Pathogenicity factors of *Phytophthora melonis* revealed by comparative proteomics

Aqeel Ahmad a, Waheed Akram b, Rui Wang a, Iqra Shahzadi c, Muhammad Umer d,e, Nasim Ahmad Yasin f and Tingquan Wu a

aInstitute of Facility Agriculture, Guangdong Academy of Agricultural Sciences (IFA, GDAAS) / Vegetable Research Institute, Guangdong Academy of Agriculture Sciences / Guangdong Key Laboratory for New Technology Research of Vegetables, Guangzhou, People’s Republic of China; bDepartment of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Lahore, Pakistan; cHubei Key Laboratory of Biomass Resource Chemistry and Environmental Biotechnology, Hubei International Scientific and Technological Cooperation Base of Sustainable Resource and Energy, Hubei Engineering Center of Natural Polymers-based Medical Materials, School of Resource and Environmental Science, Wuhan University, Wuhan, People’s Republic of China; dForestry College, Research Center of Forest Ecology, Guizhou University, Guiyang, People’s Republic of China; eKey Laboratory of Karst Georesources and Environment, Ministry of Education, Guizhou University, Guiyang, People’s Republic of China; fRO II Wing, University of the Punjab, Lahore, Pakistan

ABSTRACT

Deciphering the transcriptional activities of pathogenicity genes is critical to understand pathogenesis mechanisms of *Phytophthora melonis*, a devastating pathogen of cucurbits. In this study, we have performed comparative proteomics among *P. melonis* mutants to evaluate the interacting pathogenicity proteins. Structural characterization of all detected proteins discovered the physicochemical factors behind their pathogenic behavior. Results revealed that gamma radiations have an extreme potential to mutate *P. melonis* spores, and can be a safe alternative of chemical mutagens. *P. melonis* is accomplished with seventy-three differentially expressed proteins among which, seven proteins were ranked as strong pathogenicity factors, i.e. Q9AT01 (Elicitin), DONX1 (Oligopeptidase A, putative), DONB60 (Secreced RXLR effector peptide protein, putative), DONIK4 (IpiB3-like protein), DONIZ1 (Carboxypeptidase), H3GZF4 and H3GZF6. The Ramachandran plot determined the positions of secondary structures (α helix and β sheets) in the 3D structures of pathogenicity proteins. Furthermore, the glycine residues analysis highlighted that six out of seven proteins were functionally and structurally more stable than DONIK4 protein. The study concluded that the pathogenic proteins must have negative φ angles greater than 63°, and ψ angles ≥80° for their proper functioning. This study establishes functional connections between pathogenicity genes and the process of disease establishment.

INTRODUCTION

The genus *Phytophthora* contains a number of phytopathogenic species causing devastating diseases on many economically important crops, resulting in billions of dollars loss annually (Kovacs et al. 2011). *Phytophthora* is classified in a group Oomycetes, also known as water molds. The importance of water molds can be assessed from the fact that the causal agent of potato late blight (*Phytophthora infestans*) also belongs to this group. *P. infestans* is responsible for the Irish potato famine during the mid-nineteenth century and caused millions of deaths. The genus contains approximately 140 species, and most of them have been reported as pathogens at various crops (Vetukuri et al. 2001; Guharoy et al. 2006). Generally, *P. melonis* is a heterothallic species and has two distinct mating types (i.e. A1 and A2). For sexual reproduction, pairing is held between strains of opposite mating types. Sexual reproduction is not the only way; there is an option of asexual reproduction In the absence of compatible mating types (Wetzel 2011). However, in suitable circumstances, sexual reproductive structures, oogonia and antheridia, are formed by the interaction between opposite mating types. Sexual reproduction bears the advantage of producing more resistant sexual spores (oospores) and sexual recombination. *P. melonis* oospores have the tendency to tolerate harsh environmental conditions which is imperative to survive and remain viable in the soil for long periods (Wu et al. 2016). Additionally, Cucurbit blight is one of the most deleterious diseases of wax gourd, *Benincasa hispida* Cogn. and cucumber crop. The losses are amplified exponentially during each passing day of a rainy season. The pathogen has been reported from all over the globe with serious disease severities in Asian countries, i.e. Japan, Iran, India and China. However, the geographic origin of *P. melonis* is still unclear (Mirabolfathy et al. 2001; Guharoy et al. 2006). Generally, *P. melonis* is a heterothallic species and has two distinct mating types (i.e. A1 and A2). For sexual reproduction, pairing is held between strains of opposite mating types. Sexual reproduction is not the only way; there is an option of asexual reproduction In the absence of compatible mating types (Wetzel 2011). However, in suitable circumstances, sexual reproductive structures, oogonia and antheridia, are formed by the interaction between opposite mating types. Sexual reproduction bears the advantage of producing more resistant sexual spores (oospores) and sexual recombination. *P. melonis* oospores have the tendency to tolerate harsh environmental conditions which is imperative to survive and remain viable in the soil for long periods (Wu et al. 2016). Additionally,
sexual reproduction plays a pivotal role in bringing genotypic variability in *P. melonis*. The genetic variability lays a foundation stone for variable virulence and elevated pesticide resistance in the pathogen. A detailed elucidation of genetic variations among pathogenicity genes of *P. melonis* is necessarily needed to control the pathogen.

Previously, pathogenicity-related research work has been significantly dependent upon the availability of relevant mutants (Masuda et al. 2004), and chemical mutagens had been utilized as an effective source of mutation. Irrespective of their efficiency (Akram et al. 2014a; Khan et al. 2017b). The chemical mutagens had been a stable source of mutations in untargeted objects when released in the environment (Shafique et al. 2014). Their use was very limited due to the long list of associated risks with them (Ahmad et al. 2018). On the other hand, an ion beam technique has been introduced for the production of a wide spectrum *Arabidopsis thaliana* (Kazama et al. 2011). However, microbial cultures have not been targeted by these ion beams, and no attempts have been made to expand the genetic diversity of the pathogen genome using this new mutagenic procedure.

Pathogens are always accomplished with lytic enzymes or toxins to invade into plant tissues and to kill the plant cells (Akram et al. 2014b; Ahmad et al. 2020a). During the infection process, pathogenicity proteins play a key role in pathogen invasion and establishment in the host tissue (Rep 2005; Bashir et al. 2016). Understanding the proteins and their structural characterization is critical to reveal the mechanics behind the pathogenicity mechanisms (Khan et al. 2019; Ahmad et al. 2020b). However, most of the research done determines the pathogenicity gene sequences (Ahmad et al. 2019; Ahmad et al. 2020a).

There is a dearth of studies covering pathogenicity proteins and their structural mechanics (Ahmad et al. 2013; Ahmad et al. 2014b). This study describes the translational products of the pathogenicity genes and their structural properties. Furthermore, it utilizes ion beams as a fundamental basis for genetically variable isolates of *P. melonis*. Overall, comparative proteomics and irradiating ion beams are advanced techniques. The study is an important step towards the understanding of the infection mechanism of the pathogen within an agroecosystem. This would also support the research programs of developing new varieties of cucurbits with improved resistance against *P. melonis*. The study will be a milestone to understand the genetic basis of the pathogenic behavior of *P. melonis* and will generate some recognition of its elicitors. Using the outcome data, the program of producing resistant cultivars against the pathogen will be strengthened.

**Results**

**Irradiation assay**

The germination rate of the spores was decreased with the increase in irradiation time. Maximum spore germination (92.14%) was recorded in the case of 1-minute irradiation. However, the minimum germination rate (57.36%) was shown by 5 minutes of irradiation treatments. The same treatment (5 minutes irradiation) exhibited the maximum germination reduction (42.47%) followed by the 3 minutes irradiation treatment (25.28%). The linear trend-line showed almost complete loss of germination around $\approx 10$ minutes of irradiation period (Figure 1A).

