F₁ leaves at the 13th day after inoculation with *P. xanthii* (Fig. 1B), whereas a large number of disease spots were observed on the surface of Jin₁₂ F₁ leaves, even a layer of white powdery mildew was covered on the leaves (Fig. 1A) in comparison to the mock-inoculated Jin₁₂ F₁ seedlings (Fig. 1C). However, the cultivars of Jin₁₂ F₁ (Fig. 1C) and Sixing F₁ (Fig. 1D) leaves have no disease spots occurred in the control group.

Disease severity of *Cucurbita pepo* after inoculation with *Podosphaera xanthii*

Compared with pumpkin leaves that were inoculated with sterile water but not *P. xanthii*, the cultivars of Jin₁₂ F₁ and Sixing F₁ were begun to show the disease symptoms after inoculated with the pathogen of *P. xanthii* at the 5th and 7th days, respectively. However, the disease incidence and index were significantly different between the cultivars of Sixing F₁ and Jin₁₂ F₁ after inoculation with *P. xanthii*. In contrast, the mock-inoculated seedlings of Sixing F₁ and Jin₁₂ F₁ were grown normally, and have no disease symptoms at the recorded days after inoculation, respectively.

Increased the inoculation time (7, 9, 11 and 13 days) significantly and continuously increased the disease incidence and index of two cultivars pumpkin seedlings. The disease incidence and index of Jin₁₂ F₁ were significantly higher than the cultivar of Sixing F₁. At Day 13, the disease incidence and index of Jin₁₂ F₁ were 80.0% and 72.6, respectively, whereas the cultivar of Sixing F₁ was 22.3% and 17.7, respectively. In addition, the cultivar of Jin₁₂ F₁ began to show symptoms at the 5th day after inoculation, while the cultivar of Sixing F₁ at the 7th day after inoculation. Furthermore, the speed of disease spots expansion of the cultivar Jin₁₂ F₁ was faster than Sixing F₁ with the increase of inoculation time (Table 1 and Table 2).

Table 1

The disease incidence of different cultivars of *Cucurbita pepo* after inoculation with *Podosphaera xanthii*

Cultivars	Treatments	Days post inoculation (dpi)						
		1	3	5	7	9	11	13
		—————Disease incidence (%)—————						
Sixing F ₁	Treatment	0.0 a	0.0 a	0.0 b	6.7 b	16.7 b	21.3 b	22.3 b
	Control	0.0 a	0.0 a	0.0 b	0.0 c	0.0 c	0.0 c	0.0 c
Jin ₁₂ F ₁	Treatment	0.0 a	0.0 a	3.3 a	26.7 a	60.0 a	76.7 a	80.0 a
	Control	0.0 a	0.0 a	0.0 b	0.0 c	0.0 c	0.0 c	0.0 c
Data are means of twelve replicates. Different letters in the same column denote significant differences at the <i>p</i> < 0.05 level by Duncan's new multiple range test (n=12). In the two treatments, pumpkin seedlings leaves were inoculated with <i>P. xanthii</i> , whereas in the two controls, pumpkin seedlings leaves were inoculated with sterile water but not <i>P. xanthii</i>								

Table2

The disease index of different cultivars of *Cucurbita pepo* after inoculation with *Podosphaera xanthii*

Cultivars	Treatments	Days post inoculation (dpi)						
		1	3	5	7	9	11	13
—————Disease index—————								
Sixing F ₁	Treatment	0.0 a	0.0 a	0.0 b	4.7 b	13.3 b	16.4 b	17.7 b
	Control	0.0 a	0.0 a	0.0 b	0.0 c	0.0 c	0.0 c	0.0 c
Jin ₁₂ F ₁	Treatment	0.0 a	0.0 a	3.3 a	16.7 a	40.7 a	46.0 a	72.6 a
	Control	0.0 a	0.0 a	0.0 b	0.0 c	0.0 c	0.0 c	0.0 c
Data are means of twelve replicates. Different letters in the same column denote significant differences at the <i>p</i> < 0.05 level by Duncan's new multiple range test (n=12). The treatments are detailed in the footnote of Table 1								

Hydrogen peroxide (H₂O₂) and lipid peroxidation (MDA) contents in pumpkin seedling

The H₂O₂ and MDA contents of Sixing F₁ and Jin₁₂ F₁ seedling leaves after inoculated with *P. xanthii* were increased with the increase of days post inoculation from 1 to 9 or 11 days, peaked at the 9th and 11th days, and then declined gradually. The H₂O₂ and MDA contents of Jin₁₂ F₁ were significantly higher

than Sixing F₁ with the increase of days post inoculation. The maximum H₂O₂ and MDA contents of Sixing F₁ were increased significantly by 26.83% and 26.42% at the 9th and 11th days after inoculation, respectively, whereas 27.08% and 28.32% increased in Jin₁₂ F₁ seedling leaves, compared with each of control under sterile water treatment, respectively (Table 3).

