The α -Subunit of the Chloroplast ATP Synthase of Tomato Reinforces Resistance to Gray Mold and Broad-Spectrum Resistance in Transgenic Tobacco

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ABSTRACT

Chloroplast ATP synthase (cpATPase) is responsible for ATP production during photosynthesis. Our previous studies showed that the cpATPase CF1 α subunit (AtpA) is a key protein involved in *Clonostachys rosea*-induced resistance to the fungus *Botrytis cinerea* in tomato. Here, we show that expression of the tomato *atpA* gene was upregulated by *B. cinerea* and *Clonostachys rosea*. The tomato *atpA* gene was then isolated, and transgenic tobacco lines were obtained. Compared with untransformed plants, *atpA*-overexpressing tobacco showed increased resistance to *B. cinerea*, characterized by reduced disease incidence, defense-associated hypersensitive response-like reactions, balanced reactive oxygen species, alleviated damage to the chloroplast ultrastructure of leaf cells, elevated levels of ATP content and cpATPase activity, and enhanced expression of genes related to

carbon metabolism, photosynthesis, and defense. Incremental Ca^{2+} efflux and steady H^+ efflux were observed in transgenic tobacco after inoculation with *B. cinerea*. In addition, overexpression of *atpA* conferred enhanced tolerance to salinity and resistance to the fungus *Cladosporium fulvum*. Thus, AtpA is a key regulator that links signaling to cellular redox homeostasis, ATP biosynthesis, and gene expression of resistance traits to modulate immunity to pathogen infection and provides broad-spectrum resistance in plants in the process.

Keywords: α subunit of cpATPase, abiotic disorders, ATP, *B. cinerea* resistance, biotechnology, broad-spectrum resistance, disease resistance, molecular, plant immune responses, plant stress, tomato

Gray mold of tomato (*Solanum lycopersicum*) is a devastating disease caused by the necrotrophic fungal pathogen *Botrytis cinerea*. Previous studies suggest that *B. cinerea* is not restrained by host-mediated localized hypersensitive responses (HRs), but rather by necrotic tissues (Patel et al. 2015). However, Audenaert et al. (2002) showed that the functional deletion of abscisic acid empowered *sitiens* tomato mutants and increased resistance to *B. cinerea* via a quick epidermal HR and associated cell wall fortifications derived from the phenylpropanoid pathway. Resistance to *B. cinerea*, as with most necrotrophs, depends on the balance between cell death and survival (Mengiste 2012). Yet the effects of HR-mediated defense tactics in plants against *B. cinerea* remain unknown (Seifi et al. 2013).

Because of the lack of tomato cultivars with resistance against gray mold, no such varieties have yet been bred. Defense priming can be induced by phytopathogens and nonpathogenic beneficial organisms, among others, some of which can elicit the gene expression of defensive traits (Conrath et al. 2015; Martinez-Medina et al. 2016; Verhagen et al. 2009). *Clonostachys rosea* is an effective antagonistic fungus to *B. cinerea*. In our previous studies, both the disease incidence and the disease index were significantly reduced in *Clonostachys rosea*-inoculated tomato compared with *B. cinerea* inoculation alone by the regulation of phytohormone contents, secondary messengers, and defense-related enzymes activities in tomato; notably, the AtpA was identified as the abundant protein in tomato treated with *Clonostachys rosea* (Gong et al. 2017; Mouekouba et al. 2014). Those results indicated that the enhanced defense of tomato to *B. cinerea* infection was primed by *Clonostachys rosea* with AtpA perhaps involved in *B. cinerea* resistance. However, the mechanisms by which AtpA might suppress *B. cinerea* and influence subsequent host-defense response patterns have yet to be comprehensively explored.

Plants have evolved a sophisticated innate immune system that can be activated when they are attacked by one or more pathogens. Generally, this plant innate immune system can be summarized in a four-phase “zigzag” model (Jones and Dangl 2006). In phase 1, plants' first line of defense relies on recognizing pathogen-associated molecular patterns (PAMPs) by specific extracellular surface pattern-recognition receptors, which leads to PAMP-triggered immunity (PTI) that limits pathogen colonization of the host (Dangl et al. 2013). In phase 2, successful pathogens can suppress the host's PTI response via the production of effectors, which leads to the onset of disease, and this results in effector-triggered

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susceptibility. In phase 3, plants in turn evolve a group of specific disease resistance genes (*R* genes), some of which can specifically recognize a given effector, and this triggers a second-tier immune response called effector-triggered immunity (ETI) (Xu et al. 2019). In phase 4, pathogens are able to suppress ETI by shedding or diversifying the recognized effector gene or by acquiring new effectors. Natural selection drives new plant *R* genes to specially recognize one of the newly acquired effectors so that ETI can be triggered again in the host (Jones and Dangl 2006).