The maximum average growth rate of *Phytophthora* colonies (8.79 cm/5 days) was observed in the control treatment (0 min irradiation period). The average growth rate of colonies was decreased after 1-minute irradiation (8.35 cm) and 3 minutes irradiation (5.44 cm/5 days). However, the 5 minutes irradiation treatment recorded the minimum growth rate of 3.69 cm after five days of the incubation period (Figure 1B).

**Pathogenicity assay**

Pathogenicity assay was performed according to disease rating scale (Figure 1C), and it screened out 3 *Phytophthora* strains (i.e. Pmhp 01, Pmhp 02, Pmhp 03) as highly virulent, while three strains (i.e. pmlp 01, Pmlp 02, Pmlp 03) as the least pathogenic. Two *P. melonis* strains, i.e. Pmhp 01 and Pmhp 03, showed the maximum disease index (100) on cucumber leaves. Another *Phytophthora* strain Pmlp 02, exhibited a disease value of 96.67. However, Pmlp 01 showed the minimum disease index (3.33) followed by a 4.17 disease index value of Pmlp 03. While Pmlp 02 recorded the maximum disease index (6.67) among *Phytophthora* strains of low pathogenicity (Figure 1D).

The morphological features of *P. melonis* high pathogenicity (Pmhp) were close to Wild type, showing irregular margins and flabby surface. Colonies were white and also had wavy, radial ornamentation. The *P. melonis* low pathogenicity (Pmlp) exhibited smooth margins and a waxy upper surface with an off-white appearance and no ornamentation (Figure 1E).

Representative strains: Strains with the maximum and the minimum pathogenicity; Pmhp (*P. melonis* high pathogenicity); Pmlp (*P. melonis* low pathogenicity); WT (Wild type).

Protein profile analysis of representative *P. melonis* stains determined 73 differentially expressed proteins, among which seven proteins were screened out by Principal Component Analysis (PCA) based on their correlation with disease development. Protein Q9AT01 (Elicitin), D0MX11 (Oligopeptidase A, putative), D0NB60 (Secreted RXLR effector peptide protein, putative), D0NIK4 (IpiB3-like protein), D0NZ1 (Carboxypeptidase), and H3GZF4 (Uncharacterized protein), H3GZF6 (Uncharacterized protein) occupied a distinct position among protein profile due to high pathogenicity (Table 1).

A total of 7 proteins Q9AT01 (Elicitin), D0MX11 (Oligopeptidase A, putative), D0NB60 (Secreted RXLR effector peptide protein, putative), D0NIK4 (IpiB3-like protein), D0NZ1 (Carboxypeptidase), and H3GZF4 (Uncharacterized protein), H3GZF6 (Uncharacterized protein) were categorized under high pathogenicity zone. Oligopeptidase A, putative protein D0MX11, had the highest pathogenicity coefficient of 2.64. The Log P-value for an uncharacterized protein H3GZF4 was calculated the maximum, 2.64; however, its pathogenicity coefficient value was 2.45 (Figure 2).

A total of seven proteins were screened with high pathogenicity, among which two proteins (Q9AT01 and G4ZP65) had been previously classified as toxins. Protein D0MX11 was a hydrolase, and protein D0NZ1 belonged to the carboxypeptidase family. D0NIK4 was classified as an IpiB3-like protein. However, two proteins (H3GZF4 and H3GZF6) had not been previously classified into any specific group.
All the 3D models of pathogenicity proteins were constructed with a confidence level of ≤90% except D0NIK4 against which the minimum confidence level was 82% (Table 2).

Three-dimensional structural analysis of the protein Q9AT01 revealed two significant zones of $\phi, \psi$ angles, along with one minor zone. The concentrated glycines residues could only be observed at one zone of 90° $\phi$ and approximately 0° $\psi$. The preproline residues were observed at 40°, 100° and 140° $\psi$, among which two had a negative value of $\phi$ (−60° and −90°). All the four proline residues were recorded with negative $\phi$ values ranging from −60° to −70°, among which three had a $\psi$ angle of $< 150°$ (Figure 1S).

The structural pairs of the angles $\phi, \psi$ were found distributed into two major and four minor zones in the 3D structure of D0MX11 protein. More than 90% of the pairs possessed a negative $\phi$ value. A similar trend was recorded in the case of glycine contents of D0MX11, with the only difference being that glycine contents were grouped at two distinct sites. Furthermore, no preproline contained a positive $\phi$ value. Preproline contents developed two distribution patterns in the Ramachandran plot, with most of the contents concentrated in the vicinity of zero $\psi$. However, the masses of the preproline contents had a balance between negative and positive values of $\phi$ (−90° and 90°). No proline content was observed on the positive side of $\phi$, while the contents with negative $\phi$ were mainly had two major groups and one minor. The first proline group had a $\psi$ value $\geq 150°$, while the second major group was found between 0° $\psi$ to 60° $\psi$. The minor group of proline contents had a 56° $\psi$ (Figure 3). Ramachandran analysis for the rest of the five proteins has been provided in Supplementary Data Set 1.

The paired angular structures were abundantly found in the fourth quadrate of the Ramachandran Plot (negative $\phi$ value) with more than 90% of the contents concentrated in the vicinity of zero $\psi$. Moreover, the preproline contents had a balance between negative and positive values of $\phi$ (−90° and 90°). No proline content was observed on the positive side of $\phi$, while the contents with negative $\phi$ were mainly had two major groups and one minor. The first proline group had a $\psi$ value $\geq 150°$, while the second major group was found between 0° $\psi$ to 60° $\psi$. The minor group of proline contents had a 56° $\psi$ (Figure 3). Ramachandran analysis for the rest of the five proteins has been provided in Supplementary Data Set 1.