Table 3								
H ₂ O ₂ and MDA content in different cultivars of <i>Cucurbita pepo</i> seedlings after inoculation with <i>Podosphaera xanthii</i>								
Cultivars	Treatments	Days post inoculation (dpi)						
		1	3	5	7	9	11	13
—————H ₂ O ₂ (μmol g ⁻¹ FW)—————								
Sixing F ₁	Treatment	0.25 c	0.28 b	0.39 a	0.46 b	0.52 b	0.47 b	0.38 c
	Control	0.23 d	0.25 c	0.32 c	0.37 d	0.41 d	0.43 c	0.35 d
Jin ₁₂ F ₁	Treatment	0.29 a	0.31 a	0.37 b	0.48 a	0.61 a	0.58 a	0.49 a
	Control	0.27 b	0.28 b	0.32 c	0.41 c	0.48 c	0.48 b	0.44 b
—————MDA (nmol g ⁻¹ FW)—————								
Sixing F ₁	Treatment	2.45 c	2.91 b	3.45 b	4.05 c	4.61 c	4.69 c	4.35 c
	Control	2.21 d	2.45 c	2.88 c	3.27 d	3.68 d	3.71 d	3.57 d
Jin ₁₂ F ₁	Treatment	2.87 a	3.35 a	3.68 a	5.07 a	5.31 a	6.66 a	5.98 a
	Control	2.64 b	2.85 b	3.56 b	4.68 b	5.08 b	5.19 b	4.94 b
Data are means of twelve replicates. Different letters in the same column denote significant differences at the <i>p</i> < 0.05 level by Duncan's new multiple range test (n=12). The treatments are detailed in the footnote of Table 1								

Levels of defense enzyme expression

The activity of defense enzyme PAL in different cultivars and tissues of pumpkin (Sixing F₁ and Jin₁₂ F₁) was increased significantly at the 5th day after inoculated with the pathogen of *P. xanthii*, peaked at the 7th and 9th days, and then declined gradually (Fig. 2 and Fig. 3). However, the activity of PAL differed significantly between the cultivars of Sixing F₁ and Jin₁₂ F₁. Higher level of PAL activity was expressed in the cultivar of Sixing F₁ leaves after inoculation in comparison to the Jin₁₂ F₁. Compared with control, PAL activity of Sixing F₁ was increased by 39.15% and 32.52% in leaves (Fig. 2A), as well as 27.19% and 22.31% in petioles (Fig. 2B), and 13.83% and 13.75% in stems (Fig. 2C) at the 7th and 9th days,

respectively. In contrast, the PAL activity of Jin₁₂ F₁ was increased by 9.27% and 12.84% in leaves (Fig. 3A), as well as 11.86% and 11.75% in petioles (Fig. 3B), 7.12% and 6.16% in stems (Fig. 3C) at the 7th and 9th days, respectively. In addition, the expression level of *PAL* activity was higher in leaves, lower in petioles and stems. Overall, the control treatment followed a similar trend as the other treatments in terms of the change of PAL activity during the course of the experimental period, but the control treatment had significantly lower PAL activity at a given measurement date starting at the 1st day after inoculation.