Many effectors can suppress one or more components of PTI and ETI responses and these contribute to the enhancement of pathogen virulence (Jones and Dangl 2006; Xu et al. 2019). For example, haustorium-specific protein Pst_12806 was identified as a *Puccinia striiformis* f. sp. *tritici* effector that interacted with TaLSP, a putative component of the cytochrome b6-f complex, and attenuates the rate of photosynthesis and inhibits plant defenses (Xu et al. 2019). Effector Pst18363 was shown to target Nudix hydrolase 23 to facilitate *Puccinia striiformis* f. sp. *tritici* infection (Yang et al. 2020). The majority of *R* proteins contain a nucleotide binding site (NBS) and leucine-rich repeat domains. The NBS domain of *R* proteins is a conserved region that is responsible for the binding and hydrolysis of ATP or GTP, which is essential for their biochemical activity (Ameline-Torregrosa et al. 2007; Tameling et al. 2002). The NBS region is involved in signal transduction cascades involving phosphorylation/dephosphorylation with GTP or ATP (Liu et al. 2007). ETI is qualitatively stronger and faster and is often characterized by a HR, but we generally need a better understanding of both PTI and ETI defense responses (Dodds and Rathjen 2010; Jones and Dangl 2006; Landoni et al. 2013). Both biotic and abiotic stresses can induce the release of ATP, which can also serve as a PAMP recognized by plants (Dodds and Rathjen 2010; Tanaka et al. 2014). ATP plays an important role in ETI defense response, but how the ATP-releasing systems are regulated by environmental stresses remains unclear (Tanaka et al. 2014).

ATP is synthesized or hydrolyzed by cpATPase, utilizing or generating a proton gradient by H⁺ translocation. The cpATPase involved in photosynthesis is responsible for the synthesis of ATP from ADP in a process driven by the transthylakoid proton gradient (Kangasjärvi et al. 2014; Kramer et al. 2004). Of cpATPase's two domains, the CFI domain contains five subunits, denoted α (AtpA), β (AtpB), γ (AtpC), δ (AtpD), and ϵ (AtpE) (von Ballmoos et al. 2009). ATP binding sites located in α - and β -subunits function in regulation and catalysis, respectively, and their conformational changes result in the synthesis/hydrolysis of ATP (Leyva et al. 2003). In particular, the AtpA subunit plays a pivotal role in plant energy metabolism.

Photosynthetic activity significantly affects the redox status and energy state by supplying ATP, NADPH, and carbon skeletons, which provide a basis for plant growth and trigger responses against external stress and their maintenance (Kangasjärvi et al. 2014). Plants have evolved multiple mechanisms to optimize photosynthesis under environmental fluctuations in order to balance growth with metabolism during costly defense responses (Kangasjärvi et al. 2014). Previous findings indicate that disease resistance is related to photosynthetic parameters, such as wheat Fusarium head blight, Jujube witches' broom disease, and powdery mildew in cucumber (Lindenthal et al. 2005; Xue et al. 2018; Yang et al. 2016). However, significant uncertainties remain in our understanding of how AtpA as a photosynthetic parameter is involved in stress response to pathogens and abiotic stress. Since AtpA was found enriched in nonfungus-infested leaves of mite-infested maize by leaf proteome analysis in maize, it suggests that a higher content of ATP could be required to improve stress tolerance in leaf of maize, and by extension perhaps plant resistance against enemies (Dworak et al. 2016). Furthermore, AtpA was accumulated under a flg22 treatment, a well-characterized PAMP, which is a peptide derived from the bacterial motor protein flagellin (Göhre et al. 2012). Song et al. (2014) reported that *atpA* was upregulated 5.01-fold in poplar

(*Populus simonii*) plants after 6 h of imposed heat stress, suggesting that this gene might play important roles in photosynthesis under conditions of heat stress. In other work, the relative expression of photosynthetic complex protein AtpA was increased under saline conditions in *Desmostachya bipinnata*; hence, accumulating more AtpA may promote the synthesis of ATP to meet the greater energy demand for sustained salt resistance in plants (Asrar et al. 2017). Despite its expression patterns being well known during different stress conditions, AtpA's biological functioning and mechanism(s) remain poorly understood. In this study, gene expression of *atpA* in tomato was induced significantly by *B. cinerea* and/or *Clonostachys rosea* at 96 h posttreatment (Supplementary Fig. S1).