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| No. | Uniprot ID | Species | Protein description | Gene ID | References | Function | Relative expression (%) | Role in disease |
|-----|------------|---------|---------------------|---------|------------|----------|------------------------|----------------|
| 01  | G4ZKR2     | *Phytophthora sojae* Soybean stem and root rot agent (*Phytophthora megasperma* f. sp. *Glycines*) | Avr1b-1 avirulence-like protein | Avh1b76 PHYSODRAFT_508046 | (Tyler 2006) | Unknown | 67.44 | ^ |
| 02  | D0NU4U8    | *P. infestans* Potato late blight fungus | Serine hydroxymethyltransferase | PITG_06427 | (Haas et al. 2009) | Amino acid metabolism | 55.58 | |
| 03  | D0NRW9     | *P. infestans* | Glycine cleavage system P protein | PITG_15850 | (Haas et al. 2009) | Amino acid metabolism | 31.29 | |
| 04  | H3GR14     | *Phytophthora ramorum* (Sudden oak death agent) | Avr1b-1 avirulence-like protein | N/A | (Tyler 2006) | Unknown | 32.54 | ^ |
| 05  | D0NLS5     | *P. infestans* | Asparagine synthetase | PITG_13399 | (Haas et al. 2009) | Unknown | 78.22 | |
| 06  | H3GRB0     | *P. ramorum* (Sudden oak death agent) | Avr1b-1 avirulence-like protein | N/A | (Tyler 2006) | Unknown | 57.40 | ^ |
| 07  | G4ZL2      | *P. sojae* Soybean stem and root rot agent | Transmembrane 9 super-family | PITG_03376 | (Haas et al. 2009) | Adhesion | 66.04 | |
| 08  | D0N036     | *P. infestans* Potato late blight fungus | Transglutaminase elicitor M81D | PITG_16959 | (Haas et al. 2009) | Cell wall | 57.45 | |
| 09  | D0N1H2     | *P. infestans* | Berberine-like protein | PITG_02935 | (Haas et al. 2009) | FAD binding; Oxidoreductase activity | 38.48 | |
| 10  | D0MIX2     | *P. infestans* Potato late blight fungus | Glyoxylate/hydroxypruvate reductase A, putative | PITG_16076 | (Haas et al. 2009) | Amino acid metabolism | 77.57 | |
| 11  | D0NST9     | *P. infestans* | Anthranilate phosphoribosyltransferase, putative | PITG_17032 | (Haas et al. 2009) | Amino acid metabolism | 51.01 | |
| 12  | D0NUM7     | *P. infestans* | Cell 5A endo-1,4-beta-glucanase, putative | PITG_08613 | (Haas et al. 2009) | Cell wall | 13.09 | |
| 13  | D0NB21     | *P. infestans* | Berberine-like protein | PHYSODRAFT_74098 | (Tyler 2006) | FAD binding; Oxidoreductase activity | 78.22 | |
| 14  | G4YQ65     | *P. sojae* Soybean stem and root rot agent | Translocation protein SEC63 | PITG_02467 | (Haas et al. 2009) | Cellular trafficking | 77.30 | |
| 15  | D0MWE3     | *P. infestans* | Putative chitin synthase (Chitin-UDP-Glchacintransferase) | PITG_02050 | (Haas et al. 2009) | Cell wall | 69.74 | |
| 16  | D0MUR7     | *P. infestans* | Ubiquitin-ribsosomal fusion protein, putative | PITG_09555 | (Haas et al. 2009) | Cellular trafficking | 68.96 | |
| 17  | D0NC98     | *P. infestans* | Lipase, putative | PITG_01186 | (Haas et al. 2009) | Energy/metabolism | 69.12 | |
| 18  | D0MUV1     | *P. infestans* | Berberine-like protein | PHYSODRAFT_253342 | (Tyler 2006) | FAD binding; Oxidoreductase activity | 28.48 | |
| 19  | G4ZL73     | *P. sojae* Soybean stem and root rot agent | Glycosyl transferase, putative | PITG_04125 | (Haas et al. 2009) | Steroid binding | 75.16 | |
| 20  | D0NOL9     | *P. infestans* | Beta-elicitin cinnamomin | N/A | (Rodrigues et al. 2006) | Steroid binding | 35.40 | |
| 21  | P156269    | *P. cinnamomi* Cinnamomum fungus | Glucokinase, putative | PITG_06016 | (Haas et al. 2009) | Energy/metabolism | 71.36 | |
| 22  | D0N663     | *P. infestans* | Quinolinate synthetase A protein, putative | PITG_06783 | (Haas et al. 2009) | Sulfur cluster binding | 49.73 | |
| 23  | D0N839     | *P. infestans* | Carbonic anhydrase | PHYSODRAFT_337083 | (Tyler 2006) | Carbonate dehydratase activity; Zinc ion binding | 68.32 | |
| 24  | G4Z09      | *P. sojae* Soybean stem and root rot agent | Mitochondrial Carrier (MC) Family | PITG_12854 | (Haas et al. 2009) | Energy/metabolism | 27.61 | |
| 25  | D0NLB4     | *P. infestans* | L-gulonolactone oxidase, putative | PITG_05465 | (Haas et al. 2009) | D-arabino-1,4-lactone oxidase activity; FAD binding; galactonolactone dehydrogenase activity | 52.41 | |

(Continued)
| No. | Uniprot ID | Species | Protein description | Gene ID | References | Function | Relative expression (%) | Role in disease |
|-----|------------|---------|---------------------|---------|------------|----------|-------------------------|----------------|
| 27  | H3GJU0     | P. ramorum Sudden oak death agent | Carbonate dehydratase | N/A     | (Tyler 2006) | Carbonate dehydratase activity; Zinc ion binding | 35.10          |
| 28  | D0NY08     | P. infestans | UDP-glucose 6-dehydrogenase | PITG_18375 | (Haas et al. 2009) | Energy/metabolism | 34.38          |
| 29  | O42830     | P. parasitica Potato buckeye rot agent | CBEL protein, formerly GP34 cbel1 | N/A     | (Mateos et al. 1997) | Cellulose binding | 91.64          * |
| 30  | D0MQM5     | P. infestans | Haustorium-specific membrane protein, putative | PITG_00375 | (Haas et al. 2009) | Unknown | 101.25         |
| 31  | D0N841     | P. infestans | Glucanase | PITG_06788 | (Haas et al. 2009) | Cell wall | 71.36          |
| 32  | Q9AT01     | P. capsici, Phytophthora plurivora | Elicitin | N/A     | (Severino et al. 2014) | Pathogenesis | 84.08          ** |
| 33  | D0MQW5     | P. infestans | Pyriodoxal biosynthesis lyase pdks | PITG_00471 | (Haas et al. 2009) | Lyase activity | 75.85          |
| 34  | Q3L578     | P. megakarya | N,N-acetylglucosaminyltransferase 1 | NEP1 | (Bae et al. 2005) | Unknown | 62.91          |
| 35  | DONAR3     | P. infestans | 12-oxophytodienoate reductase, putative | PITG_08491 | (Haas et al. 2009) | FMN binding; oxidoreductase activity | 77.32          |
| 36  | D0MYL7     | P. infestans | Uncharacterized protein | PITG_03822 | (Haas et al. 2009) | Transport | 22.67          |
| 37  | Q3L570     | P. megakarya | N,N-acetylglucosaminyltransferase 7 | NEP7 | (Bae et al. 2005) | Unknown | 61.07          |
| 38  | D0MX11     | P. infestans | 12-oxophytodienoate reductase, putative | PITG_02711 | (Haas et al. 2009) | Pathogenicity | 103.08          ** |
| 39  | D0MW88     | P. infestans | Folate-Bioprotein Transporter (FBI) Family | PITG_02409 | (Haas et al. 2009) | Transport | 98.05          |
| 40  | G4ZP65     | P. sojae Soybean stem and root rot agent | N,N-acetylglucosaminyltransferase 1 | PHYSODRAFT_334498 | (Tyler 2006) | Unknown | 56.96          |
| 41  | D0NB60     | P. infestans | Secreted RXLR effector peptide protein, putative Uncharacterized protein | PITG_09218 | (Haas et al. 2009) | Pathogenicity | 118.08          ** |
| 42  | D0M1U5     | P. infestans | Secreted RXLR effector peptide protein, putative Uncharacterized protein | PITG_01973 | (Haas et al. 2009) | Signaling | 87.40          |
| 43  | Q8LX08     | P. sojae Soybean stem and root rot agent | N,N-acetylglucosaminyltransferase 1 | N/A     | (Qutob et al. 2002) | Unknown | 50.63          |
| 44  | D0NIK4     | P. infestans | IpI3-like protein | PITG_11341 | (Ah-Fong et al. 2017; Zhang et al. 2019) | Pathogenicity | 93.09          ** |
| 45  | D0NXS5     | P. infestans | Glutathione peroxidase | PITG_18316 | (Haas et al. 2009) | Redox | 78.22          |
| 46  | T2FFK2     | P. capsici | NLP effector | N/A     | (Chen et al. 2013) | Unknown | 77.30          |
| 47  | D0N1Z1     | P. infestans | Carboxypeptidase | PITG_11525 | (Haas et al. 2009) | Pathogenicity | 99.74          ** |
| 48  | D0NPZ7     | P. infestans | N,N-acetylglucosaminyltransferase 1 | PITG_14492 | (Haas et al. 2009) | Redox | 68.96          |
| 49  | G2XK62     | P. capsici | Pectate lyase | pcpel1 | (Fu et al. 2015) | Lyase activity | 99.12          |
| 50  | D0N1T7     | P. infestans | Pyridoxal biosynthesis lyase | PITG_04690 | (Haas et al. 2009) | Redox | 28.48          |
| 51  | D0N5D0     | P. infestans | Secreted RXLR effector peptide protein, putative Uncharacterized protein | PITG_06657 | (Haas et al. 2009) | Redox | 75.16          |
| 52  | T1XKE7     | P. capsici | Pectate lyase | pel15 | (Feng et al. 2014) | Lyase activity | 40.50          |
| 53  | D0NY6      | P. infestans | Uncharacterized protein | PITG_12958 | (Haas et al. 2009) | Redox | 72.10          |
| 54  | G2XKU9     | P. capsici | Pectinesterase | pcpme2 | (Reitmann et al. 2017) | Catalytic activity of pectin degradation | 84.77          * |
| 55  | D0N939     | P. infestans | Vacular amino acid transporter, putative Uncharacterized protein | PITG_06611 | (Haas et al. 2009) | Transport | 68.83          |
| 56  | D0N9H2     | P. infestans | Vacular amino acid transporter, putative Uncharacterized protein | PITG_11891 | (Haas et al. 2009) | Unknown | 79.72          |
| 57  | G2XKV5     | P. capsici | Pectinesterase | pcpme7 | (Reitmann et al. 2017) | Catalytic activity of pectin degradation | 90.70          * |
| 58  | H3GDN4     | SCP-like extracellular protein | N/A     | N/A     | (Tyler 2006) | Unknown | 31.06          |