Levels of defense gene expression

Higher transcript level of *PAL* gene expression was observed on different cultivars and tissues of pumpkin (Sixing F₁ and Jin₁₂ F₁) after inoculated with the pathogen of *P. xanthii* (Fig. 4 and Fig. 5). Compared with pumpkin leaves that were inoculated with sterile water but not *P. xanthii*, the level of *PAL* gene expression in different tissue (leaves, petioles and stems) of two cultivars Sixing F₁ (Fig. 4) and Jin₁₂ F₁ (Fig. 5) were up-regulated and increased significantly after inoculation with *P. xanthii* at different time points. Also, there were significant differences at the level of *PAL* gene expression between the cultivars of Sixing F₁ and Jin₁₂ F₁ at different time points after inoculation. The level of *PAL* gene expression in different tissues of Sixing F₁ was significantly higher than the cultivar of Jin₁₂ F₁. Similarly, among the three different tissues of the plant, the expression level of *PAL* gene in different tissues was higher in leaves (Fig. 4A and Fig. 5A), lower in petioles (Fig. 4B and Fig. 5B) and stems (Fig. 4C and Fig. 5C). The level of *PAL* gene expression reached its maximum at the 7th and 9th days after inoculation and thereafter it declined gradually in all the treatments. Our results indicate that the level of *PAL* gene expression in different tissues of cultivars showed a trend of increased first, and then decreased with the increase of inoculation time.

Enhancement expression of *PAL* gene after inoculation with *Podosphaera xanthii*

The enhancement changes of *PAL* gene expression were significantly different in different cultivars and tissues of pumpkin (Sixing F₁ and Jin₁₂ F₁) after inoculated with *P. xanthii* (Fig. 6). However, the increased *PAL* gene expression in Sixing F₁ was significantly higher than the cultivar of Jin₁₂ F₁, as well as higher in leaves (Fig. 6A) than the petioles (Fig. 6B) and stems (Fig. 6C). The enhancement changes of *PAL* gene expression in different tissues and cultivars of pumpkin also showed a trend of increased first, and thereafter it declined with the increase of inoculation time. The enhancement expression of *PAL* gene reached its maximum at the 9th, 7th and 7th days in leaves (Fig. 6A), petioles (Fig. 6B) and stems (Fig. 6C) after inoculation and thereafter it declined.

Lignin and total phenolics contents in pumpkin seedlings

The cultivars of Sixing F₁ and Jin₁₂ F₁ with *P. xanthii* treatment increased the lignin and total phenolics contents in seedling leaves from 1 to 9 or 11 days, and peaked at the 9th and 11th days. The level of lignin and total phenolics contents of Sixing F₁ significantly higher in comparison to the cultivar of Jin₁₂ F₁. The average contents of lignin and total phenolics were significantly increased by 21.24% and 21.09% in Sixing F₁ seedlings leaves from 9 to 11 days after inoculation in comparison to the control under sterile water treatment, respectively. However, in the cultivars of Jin₁₂ F₁, the contents of lignin and total phenolics in the seedling leaves were increased by 12.38% and 18.65% from 9 to 11 days after inoculation, compared with the control under sterile water treatment, respectively (Table 4).

Table 4

Lignin and total phenolic content in different cultivars of *Cucurbita pepo* seedlings after inoculation with *Podosphaera xanthii*

Cultivars	Treatments	Days post inoculation (dpi)						
		1	3	5	7	9	11	13
————Lignin content (% of cell wall dry weight)————								
Sixing F ₁	Treatment	2.56 a	2.89 a	3.28 a	5.08 a	6.56 a	6.52 a	6.13 a
	Control	2.43 b	2.78 b	3.09 b	4.25 b	5.34 b	5.45 b	5.25 b
Jin ₁₂ F ₁	Treatment	2.12 c	2.24 c	2.67 c	3.11 c	3.98 c	4.02 c	3.96 c
	Control	2.01 c	2.15 c	2.48 d	2.81 d	3.51 d	3.61 d	3.74 d
————Total phenolics content (g FW)————								
Sixing F ₁	Treatment	3.62 a	3.85 a	3.98 a	4.29 a	4.79 a	4.81 a	4.25 a
	Control	3.35 b	3.42 b	3.62 b	3.98 b	4.02 b	3.91 c	4.06 b
Jin ₁₂ F ₁	Treatment	3.34 b	3.57 b	3.72 b	3.89 b	4.21 b	4.33 b	3.98 bc
	Control	3.01 c	3.12 c	3.23 c	3.45 c	3.64 c	3.56 d	3.54 c
Data are means of twelve replicates. Different letters in the same column denote significant differences at the <i>p</i> < 0.05 level by Duncan's new multiple range test (n=12). The treatments are detailed in the footnote of Table 1								

Table 1
Specific PCR primers for *PAL* gene and *Actin* gene

Number of primers	Primers sequence
<i>PAL</i> (F)	5'-AACTTCTCCTCAATGGCTTGGT-3'
<i>PAL</i> (R)	5'-TGAAACATCAATCAAAGGGTTG-3'
<i>ACT</i> (F)	5'-TgYgACAATggAACWggAATg-3'
<i>ACT</i> (R)	5'-CATCTgYTggAARgTgCTgAg-3'
Note: F represents forward, R represents reverse	