Various signaling molecules, such as reactive oxygen species (ROS), nitric oxide (NO), hydrogen peroxide (H₂O₂), and Ca²⁺, are involved in the regulation of multiple pathways controlling plant stress responses (Govrin and Levine 2000; Seifi et al. 2013). Antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and phenylalanine ammonia lyase (PAL), are crucial for plants' resistance against *B. cinerea* infection (Bui et al. 2019; Govrin and Levine 2000; Jing-Yu et al. 2020). To evaluate the physiological effects and functional mechanisms of *atpA* on *B. cinerea* resistance, we compared changes in ATP and ROS content, activity of ATP synthase and antioxidant enzymes, resistance-related genes, Ca²⁺ and H⁺ levels, and chloroplast structure in transgenic and control tobacco plants. These results broaden our understanding of the resistance mechanisms against *B. cinerea* and broad-spectrum resistance as mediated by AtpA. Furthermore, these findings could potentially be useful for improving disease resistance traits in tomato cultivars.

MATERIALS AND METHODS

B. cinerea strains, plant material, and growth conditions.

The *B. cinerea* strain used was isolated from a diseased tomato plant (Wang 2013; Wang et al. 2019) and cultured on potato dextrose agar plates at 25°C for 7 days. Conidial suspensions of the strain were prepared as previously described (Gong et al. 2017).

Seeds of homozygous tomato variety 09888-6 and cultivated tobacco K326 were provided by the Northeast Agricultural University Tomato Research Institute in China. The tomatoes were grown in a mixture of perlite/vermiculite/plant ash (1:6:2) in growth chambers at 25°C/22°C (day/night) and 75% relative humidity under a 16-h/8-h (day/night) photoperiod. Tobacco seeds were germinated on Murashige and Skoog (MS) medium (M519; PhytoTechnology Laboratories, Shawnee Mission, KS). Seedlings were grown at 25°C/22°C (day/night) and 60% relative humidity under a 16-h/8-h photoperiod in a phytotron. When their fifth leaves were fully developed, the tobacco plants were ready for use in the *Agrobacterium*-mediated transformations.

Cloning and sequencing of *atpA*. Total RNA was extracted from tomato leaves and stems using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and then treated with RNase-free DNase I to remove any contaminating genomic DNA. First-strand cDNA was synthesized using 2 μ g of RNA with the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific Co., Ltd., Shanghai, China). The full-length sequence of *atpA* was amplified from the cDNA by using forward primer 5'-ATGGTAACCATTCGAGCTGACG-3' and reverse primer 5'-TTATGCTTGTTCCTGAAGTATAAAACG-3'. The PCR amplification products were inserted into the pMD19-T vector (Takara, Dalian, China). The construct was then introduced into chemically competent *Trans5 α* *Escherichia coli* cells (TransGen Biotech, Beijing, China) according to the manufacturer's instructions and verified by sequencing.

***Agrobacterium*-mediated transformation of tobacco plants.** The full-length sequence (1,524 bp) of tomato *atpA* was inserted into the pCAMh+p expression vector (structured by pBI121 and

pCAMBIA2300) driven by the *Cauliflower mosaic virus* 35S (35S-CaMV) promoter between *Bam*HI and *Sac*I (Supplementary Table S1). pCAMh+p-*atpA* was introduced into *A. tumefaciens* strain LBA4404 by a freeze-thaw method. Transgenic tobacco plants were generated by a *Agrobacterium*-mediated leaf-disc transformation process (Horsch et al. 1985). DNA and RNA obtained from the T₀ 100 mg/liter of kanamycin-resistant tobacco and untransformed tobacco (the wild type [WT]) were extracted for PCR and quantitative reverse transcription PCR (qRT-PCR) examinations (Supplementary Fig. S2). Genomic DNA was isolated from the tobacco leaves using a modified cetrimonium bromide method (Murray and Thompson 1980). The T₀ plants were self-fertilized and the progeny obtained from the T₁ generation was named T₂. qRT-PCR results showed that all 100 mg/liter of kanamycin-resistant plants had significantly higher mRNA expression levels than their WT counterparts. The T₂ generation of transgenic tobacco was considered homozygous (Wang et al. 2014). The T₂ seeds were then germinated on MS medium without any growth regulators or antibiotics; the plants were used for physiological and molecular analyses (Jing et al. 2015) to further estimate plant resistance to *B. cinerea*.