(Continued)
| No. | Uniprot ID | Species | Protein description | Gene ID | References | Function | Relative expression (%) | Role in disease |
|-----|------------|---------|---------------------|---------|------------|----------|------------------------|----------------|
| 59  | G4ZZW1     | P. sojae | Transglutaminase elicitor | PHYSODRAFT_563970 | (Tyler 2006) | Transferase activity, transferring amino-acyl groups | 52.93 | |
| 60  | D0NSE6     | P. infestans | Major Facilitator Superfamily (MFS) | PITG_06673 | (Haas et al. 2009) | Transport | 45.07 | |
| 61  | D0N8C1     | P. infestans | Uncharacterized protein | PITG_07409 | (Haas et al. 2009) | Unknown | 53.76 | |
| 62  | GSAO54     | P. sojae Soybean stem and root rot agent | Transglutaminase elicitor MB1C | PHYSODRAFT_563993 | (Tyler 2006) | Transferase activity, transferring amino-acyl groups | 37.31 | |
| 63  | D0NY89     | P. infestans | ATP-binding Cassette (ABC) Super-family | PITG_07717 | (Haas et al. 2009) | Transport | 69.97 | |
| 64  | G4ZZW4     | P. sojae Soybean stem and root rot agent | Transglutaminase elicitor | PHYSODRAFT_261536 | (Tyler 2006) | Transferase activity, transferring amino-acyl groups | 43.49 | |
| 65  | D0NMB1     | P. infestans | ATP-binding Cassette (ABC) Super-family | PITG_13575 | (Haas et al. 2009) | Transport | 43.59 | |
| 66  | D0NMB4     | P. infestans | ATP-binding Cassette (ABC) Super-family | PITG_13579 | (Haas et al. 2009) | Transport | 28.22 | |
| 67  | H3GZ64     | P. ramorum Sudden oak death agent | Uncharacterized protein | N/A | (Tyler 2006) | Transferase activity, transferring amino-acyl groups | 95.61 ** | |
| 68  | H3GZ66     | P. ramorum Sudden oak death agent | Uncharacterized protein | N/A | (Tyler 2006) | Transferase activity, transferring amino-acyl groups | 105.67 ** | |
| 69  | D0RLV7     | P. infestans Potato late blight fungus | Transglutaminase elicitor-like protein, transferring amino-acyl groups | PITG_22117 | (Haas et al. 2009) | Transferase activity, transferring amino-acyl groups | 68.83 | |
| 70  | D0NK23     | P. infestans | Glucose transporter, putative | PITG_13003 | (Haas et al. 2009) | Transport | 43.38 | |
| 71  | Q6XDM3     | P. infestans Potato late blight fungus | Uncharacterized protein | N/A | (Fabritius and Judelson 2003) | Transferase activity, transferring amino-acyl groups | 23.11 | |
| 72  | G4ZZV6     | P. sojae Soybean stem and root rot agent | Transglutaminase elicitor | PHYSODRAFT_520208 | (Tyler 2006) | Transferase activity, transferring amino-acyl groups | 65.79 | |
| 73  | D0N0GB     | P. infestans | Glycerol-3-phosphate dehydrogenase [NAD(+)] | PITG_04065 | (Haas et al. 2009) | Energy/metabolism | 71.39 | |

^ represents the protein detectable to the host defense system making the infection less likely to occur.
* represent the protein of pathogen supporting the infection and facilitating pathogenic invasion.
** represents the proteins directly pathogenic to host plants.
Table 2. List of pathogenicity proteins detected in Phytophthora melonis along with their encoding genes, 3D structures, international identifiers, and relevant classes.

| Protein Number | Protein ID | Gene Number | 3D Structure | PDB Code | Classification |
|----------------|------------|-------------|--------------|----------|----------------|
| 32             | Q9AT01     | N/A         | 1LR1A        | Toxin    |
|                | AAK32727.1 |             |              |          |                |
|                | AF361169.1 |             |              |          |                |
| 38             | D0MX11     | PITG_02711  | 1Y791        | Hydrolase|
|                | EEY64174.1 |             |              |          |                |
| 41             | G4ZP65     | PHYSODRAFT_334498 | 3st1A | Toxin |
|                | EGZ16304.1 |             |              |          |                |
| 44             | D0NIK4     | PITG_11341  | 3kud.1.B    | IpiB3-like protein |
|                | XM_002900902.1 |             |              |          |                |
| 47             | D0NZI1     | PITG_11525  | 4ci9.1.A    | Carboxypeptidase |
|                | XM_002901039.1 |             |              |          |                |
| 67             | H3GZF4     | Phyra83167  | 3tw5.1.A    | Unclassified |
|                | D5S66080.1 |             |              |          |                |

(Continued)
and positive $\psi$) of the protein G4ZP65. The third quadrate (negative $\phi$ and negative $\psi$) had a relative concentration of pairwise angles. However, a group of angles was found distributed on both sides of the $\psi$ axis line, not allowing most of the pairs a high value of negative $\psi$. In the positive quadrates of $\phi$ (Q1 and Q2), only one minor group of angular pairs was observed equally distributed in the area close to $\psi$ axis with a $\phi$ range of 60° to 90°. The protein G4ZP65 had very few glycines contents, all with a negative $\phi$ and a positive $\psi$ value. The total preproline contents could be divided into five different groups scattered away from the $\phi$ axis. However, two groups were in the close area of $\psi$ axis, and $\psi$ values of the rest of the three groups were close to either extreme ($-180^\circ$ or $180^\circ$). None of the very few proline contents of protein G4ZP65 possessed a positive $\phi$. With reference to the $\psi$ axis, the proline contents were distributed among two quadrates (3rd and 4th). However, a higher positive $\psi$ value was recorded in the case of most of the proline contents (Figure 2S).