Discussion

Previous studies have been demonstrated that the disease resistance screening in introduced germplasm was important to get resistant and tolerant germplasm for breeding new cultivars to control powdery mildew in field and greenhouse-grown pumpkins [27], but the mechanisms of pumpkin germplasm in resistance to *Podosphaera xanthii* infection remain unresolved because specific information about the improvement of antioxidative defense system and gene expression in different cultivars are virtually unknown at the physiological, biochemical and molecular levels. In the present study, we evaluated the ability and unveiled the mechanisms of two commercial pumpkin cultivars in resistance to *P. xanthii* infection. Interestingly, we discovered that the cultivar of Sixing F₁ exhibited higher ability in resistance to *P. xanthii* infection, and less ROS accumulation, lower rates of lipid peroxidation, and higher level of PAL activity and gene expression, lignin and total phenolics contents than Jin₁₂ F₁. Thus, our results indicate that Sixing F₁ and Jin₁₂ F₁ can be considered as the resistant cultivar and susceptible cultivar, respectively, and the mechanism for their resistance to *P. xanthii* infection through enhancing the level of PAL activity and gene expression to reduce the ROS accumulation, and increase the lignin and total phenolics contents in their tissues to activate the defense system to prevent the pathogens infection. Our results will provide a vital theoretical basis and new insight for the mechanisms of different cultivars of pumpkin in resistance to *P. xanthii* infection. To the best of our knowledge, this is the first report suggesting that the cultivars of pumpkin in resistance to *P. xanthii* infection through stimulating the defense system response in different tissues.

De Oliveira Rabelo et al. (2017) reported that breeding for resistance is one of the best strategies to decrease powdery mildew damage, and for the selection of cucurbits cultivars in resistance to powdery mildew [28]. Our results showed that the cultivar of Sixing F₁ exhibited higher ability in resistance to *P. xanthii* infection, whereas the cultivar of Jin₁₂ F₁ exhibited lower ability. The disease incidence and index of Jin₁₂ F₁ were significantly higher than Sixing F₁ at different time points after inoculation. Yan et al. [29] showed that the disease incidence of the cultivar of Guangban was significantly higher than the cultivars of Sanxing, Erxing, and Hongfu after inoculation with the pathogen of *P. xanthii*. However, our results indicate that the cultivar of Sixing F₁ presented higher ability in resistance to *P. xanthii* infection than

Jin₁₂ F₁. Thus, the cultivar of Sixing F₁ can be considered as the resistant cultivar in resistance to *P. xanthii* infection, whereas the cultivar of Jin₁₂ F₁ can be considered as the susceptible cultivar.

Furthermore, some previous studies have been reported that plants can produce the constitutive and induced mechanisms to defend themselves against pathogens attack [30, 31], such as i.e. the rapid generation of hydrogen peroxide (H₂O₂) and lipid peroxidation (MDA) have been considered as the important types of ROS and key biochemical indicators of oxidative damage in plants against pathogens infection [32–35]. In the present study, a significant increase in H₂O₂ and MDA generation in both pumpkin cultivars in response to *P. xanthii* infection, and the greatest accumulation of H₂O₂ and MDA were found in the susceptible cultivar Jin₁₂ F₁. In addition, it was interesting that a positive relationship was discovered between the high contents of H₂O₂ and MDA, and disease incidence and index. Similarly, Patykowski and Urbanek (2003) reported that the ability of tomato in resistance to *Botrytis cinerea* infection resulted from the early induction of H₂O₂ [14]. Meanwhile, the excess ROS can lead to the peroxidation of unsaturated lipids of membranes in plants [36], such as the accumulation levels of ROS led to the increased contents of MDA in both the resistant and susceptible cultivars of faba bean during the interaction of *B. fabae* [37]. Our results indicate that the induction of H₂O₂ and MDA in inoculated plants may be one of the pumpkin defense mechanisms against the invading pathogen infection, and the lower levels of ROS and MDA correlated with the susceptibility of pumpkin leaf tissues to infection with *P. xanthii*. El-Komy (2014) demonstrated that the resistant cultivar showed less ROS accumulation, a lower rate of lipid peroxidation and higher activity of the enzymatic ROS scavenging system compared with susceptible cultivar during the interaction of *B. fabae* [37].