The 3D structure of the protein D0NIK4 contained a few pairs of $\phi$ and $\psi$, and the distribution pattern put all of them (except one) in the two quadrates of negative $\phi$ (3rd and 4th). One group of $\phi,\psi$, was located in the first quadrate (positive $\phi$ and positive $\psi$) with a value of $70^\circ, 12^\circ$ ($\phi,\psi$). Although some suitable areas were available, no preproline or proline

| Protein Number | Protein ID | Gene Number | 3D Structure | PDB Code | Classification |
|----------------|------------|-------------|--------------|----------|----------------|
| 68             | H3GZF6     | DS566080.1  | 4dt5.1.A     | Unclassifi ed |

Table 2. Continued.

Figure 3. 3D structural analysis D0MX11 pathogenicity protein a generalized pairwise positioning of $\phi,\psi$ (A), among glycine residues (B), among generalized proline favored preproline (C), and among proline residues (D). Plots were constructed using MolProbity v4.4.
content was detected in the structure of the protein D0NIK4 (Figure 3S).

The 3D structure of the protein D0NIZ1 was rich in $\phi, \psi$ pairs, and the distribution was much more scattered than the other pathogenicity proteins of *P. melonis*. A total of 5 groups of $\phi, \psi$ pairs were observed among which the biggest group was located in the fourth quadrate, and its $\psi$ value was $\geq 90^\circ$. The second-largest group was close to the axis $\psi$, but the $\phi$ values of its members ranged from 40° to 155° $\phi$. The glycine residues were plotted in the three quad rates of the Ramachandran plot (One quadrate, one glycine group), except the second quadrate (positive $\phi$ and negative $\psi$) in which no glycine residue could take its place. The preproline contents had four distinct groups, among which two were close to the $\psi$ axis, each on the negative and positive side of the $\phi$ axis. The proline contents were divided into two groups, and both were located at the negative side of the $\phi$ axis. However, one group had a $\psi$ range of 5° to 40°, while the $\psi$ values for the second group was $\geq 123^\circ$ (Figure 4S).

The pattern of $\phi, \psi$ pairs in the 3D structure of protein H3GZF4 was similar to the respective pattern in protein D0MX11, except one extra group with the highest $\phi$ and $\psi$ values ($\geq 167^\circ$). In this way, the protein H3GZF4 had a total of six groups of $\phi, \psi$ pairs. The glycine contents occupied three quad rates (1st, 3rd, and 4th). However, the glycine groups in the first and third quadrate had only one member each. The compound glycine group of fourth quadrate had $\phi$ and $\psi$ values ranging from 42° to 168° and 111° to 176°, respectively. Just similar to the $\phi$ and $\psi$ pairs, preproline contents were also divided into six groups. Four groups occupied the extreme positions of the four quad rates (one group each quadrate), while two were placed at $\psi$ axis on both sides of $\phi$ axis (with $\phi$ values $\geq 40^\circ$ and $\leq -40^\circ$). The protein D0MX11 had relatively higher proline contents, all having negative $\phi$ values (between $-30^\circ$ to $-82^\circ$). However, concerning angle $\psi$, the proline contents were divided into two groups, one with $\psi$ value 98° to 176° and the second with $-10^\circ$ to $-55^\circ$ (Figure 5S).

The $\phi$ and $\psi$ pairs were divided into a total of five groups in the protein H3GZF6. The largest group was located in the quadrate with negative $\phi$ and positive $\psi$ values. However, the two groups were placed around the $\psi$ axis, both on the range positive and negative $\phi$ values. There was only one glycine group detected in the protein H3GZF6. The group had negative $\phi$ ($-40^\circ$ to $-126^\circ$), but positive $\psi$ values ($42^\circ$ to $133^\circ$). There were a few preproline contents, distributed so apart to generate three separate groups. The most crowded group had the $\phi$ range of $-100^\circ$ to $-136^\circ$, and $\psi$ range of $131^\circ$ to $178^\circ$. The proline contents were classified into three groups, with no proline residue having a positive $\phi$ value (Figure 4).

The study provided the first evidence regarding gamma irradiation as an efficient technique to mutate zoospores. Further, the protein profile analysis, accompanied by the pathogenicity data, screened out seven pathogenicity related proteins of *P. melonis*. The structural analysis provided detailed information about the conserved regions of the

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**Figure 4.** 3D structural analysis H3GZF6 pathogenicity protein, a generalized pairwise positioning of $\phi, \psi$ (A), among glycine residues (B), among generalized proline favored preproline (C), and among proline residues (D). Plots were constructed using MolProbity v4.4. µm.
proteins, mainly defining the pathogenicity of the proteins (Figure 5).

Discussion

According to the best of our knowledge, the study is the first time establishing a method of producing mutants of a microbe (P. melonis) through irradiation technique and then utilizing those mutants to determine pathogenicity conferring tools. Previously, several studies can be found to identify the pathogenicity related genes by evaluating the genome sequences. Some studies have used chemical mutagens to confer the pathogenic behavior of the genes (Oladosu et al. 2016; Khan et al. 2018; Yasin et al. 2018). However, the study has successfully used gamma radiations to get the pathogen mutated, which is a safer technique than using chemical mutagens. This confirms that irradiation of the spores is an effective method to mutate the spores and then to study the differential pathogenicity of the spores.

Pathogens have evolved a myriad of virulence factors that allow them to manipulate host cellular pathways to colonize plants successfully. Pathogens use them to gain entry into, multiply, move within, and eventually exit the host plant for a new infection cycle. Researchers have focused on the characterization of pathogenicity factors, providing unique insights into basic pathogenicity mechanisms (Abbas et al. 2020). Identification of the additional pathogenicity factors promises to continue shedding light on fundamental cellular mechanisms in plants and ensures future food security. It also enhances our understanding of plant signaling, metabolism, and cell biology. The current study has highlighted a total of seven pathogenicity factors of P. melonis. It means that the pathogen targets the host cell in seven different ways, and each of the pathogenicity tools has its limitations and advantages. It means that a successful breeder has to develop a cultivar resistant against all the seven pathogenicity tools of the pathogen. If there is any viable pathogenicity tool available to P. melonis, it can put the agricultural crop yield into a severe threat.

Dozens of plant pathology studies have uncovered a remarkable assortment of toxins used as pathogenicity factors by plant pathogens. This study discovered the toxic roles of two proteins produced by P. melonis. Although the secretory system of these toxins is yet to be determined, the findings are alarming because of the difficulty in controlling toxins (Khan et al. 2017a; Yasin et al. 2018; Zaheer et al. 2018). The toxins produced by plant pathogens are often key determinants of their pathogenicity. Not only do proteinaceous factors act on the host metabolic pathways, but they also can exploit the activity of E3 ubiquitin ligases to promote disease (Bray Speth et al. 2007). Therefore, it seems that in the presence of two distinct proteins with cytotoxic properties, it would be challenging for future studies to control the pathogen.

The study has revealed that the presence of multiple pathogenicity tools enables the pathogen to employ various and complex mechanisms for penetration, colonization, host-pathogen interaction, and expansion in the host (Yasin et al. 2018). To control the pathogen, researchers have to find out the neck of the bottle and
then devise some strategy to block the pathogenic activities (Yasin et al. 2019). For instance, it would be wise to block the enzyme used for cell degradation than to chock the kinase and oligopeptidase, which are used to modulate host cellular machinery after infection. Otherwise, it would be challenging to deal with an established pathogen with all of its pathogenicity tools and complex pathogenic mechanisms.

Three-dimensional protein structures usually contain regions of local order, called secondary structures, such as α-helices and β-sheets. The secondary structure is characterized by the local rotational state of the protein backbone, quantified by two dihedral angles called ϕ and ψ (Mannige et al. 2016). It is imperative to determine the rotational points (ϕ and ψ) of the protein to understand the functions and mechanics of the protein. The current study defines the rotational angles and the rotational position of secondary structures in all the seven pathogenicity proteins. The data will help in drawing the supramolecular mechanisms of pathogenicity by these proteins.

The glycine residues are thought to be highly conserved among the members of individual structural protein families and super-families. As glycine takes a strict and prime part in folding the protein, the structure conservation is mainly dependent upon glycine residues in a protein (Purushottam et al. 2019). Glycine residues cover a petite size and the minimal steric hindrance of side chains; thus, they don’t hinder the folded structure of the protein (Parrini et al. 2005). Consequently, the presence of glycine residues at defined places keeps the function of the protein intact. The current study has positioned the glycine residues in the pathogenicity protein of P. melonis. Any change in the glycine position of the protein can be used to assess the mutation in the protein. Overall, it has developed a very precious database of pathogenic functions of proteins.

Furthermore, by comparing the glycine residues of all the pathogenic proteins, we can conclude that the pathogenic proteins must have negative ϕ angles in their structure with a value ≤ of 63°. In contrast, the value of angle ψ should be ≥80°. In the results, the six proteins strictly followed this pattern. However, one IpiB3-like protein (D0NIK4) with a domain XM_002900902.1, and encoded by gene PITG_11341, doesn’t have any glycine residue. By considering the glycine properties, we conclude that the structural and functional conservation of protein D0NIK4 mainly depends upon its proline contents. Proline residues also define loop structures of proteins and play an important role during chain compaction early in folding. Loop formation is significantly slower around trans prolyl peptide bonds of proline but much faster around glycine residues (Krieger et al. 2005). However, short loops are formed fastest around cis prolyl bonds with a time constant of 6 ns for end-to-end contact formation in a four-residue loop. Moreover, the altered dynamics around glycine and trans prolyl bonds can be mainly ascribed to their response against the activation energy of loop formation. Therefore, proline contents become important in the absence of glycine residues. However, it needs to design detailed studies to discover the supramolecular mechanisms of the pathogenic behavior of D0NIK4 protein.

### Experimental procedures

#### Sample collection

The method and preservation measures of Ahmad et al. (Ahmad et al. 2014) were strictly followed during the sampling of the study material. Infected cucurbit plants were collected from the experimental fields of Guangdong Academy of Agricultural Sciences, Guangzhou. Each sample was stored in a paper envelope and kept in a cool box with dry ice before its transportation to the lab. In the lab, the samples were washed and cut into small blocks (0.5 cm²) for isolation of the pathogen.

#### Isolation and identification of P. melonis

A well-optimized method of Davison (1998) was used to isolate and identify P. melonis. For isolation of P. melonis, the infected sample blocks were rinsed with several changes of sterilized distilled water. After desiccation by filter paper, blocks were placed onto Petriplates of the selective medium, V8 agar (Buffered and clarified with pH: 6.7). The Petri plates were incubated at 28 °C in the dark. The identification of P. melonis was made based on species description in previous literature (Davison 1998).

#### Pathogenicity assay of Phytophthora sp. on cucurbits

The pathogenicity of P. melonis isolates obtained from cucurbits was tested on seedlings of healthy cucumber (Cucumis sativum) through pot experiment studies (Davison 1998). Each pot was filled with 400 g of autoclaved soil mixed with 10 mL of 6 days old cultures of each strain grown in V-8 juice agar at 25 °C. The seedlings were planted (one seedling in one pot) in infested soils, and the experiments were carried out in the plant house with day and night temperatures of 30–35 and 23–30 °C, respectively. Sterilized soils without inoculum were served as control. Three biological replications were maintained for each strain, and the experiment was repeated twice. The inoculated pathogen was re-isolated from the infected plants to prove the Koch’s postulates.

#### Mutation assay

**Irradiation of spores**

Spores suspension (400 µL) of pathogenic Phytophthora was prepared in distilled sterilized water, containing 10000 spores per 100 µL (Tingquan 2019). The spore suspension was shaken thoroughly, and the concentration was adjusted using a haemocytometer. The suspension was divided into four irradiation treatments, i.e. 0 minutes, 1 min, 3 , and 5 minutes. Irradiation of the spores was conducted using an irradiation system of ARTP, Breeding Mutagenesis Machine, Model: ARTP-P; Manufacturer: TMAXTREE Biotechnology Co. Ltd. (Masuda et al. 2004). Spore irradiation apparatus was connected to a vertical beamline of the AVF-cyclotron in conjunction with a 50-MeV helium ion irradiation. All the 10000 spores of every single treatment were dispersed in a thin film of sterilized water to make a monolayer of spores for homogenous irradiation. The spores were irradiated under atmospheric pressure, and the spores were kept under room temperature conditions for one hour after the irradiation. The irradiated spores were subjected to germination on V8 agar medium Petri plates to establish
distinct colonies. The rate of spore germination and growth rates of developing colonies were recorded to compare the effect of mutation treatment. The radius of each colony was measured from 30° angular dimensions to eliminate the error of differential growth rates in different directions. Then the average of the radii was calculated to determine the colony growth rate. The experiment contained three biological replicates to ensure the reliability of the results.

Pathogenicity assay of P. melonis mutants on cucumber

All the mutated strains were tested for their pathogenicity on apparently healthy cucumber leaves (Anjum et al. 2017). For inoculation, each strain of P. melonis was grown on V8 medium plates. Twenty (20) days old seedlings of cucumber were brought to the laboratory, and their cotyledonous leaves were detached into Petri plates (9 cm diameter) already impregnated with a double layer of Whatman filter papers. The filter papers were moistened by adding 5 mL distilled sterilized water into each Petri plate. The leaves were inoculated by putting V8 medium blocks having the growth of P. melonis. The control leaves were treated with V8 medium blocks without the pathogen growth. Three biological replicates were maintained for each strain, and the experiment was repeated thrice. All the inoculated and control treatments of different cucumber leaves were placed in an incubator at 28 °C. Development of symptoms on leaves inoculated by the pathogen and controls were observed continuously every 6 hours for four days.

Disease scale of 0 - 100% was divided into six classes of disease severity (Ahmad et al. 2014a). The symbolic demonstration of the infected area percentage for the disease rating scale is shown in Figure 1.

The disease index was calculated according to the following formulae:

Disease index = \frac{\text{Sum of disease scores of observed leaves}}{\text{Maximum possible score} \times \text{Number of leaves observed}} \times 100

While,

Disease Score = \text{Score obtained by observed leaf according to disease rating scale of Figure 1.}

Disease index = \text{The highest score a leaf could get according to the disease rating scale.}

Protein profiling

Total protein profiles of Phytophthora strains were analyzed to assess the differentially expressed proteins, considering them the active factor of pathogenicity against the host plant (Khan et al. 2019). Total protein contents of three biological replicates of each treatment were extracted by adopting the phosphate buffer saline method (Ahmad et al. 2014b). Hence, the extraction solvent phosphate buffer saline (PBS) contained 140 mM NaCl, 10 mM Na2HPO4, 1.8 mM NaH2PO4, and 2.5 mM KCl. Protein samples were dissolved in 8 M Urea solution prior to their Native PAGE. Electrophoresis in the second dimension was performed on 12% Native Polyacrylamide Gel (Native-PAGE). Electrophoresis in the second dimension was performed under identical conditions with the addition of SDS to attain fine resolution. Then, Coomassie blue staining of proteins was performed to visualize and record results. Digital images of protein gels were captured for their detailed analyses. The stained protein spots were identified and compared using digital software SAMEPOTS (Total-Lab Ltd., UK) and TOPSPOT, Kroger and Prehm, Berlin, Germany (Khan et al. 2019).

Protein profiles of all Phytophthora strains from each cultivar were compared with each other, and the profusion index for each protein was calculated (Bashir et al. 2016). Profusion behavior of different proteins was plotted in a matrix plot to screen the proteins by the ‘profusion index.’ It provided the proteins most pathogenic against cucumber plants.