In addition, plants can possess an inherent capacity to eliminate the harmful effects of reactive oxygen species (ROS) through the involvement of an antioxidative system that protects cell constituents from the oxidative damage [38]. Pathogenesis-related protein is one of the most important accumulated biotic components in plants to defend the pathogens after their attack and infection, including PAL, chitinase peroxidase, and other proteins [39]. Among all the pathogenesis-related proteins, PAL is the first enzyme in the phenylpropanoids pathway, which produces the precursors for lignin and phenolic secondary metabolites that may be enhanced after pathogen infection [40]. Also, *PAL* is one of the key genes in the phenylpropane synthesis pathway, and is closely related to plant resistance to external stresses [41]; *PAL* gene is among those most relevantly upregulated in plants that develop resistance to phytopathogens [24]. Our results found that PAL plays a significant role in inducing plant systemic resistance, including the PAL activity and gene expression in different tissues of pumpkin at different time points were activated by *P. xanthii* inoculation. Similar studies revealed that PAL plays a significant role in plant resistance to pathogens infection, which involved in induced systemic resistance [42]; Chen et al. (2014) reported that the expression level of PAL activity and gene in cucumber leaves were activated by *P. xanthii* inoculation [41]. Additionally, we found that the activity of PAL and the expression level of *PAL* gene in different tissues of resistant cultivar were significantly higher than the susceptible cultivar, as well as higher expression in leaves than petioles and stems. Similarly, higher transcript levels of *PAL* activity and gene expression were found in pathogens inoculated plants than the no-inoculated plants. Several

previous studies also reported that the increased levels of the gene expression or the enzymic activity have been observed in plants after inoculation with pathogenic microbe [43–45]. Thus, our results indicate that *PAL* gene plays a significant role in coding the PAL activity to induce the ability of pumpkin resist to *P. xanthii* infection. The expression level of *PAL* gene in different tissues of pumpkin cultivars reached its maximum at the 7th and 9th days after inoculation and thereafter it declined with the increase of inoculation time. Gao et al. [46] revealed that the relative expression level of *CsPAL* gene in cucumbers exhibited a trend of increased first after inoculation with powdery mildew, and thereafter decreased with the increase of inoculation time.

PAL is a key and rate-limiting enzyme in catalyzing phenylalanine to trans-cinnamic acid to improve the disease resistance of plants by promoting the synthesis of phenolic substances and lignin [47]. Additionally, phenylalanine lignification is one of the physical and biochemical changes of plant cell wall which could be induced by pathogen infection [48], and also phenolic compound plays an important role in participating in the defense mechanism against fungal infection [49, 50]. Our results found that a significant increase in lignin and total phenolics contents in seedling leaves of Sixing F₁ and Jin₁₂ F₁ after treatment with *P. xanthii*, the level of lignin and total phenolics contents in the cultivar of Sixing F₁ significantly higher in comparison to the cultivar of Jin₁₂ F₁. Similarly, Muslim et al. (2019) revealed that the lignin deposition is an important step to prevent systemically immunised cucumber plants from pathogen of *Colletotrichum orbiculare* infection [51], and also the level of total phenolics was increased in cucumber seedlings after inoculated with the *P. xanthii* [41].

Conclusions

In summary, the results of our study suggest that Sixing F₁ can be considered as the resistant cultivar, and Jin₁₂ F₁ can be considered as the susceptible cultivar. One novel possible mechanism is related to the pumpkin cultivars in resistance to *P. xanthii* infection through enhancing the level of *PAL* activity and gene expression to promote the synthesis of phenolic substances and lignin, and reduce the ROS accumulation to activate the defense system in different tissues to prevent the pathogens infection finally. However, more research is needed to determine other defense genes in pumpkins that coding the pathogenesis-related protein expression in resistance to *P. xanthii* infection in the future.

Materials And Methods

Experiments were carried out at the Laboratory of Plant Pathology, College of Plant Protection; Gansu Provincial Key Laboratory of Arid Land Crop Science, Gansu Agricultural University. All treatments in the experiments described below had twelve replicates.

Seeds treatment

Seeds of two commercial pumpkin cultivars (Sixing F₁ and Jin₁₂ F₁) were selected and kindly provided by the company of Wuwei Golden Apple co. LTD. Seeds with a uniform size were surface-sterilized with 5% NaOCl (v/v) for 3 min. After disinfection, all the seeds were rinsed with sterile water for 5 times, and then were soaked in sterile water for 12 hours for germination.

Greenhouse experiments

The experiments were carried out in the greenhouse of Gansu Agricultural University in August, 2013. Seeds of two pumpkin commercial cultivars were germinated in 9-cm diameters Petri dishes, and covered with two layers of absorbent cotton and blotter papers in a constant temperature at 25°C for germination. Thereafter, the germinated seeds with a uniform size were planted in 12-cm diameters pots that contained 500 g of sterilized soil. Each pot was planted with 8 seeds and each cultivar with 12 pots (a total of 96 plants) after germination. The experiment was arranged in a completely randomized design with twelve replications, took place in a greenhouse with the inside temperature maintained between 25°C and 20°C (day and night), the supplemental photoperiod was 16/8 hours light/dark, and the relative humidity at 60%. Irrigation was done twice weekly at each treatment and control.

Podosphaera xanthii identification and inoculum preparation

Pumpkin leaves infected with the pathogen of powdery mildew were collected from the field (Wuwei, China) in July 15, 2013 for microscopic observation of the pathogen. The conidia observed under light microscope (E200, Germany) were oidium type and conidiospore with cylindrical fibrosin bodies of conidia which was identified to be *Podosphaera xanthii* according to the earlier published papers [52, 53]. Thereafter, artificial inoculation of host plants seedlings were performed by manually through dusting the sporulated leaves and *P. xanthii* isolate in host plants were kept for 20 days in greenhouse. The tested plants were inoculated at the 4-true leaf stage with the suspension of powdery mildew fungal pathogen *P. xanthii* spores by smear method, and 5 plants with relatively consistent growth were selected for each pot, and each plant was inoculated with 3 leaves. After that, the inoculated seedlings were placed in a greenhouse at 25°C and 20°C (day and night), relative humidity of 60% and light of 16/8 hours for development of powdery mildew. Control plants (mock-inoculated) were inoculated with the same volume of sterile water and maintained separately from the inoculated plants in the same greenhouse. The seedlings disease incidence, disease index H₂O₂, MDA, lignin and total phenolics contents, and the level of PAL activity and gene expression at different time points in each treatment and control were recorded and calculated every 2 days after inoculation.

Disease incidence and index determination

Seedlings disease incidence and index were observed and recorded every 2 days post inoculation (dpi) for both the inoculated and mock-inoculated plants, and collections continued for 1, 3, 5, 7, 9, 11 and 13 dpi from both cultivars of Sixing F₁ and Jin₁₂ F₁. Five plants from each treatment and control were used as one independent replicate per time point. Disease severity was recorded on individual pot of each cultivar. Based on the powdery mildew symptoms developed on the host plants, a 1 to 9 scales of increasing disease severity were used according to the standard described by Liu et al.(2006) [54].

Scale levels:

0: no symptoms;

1: the infected areas less than 30% in the front of leaves, and no symptoms in the reverse of leaves;

3: the infected areas greater than 30% in the front of leaves, and less than 10% in the reverse of leaves;

5: the infected areas greater than 30% and 10% in the front and reverse of leaves, respectively, and a few lesions appeared on the petioles;

7: the powdery mildew covered in the front of leaves and the infected areas greater than 10% in the reverse of leaves, and more lesions appeared on the petioles and a few on the main stems;

9: the powdery mildew covered in the front of leaves, petioles and main stems, and the infected areas greater than 10% in the reverse of leaves.

The calculation formulas of disease incidence and index as follows:

$$\text{Disease incidence (\%)} = (\text{NIL}/\text{TNIL}) \times 100 \dots\dots\dots(1)$$

where NIL is number of infected leaves, and TNIL is total number of investigated leaves.

$$\text{Disease index} = (\sum \text{NDL GL}) / (\text{TNIL HGL}) 100 \dots\dots\dots(2)$$

where NDL is the number of diseased leaves in each lever; GL is grade levels; TNIL is total number of investigated leaves; HGL is the highest grade level.