Profusion Index = \sum \text{Ind/Frequency of occurrence}

\text{Ind} = \text{Number of times a protein was professed by a stimulus (in comparison to control)}

Protein orthologues were used to calculate the correlation coefficient as the measure of the relationship between protein–protein interactions. The correlation coefficient measured the relative shape of the relationship rather than absolute levels, and it captured both positive and negative interactions. All the interactions involving a protein with itself (homodimers) were discarded to avoid skewed results for a trivial reason. For a quantitative comparison of the interactions, three key statistics of the datasets were used, differences among their means, and medians were calculated. Besides, the non-parametric Kolmogorov–Smirnov (KS) test was employed to determine the underlying distribution probability. The peptides were labeled with isobaric tags by using Multiplex Kit (AB Sciex, Foster City, CA, USA). The previously established method of Wu et al. (2020) was adopted to perform this peptide labeling step.

Liquid chromatography-tandem mass spectrometry

Lyophilized peptides obtained from OFFGEL fractionation were dissolved in 8 µL of 5% CH3CN/0.1% FA; 5 µL of the resulting sample was injected for LC-MS/MS analysis. MS analysis was performed on an LTQ Orbitrap Velos Pro from Thermo Electron (San Jose, CA) equipped with a NanoAcquity UPLC system from Waters (Milford, MA, USA). Peptides were trapped on a home-made (5 µm 200 µm 100 Å Magic C18 AQ, 0.75×15 mm) column (Michrom, Auburn, CA, USA) and separated on a home-made (5 µm 100 Å Magic C18 AQ, 0.75×15 mm) column (Michrom). The analytical separation was run for 65 min using a gradient of 99.9% H2O/0.1% FA (solvent A) and 99.9% CH3CN/0.1% FA (solvent B). The gradient was run as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min at a flow rate of 220 nL/min. For MS survey scans, the OT resolution was set to 60000, and the ion population was set to 5×105 with an m/z window from 400 to 2000. A maximum of 3 precursors was selected for both the collision-induced dissociation (CID) in LTQ and the high-energy C-trap dissociation (HCD) with analysis in the OT. For MS/MS in the LTQ, the ion population was set to 7×103 (isolation width of 2 m/z). In contrast, for MS/MS detection in the OT, it was set to 2×103 (isolation width of 2.5 m/z), with a resolution of 7500, first mass at m/z = 100, and maximum injection time of 750 ms. The
normalized collision energies were set to 35% for CID and 60% for HCD (Hu et al. 2017).

**Data extraction, relative protein quantification, and database interrogation**

Peak lists were generated from raw data using the software MZMine version 2.30 (Pluskal, Okinawa, Japan). The conditions and methods were followed as used by Bashir et al. (2016). After peak list generation, the collision-induced dissociation and high-energy C-trap dissociation spectra were merged for simultaneous identification and quantification. The merged files were used for protein identification and quantification. For protein identification, parameters were specified as follows: databases = uniprot_sprot/ uniprot_trembl; taxonomy = Phytophthora; precursor error tolerance = 25 ppm; variable modification = oxidized methionine; fixed modifications = carbamidomethylated cysteine, iTRAQ-labeled amino terminus and lysine; enzyme = trypsin; potential missed cleavage = 2; cleavage mode = normal; search round = 1. Protein and peptide scores were set up to maintain the false positive peptide ratio below 5%. For protein quantification, the isotopic correction was applied to reporter intensities according to the iTRAQ reagents certificate of analysis. iTRAQ reporter peak intensities were further normalized using the spiked LAC standard. For each protein, the mean, the standard deviation, and the coefficient of variation of relative peptide intensities were obtained. The ratio of the protein was then computed as the geometric mean of all peptide ratios belonging to the protein. A Student’s t-test distribution was computed by the algorithm with a null hypothesis stating that the log2 of the protein ratio was equal to zero (confidence interval = 95%).

The protein sequence was modeled by SIMULINK®, an add-in of MATLAB®. The package constructed the structure based on its homology (comparative modeling), by constructing an atomic-resolution model. For this purpose, the amino acid sequence was run a BLAST analysis, from which the most similar protein sequence (3tw5 belonging to Phytophthora sojae) was selected. Alignment analysis was carried out between H3GZ6,5 and 3tw5 before the development of the 3D structure. After the structure development, it was compared with the structure of the homologous protein (3tw5). After the successful production of alignment mapping residues, the template structure was used to produce a structural model. For 3D simulation, protein cleavage analysis was performed to determine distance, and Terminal Selection boxes (N and C) was set from end to end as default. A built-in graphical user interface was used to visualize the constructed structure for detailed analysis. For detection of ligand attachment a-factor, and the protein subunits participated in reactions. Ligand appeared on both sides of the reaction. Ordinary differential equations (ODEs) were used to rate the model for biochemical properties, and then binding for the formation of a heterotrimeric protein complex was performed. kGa and kGd were calculated on the basis of dose–response curves (Yi et al. 2003).

The identifiers of protein detected domains were underwent a BLAST analysis with a bit score >600 to find out peptides with similar sequences. The algorithm was used at maximum threshold level 0.05, bits threshold 25, and an initiating size of six. A matrix Blosum62 was selected with Gap Costs extension 1.0 and existence 11.0. The compositional adjustments were opted to be conditional compositional score matrix adjustments in the absence of any filter and/or mask settings. The results were used to construct a dendrogram showing the taxonomic distribution of the protein in other species and higher taxa. Furthermore, a list of structurally similar proteins was developed using the ProBis algorithm based on similar interaction patterns of the binding sites (Konc and Janežič 2010).

**Ramachandran plots**

For atom pairs, two sets of allowed inter-atomic separations. Subsequently, all possible combinations of backbone φ, ψ angles were determined for an alanyl dipeptide mimetic (N-acetyl-L alanine-methyl ester). The angle φ was defined as the dihedral angle with clockwise rotation around the N-Cα bond of the backbone atoms C'-N-Cα-C', and the angle ψ was considered as the dihedral angle with clockwise rotation about the Cα-C' bond involving the backbone atoms N-Cα-C'-N. The consistent combinations of ϕ/ψ were identified with inter-atomic separations to draw Ramachandran Plots using Biopython-1.77 and R-4.0.2 (Zhou et al. 2011).

**Statistical analysis**

The data were collected from the three biological replicates of each experiment (irradiation assay, pathogenicity assay, and protein profiling). The data were statistically analyzed using SPSS, 17.0 for windows (SPSS, Chicago, IL, USA). Standard error was also calculated, and analysis of variance (ANOVA) was performed on the data to determine the least significant difference (LSD) between treatment means with the level of significance at P≤0.05. However, the genetic data obtained were analyzed using computer tools developed explicitly for molecular data analysis, as mentioned in the methodology section.

**Declaration section**

**Ethics approval and consent to participate**

Ethics and consent are not applicable to the study because there are no human or animal tissues involved.

**Consent for publication**

All the data included in the manuscript have been recorded during the current research work.

**Competing interests**

There is no financial or non-financial competing interests of authors related to this manuscript.

**Authors’ contributions**

AA, TW, IS, conceived the idea. RW, MU, IS, designed the research plan. WA, RW, acquired technical software. IS, AA, MU, performed the research work. AA, and IS analyzed the data and prepared graphical presentations. AA, WA, NAY, drafted the manuscript. NAY reviewed and corrected the manuscript. TW supervised the study.
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Data availability statement
All the data generated during the research period has been provided along with the manuscript. The protein sequences are already available at the publicly available database, www.uniprot.org, for which the accession numbers (Uniprot IDs) have been mentioned in Table 1 of the manuscript.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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Supporting Information legends
Structural analysis of the MPPs highlighting the angle pairs (φ, and ψ) in 3D protein structures and construction of Ramachandran Plots.