Hydrogen peroxide (H₂O₂) and lipid peroxidation (MDA) contents determination

For the determination of H₂O₂ contents in different cultivars of pumpkin seedling leaves, 0.5 g fresh leaf samples were homogenized in 5 ml of precooled HClO₄ (1.0 M) using the pre-chilled mortar and pestle. Thereafter, the reaction mixture was centrifuged at 10, 000 g for 10 min, and the contents of H₂O₂ in extracts were determined and calculated according to the method described by Willekens et al. (1997) [55]. The contents of H₂O₂ were expressed as μmol g⁻¹ FW.

The level of lipid peroxidation was determined by quantifying the MDA contents in different cultivars of pumpkin seedlings according to the method described by Hodges et al. (1999) [56] and Tian et al. (2015) [57] with some modifications. For the determination of the accumulation of MDA in pumpkin seedling leaves, 0.5 g fresh leaf samples were homogenized in 2.5 ml of 0.1% trichloroacetic acid and the homogenate. Afterwards, the reaction mixture was centrifuged at 10,000 g for 15 min and the absorbance of supernatant was recorded at 532 nm wavelength. The content of MDA was expressed as $\text{nmol g}^{-1} \text{FW}$.

PAL activity determination

Fresh pumpkin seedling leaf, petiole and stem samples of 0.5 g were homogenized in 6 ml ice-cold borate buffer (5 mM, pH 8.8) using pre-chilled mortar and pestle, respectively. The supernatant of homogenates were collected and used as crude extracts after centrifuging at 8,000 g for 20 min at 4°C. Thereafter, the supernatant was mixed with 0.02 M phenylalanine and distilled water to obtain the extractions. The reaction mixtures were placed in a thermostatic water bath at 30°C for 30 min and then measured at 290 nm. For the determination of PAL activity, the crude extraction was measured according to the method described by Hu et al. (2009) [61] and Ruiz et al. (1999) [62]. The activity of PAL was expressed as $\text{U min}^{-1} \text{g}^{-1} \text{FW}$.

Total RNA extraction and first strand cDNA synthesis

The samples of leaves, petioles and stems were collected every 2 days post inoculation (dpi) for both the inoculated and mock-inoculated seedlings, and the collections continued for 1, 3, 5, 7, 9, 11 and 13 dpi from both cultivars of Sixing F_1 and Jin₁₂ F_1 for total RNA extraction. Five plants from each treatment were used as one independent replicate per time point. Total RNA was extracted from control and treated frozen leaves, petioles and stems samples performed immediately using the Tiangen RNA Simple Total RNA Kit (Tiangen Biotechnology, Beijing, China) according to the manufacturer's instructions. Thereafter, total RNA was stored at -80°C until use. The quantity and quality of total RNA were measured and determined using a Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies, Waltham, MA, USA), and only high-quality RNA samples were used for subsequent experiments. First strand synthesis of total RNA was carried out employing an oligo (dT) primer mix and a random primer mix. Two microliters of total RNA was reversely transcribed using the M-MuLV First Stand DNA Synthesis Kit (Sangon Biotech, Shanghai, China) for the first-strand cDNA synthesis with oligo-dT18 primer priming method according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) analysis

The level of *PAL* gene expression was determined in pumpkin leaves, petioles and stems at different time points after inoculation with *P. xanthii* or sterile water in each treatment and control. qRT-PCR was

performed using a SYBR Premix Ex Taq kit (Takara Biotechnology, Dalian, China) following the manufacturer's instructions. The sequences of the forward and reverse primer pairs used for qRT-PCR analysis were designed according to the EST sequences of pumpkin in NCBI using Primer Express 5.0 software that amplifies the target genes. The *actin* gene of pumpkin was used as an internal control. The level of *PAL* gene expression was determined using the method of $2^{-\Delta\Delta Ct}$ [63]. The gene specific primers used for this analysis are shown in Table 5.

Leaf cell wall isolation and lignin content determination

The leaf cell wall of different cultivars of pumpkin seedlings were isolated according the method described by Eskandari et al. (2018) [58]. A 0.5 g of fresh leaf samples were frozen and ground to powder using the liquid nitrogen. The powder samples were homogenized in distilled water and then centrifuged at 10,000 g for 10 min. The precipitation was washed with absolute ethanol, and rinsed in chloroform and methanol (v/v = 1: 2) and then washed with acetone for three times. The cell wall pellet was filtered and finally dried overnight at 35°C. The residue (cell wall) was collected and kept in a desiccator at room temperature until use.