Notes on contributors
Aqeel Ahmad has completed his Ph.D. in Agricultural Sciences from the University of the Punjab, Lahore, Pakistan, and served Huazhong Agricultural University, and Guangdong Academy of Agricultural Sciences as a postdoctoral researcher. He is a young and energetic scientist with a highly talented researcher, serving the Guangdong Academy of Agricultural Sciences, due to which we could perform and conclude the study.

Rui Wang is a highly talented researcher, serving the Guangdong Academy of Agricultural Sciences. She has a previous applied experience of performing editing and review activities for multiple impact factor journals and publishers.

Iqra Shahzadi bears the affiliation of Environmental Sciences Department, Wuhan University, China. Her research record contains multiple high impact factor, international articles, and book chapters, etc. She is also performing editing and review activities for multiple impact factor journals and publishers.

Muhammad Umer has completed his Ph.D. in plant pathology from the College of Plant Sciences and Technology, Huazhong Agricultural University, Wuhan, China. He is a young and energetic researcher with an eminent research profile containing a published record of (i) 12 peer-reviewed research manuscripts. Presently, he is working as a Postdoctoral fellow at the Guizhou University, Guiyang, China.

Nasim Ahmad Yasin is an enthusiastic scientist and head of the gardening department at University of the Punjab, Lahore, Pakistan. He earned his Ph.D. in stress physiology of rose plants and presently he owns a brilliant track record of scientific research by publishing multiple high quality research articles and mentoring masters and doctoral theses.

Tingquan Wu is serving as a professor at Guangdong Academy of Agricultural Sciences. His area of research covers vegetable research, especially melons and cucumber, for which he has been searching the innovative techniques to control biotic stressors. His eminent research record contains multiple research articles, disease reports, patents, and third party fundings.

References
Abbas HMK, Huang H-X, Wang A-J, Wu T-Q, Xue S-D, Ahmad A, Xie D-S, Li J-X, Zhong Y-J. 2020. Metabolic and transcriptomic analysis of two Cucurbita moschata germplasms throughout fruit development. BMC Genomics. 21(1):365.
Ah-Fong AMV, Kim KS, Judelson HS. 2017. RNA-seq of life stages of the oomycete Phytophthora infestans reveals dynamic changes in metabolic, signal transduction, and pathogenesis genes and a major role for calcium signaling in development. BMC Genomics. 18(1):198.
Ahmad A, Akram W, Shahzadi I, Wang R, Hu D, Bashir Z, Jaleel W, Ahmed S, Tariq W, Li G, et al. 2019. Benzene dicarboxylic acid upregulates O48814 and Q8FJQ8 for improved nutritional contents of tomato and low risk of fungal attack. J Sci Food Agric. 99(14):6139–6154.
Ahmad A, Akram W, Shahzadi I, Wang R, Hu D, Li G, Yasir NA, Ahmed S, Wu T. 2020a. First report of Fusarium nelsonii causing early-stage fruit blight of cucumber in Guangzhou, China. Plant Dis. 104(5):1542.
Ahmad A, Khan TA, Mubeen S, Shahzadi I, Akram W, Saeed T, Bashir Z, Wang R, Alam M, Ahmed S, et al. 2020b. Metabolic and proteomic perspectives of augmentation of nutritional contents and plant defense in Vigna unguiculata. Biomolecules. 10(2):224.
Ahmad A, Shafique S, Shafique S. 2013. Cytological and physiological basis for tomato varietal resistance against Alternaria alternata. J Sci Food Agric. 93(9):2315–2322.
Ahmad A, Shafique S, Shafique S. 2014a. Intracellular interactions involved in induced systemic resistance in tomato. Sci Hortic. 176:127–133.
Ahmad A, Shafique S, Shafique S. 2014b. Molecular basis of antifungal resistance in tomato varieties. Pakistan J Agric Sci. 51(3):683–687. https://pajkias.com.pk/papers/2327.pdf.
Ahmad A, Shafique S, Shafique S, Akram W. 2014. Penicillium oxalicum directed systemic resistance in tomato against Alternaria alternata. Acta Physiol Plant. 36(5):1231–1240.
Ahmad A, Yasin NA, Ibrahim A, Shahzadi I, Gohar M, Bashir Z, Khan J, Khan Wu, Akram W. 2018. Modelling of cotton leaf curl viral infection in Pakistan and its correlation with meteorological factors up to 2015. Clin Dev. 10(6):520–525. https://doi.org/10.1080/17565529.2017.1318738.
Akram W, Anjum T, Ahmad A. 2014a. Basal susceptibility of tomato varieties against different isolates of Fusarium oxysporum f. sp. lycopersici. Int J Agric Biol. 16(1):171–176.
Akram W, Anjum T, Ahmad A, Moeen R. 2014b. First report of Curvularia lunata causing leaf spot on Sorghum bicolor from Pakistan. Plant Dis. 98(7):1007–1007.
Anjum T, Akram W, Shafique S, Sahique S, Ahmad A. 2017. Metabolomic analysis identifies synergistic role of hormones biosynthesis and phenylpropanoid pathways during fusarium wilt resistance in tomato plants. Int J Agr Biol. 19(05):1073–1078.
Bae H, Bowers JH, Toole PW, Bailey BA. 2005. Nep1 orthologs encode dinocyst formation and ethylene inducing proteins exist as a multigene family in tomato. Int J Agric Biol. 9(04):1385.
Bangashwani Z, Mirtalab E, 2008. Saflower seedling a selective host to discriminate Phytophthora melonis from Phytophthora drechsleri. J Phytopathol. 156(7–8):499–501.
Bashir Z, Shafique S, Ahmad A, Shafique S, Yasin NA, Ashraf Y, Ibrahim A, Akram W, Noreen S. 2016. Tomato plant proteins actively responding to fungal applications and their role in cell physiology. Front Physiol. 7:257.
Bray Speth E, Lee YN, He SY. 2007. Pathogen virulence factors as molecular probes of basic plant cellular functions. Curr Opin Plant Biol. 10(6):580–586.
Chen X-R, Xing Y-P, Li Y-P, Tong Y-H, Xu J-Y. 2013. RNA-seq reveals infection-related gene expression changes in Phytophthora capsici. PLoS One. 8(9):e74588.
Parrini C, Taddei N, Ramazzotti M, Degl'Utri FV, Rickauer M, Esquerré-Tugayé M-T. 1997. Cloning and characterization of stress-sensitive and tolerant variety of tomato treated with brassicamycin: a review. Biotechnol Biotechnol Eq. 30(1):1–16.

Parrini C, Taddei N, Ramazzotti M, Degl'Utri FV, Rickauer M, Esquerré-Tugayé M-T. 1997. Cloning and characterization of stress-sensitive and tolerant variety of tomato treated with brassicamycin: a review. Biotechnol Biotechnol Eq. 30(1):1–16.

Parrini C, Taddei N, Ramazzotti M, Degl'Utri FV, Rickauer M, Esquerré-Tugayé M-T. 1997. Cloning and characterization of stress-sensitive and tolerant variety of tomato treated with brassicamycin: a review. Biotechnol Biotechnol Eq. 30(1):1–16.

Parrini C, Taddei N, Ramazzotti M, Degl'Utri FV, Rickauer M, Esquerré-Tugayé M-T. 1997. Cloning and characterization of stress-sensitive and tolerant variety of tomato treated with brassicamycin: a review. Biotechnol Biotechnol Eq. 30(1):1–16.