The content of lignin was determined and assayed by following the procedure of Iiyama and Wallis (1990) [59] with a minor modification. A 5 mg of cell wall preparation was treated with a mixture (2.5 ml) that consisted of 5% (w/w) acetyl bromide and AcHO, and 0.1 ml of 70% HClO at 70°C for 30 min. Finally, the reaction mixture was treated with 50 ml that contained 2 M NaOH and AcHO after cooling. The lignin content was determined by measuring the absorbance at 280 nm using a specific absorption coefficient of $20.0 \text{ g}^{-1} \text{ l cm}^{-1}$.

Total phenolics content determination

The contents of total phenolics were measured according to the method described by Singleton and Rossi (1965) with a minor modification [60]. A 0.5 g fresh leaf samples were ground with a small amount of quartz sand, and thereafter extracted in 70% ethanol (10 ml) for 10 min. The reaction mixture was centrifuged at 12,000 g for 20 min. The supernatant of the reaction mixture was collected and used to measure the total phenolic content, and expressed as $\text{mg g}^{-1} \text{ FW}$.

Statistical analysis

The data were subjected to variance analysis (ANOVA) using SPSS Version 16.0 (SPSS Inc., Chicago, IL). Each experiment had twelve replications. Duncan's multiple range test was computed using standard error and T values of adjusted degrees of freedom. The significant differences between the treatments were considered at the level of $p < 0.05$. The data of disease incidence, index and gene expression levels

of two commercial pumpkin cultivars (Sixing F₁ and Jin₁₂ F₁) were calculated according the [formulas](#) as described previously.

Declarations

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Author contributions

SZ conceived the experiments with the help of BX. SZ collected and prepared the fungus and pumpkin seedling samples, and performed the mechanisms of different cultivars of pumpkin in resistance to *Podosphaera xanthii* infection and extracted the total RNA from pumpkin seedling samples. JL and SZ performed qRT-PCR, analyzed the data, and interpreted the results. SZ wrote the manuscript. SZ and BX revised and approved the final manuscript.

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Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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- Table 1 The disease incidence of different cultivars of *Cucurbita pepo* after inoculation with *Podosphaera xanthii*

Figures

Figure 1

The symptoms of different cultivars of *Cucurbita pepo* after inoculation with the pathogen of *Podosphaera xanthii* at the 13th day. Where A the cultivar of Jin12 F1 after inoculation with *P. xanthii*; B

the cultivar of Sixing F1 after inoculation with *P. xanthii*; C the cultivar of Jin12 F1 after inoculation with sterile water but not *P. xanthii*; D the cultivar of Sixing F1 after inoculation with sterile water but not *P. xanthii*

Figure 2

PAL activity in the (A) leaves, (B) petioles, and (C) stems of Sixing F1 at different time points after inoculated with *Podosphaera xanthii*. The line bars represent the standard errors of the means. Different letters denote significant difference at the p < 0.05 level by Duncan’s new multiple range test (n = 12). The treatments are detailed in the footnote of Table 1

Figure 3

PAL activity in the (A) leaves, (B) petioles, and (C) stems of Jin12 F1 at different time points after inoculated with *Podosphaera xanthii*. The line bars represent the standard errors of the means. Different letters denote significant difference at the $p < 0.05$ level by Duncan's new multiple range test ($n = 12$). The treatments are detailed in the footnote of Table 1

Figure 4

Expression levels of PAL gene in the (A) leaves, (B) petioles, and (C) stems of Sixing F1 at different time points after inoculated with *Podosphaera xanthii*. The line bars represent the standard errors of the means. Different letters denote significant difference at the $p < 0.05$ level by Duncan's new multiple range test ($n = 12$). The treatments are detailed in the footnote of Table 1

Figure 5

Expression levels of PAL gene in the (A) leaves, (B) petioles, and (C) stems of Jin12 F1 at different time points after inoculated with *Podosphaera xanthii*. The line bars represent the standard errors of the means. Different letters denote significant difference at the $p < 0.05$ level by Duncan's new multiple range test ($n = 12$). The treatments are detailed in the footnote of Table 1

Figure 6

Changes of PAL gene expression in different cultivars and tissues (A) leaves, (B) petioles, and (C) stems of *Cucurbita pepo* at different time points after inoculated with *Podosphaera xanthii*. The line bars represent the standard errors of the means. Different letters denote significant difference at the $p < 0.05$ level by Duncan's new multiple range test (n = 12)