Evaluation of *B. cinerea* tolerance. Leaves of 1-month-old seedlings of transgenic lines and WT tobacco were inoculated with the *B. cinerea* strain using a modified version of the method described in Munis et al. (2010). A small wound (2 to 3 mm in length) was gently made in the third mature leaves with blades, to which 30 µl of filtered spores (10⁷ spore/ml) was applied per plant. These inoculated plants were kept in humid and dark conditions at 22°C for 30 days postinoculation (dpi).

In the whole-plant inoculation assay, transgenic and WT tobacco individuals were infected with *B. cinerea* through a suspension (10⁷ spores/ml)-spraying process until the plants were covered with droplets, whereas the control group was sprayed with only sterile water. For each treatment, a triplicate of 20 tobacco plants was used, all incubated under humid conditions at 22°C. The disease index and the disease incidence of gray mold disease were each calculated at 15 dpi according to Gong et al. (2017), albeit with slight modifications. The degree of disease severity after inoculation with *B. cinerea* was scored as follows: 0, no lesion; 1, a few scattered lesions covering <2% of the tobacco leaf area; 2, extensive lesions covering >2% but <5% of the tobacco leaf area; 3, extensive lesions covering >5% but <25% of the tobacco leaf area, and; 4, extensive lesions covering >25% of the tobacco leaf area. The disease index for each treatment was calculated using the following formula:

$$\text{Disease index} = \left\{ \left(\sum_{i=0}^4 N_i \times i \right) / \left(4 \times \sum_{i=0}^4 N_i \right) \right\} \times 100\%$$

where *i* is the severity score (0 to 4), for which 4 is the highest level, and *N_i* is the number of tobaccos with a severity of *i*. The disease incidence was calculated at 15 dpi using the following formula:

$$\text{Disease incidence} = \left(\text{no. of tobacco plants diseased} / \text{total no.} \right) \times 100\%$$

Chloroplast ultrastructure under *B. cinerea* infection.

Leaf samples (of whole-plant inoculations) were collected close to infected sites, from green parts at the edge of necrotic lesions (cut into 2 to 3 mm). All samples were taken at 15 dpi. Samples were pinned onto 2.5% (vol/vol) glutaraldehyde and placed in a vacuum for 3 h. The prefixed samples were washed three times with 0.1 M of phosphate-buffered saline (PBS; pH 6.8) for 15 min each time. Next, the samples were briefly postfixed in 1% osmium tetroxide, then rinsed again in 0.1 M of PBS (pH 6.8) three times for 15 min each time. These specimens were dehydrated with 50, 70, 90, and 100% ethanol and soaked in a mixture of ethanol and acetone (1:1)

for 10 min. The above steps were carried out at 4°C. Then the samples were dehydrated with 100% acetone at room temperature, fixed in 100% resin overnight, and embedded in Spurr's medium. Thin sections (50 to 60 nm) were cut using an LKB-V ultramicrotome. Sections were dyed with lead citrate and uranium acetate, and each sample was observed under a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan).

Measurements of physiological parameters and secondary messengers. Transgenic and WT tobacco plants were sprayed with a *B. cinerea* suspension, as described above. Their third mature leaves were collected at 0, 0.5, 3, 6, 12, 24, 48, 72, and 96 h postinoculation (hpi) and cleaned with sterile water. Defense-related enzyme (CAT, SOD, POD, PAL) activity and secondary messengers (NO, O₂⁻, H₂O₂) were measured using a commercial chemical assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). ATP content and cpATPase enzyme activity were measured with a commercial chemical assay kit (Jiangsu Keming Biotechnology Institute, Suzhou, China) per the manufacturer's manual.

Chlorophyll was extracted from leaves following Arnon (1949), with minor modifications. Leaf disks (each 0.04 g) were homogenized in 0.6 ml of 95% acetone on ice and the ensuing homogenate was then centrifuged at 3,000 × *g* for 10 min. The supernatant was retained, to which 95% ethanol was added for a total volume of 5 ml. Absorbance was recorded at 663, 645, and 470 nm.

Flux measurement of net Ca²⁺ and H⁺. Transgenic and WT tobacco plants were infected with a *B. cinerea* suspension as described above. The third mature leaves from 1-month-old seedlings of tobacco individuals were used for net Ca²⁺ and H⁺ flux measurement, performed with noninvasive microtest technology (NMT) (NMT100 Series, YoungerUSA LLC, Amherst, MA; Xuyue Sci. & Tech. Co., Ltd., Beijing, China) at 0, 0.5, and 3 hpi, with four biological replicates per time point. Net fluxes of H⁺ and Ca²⁺ were measured by moving microelectrodes repeatedly between two positions (5 and 35 µm) adjacent to the exposed tobacco leaf mesophyll cells and calculated by applying Fick's law of diffusion (Liu et al. 2017).

RNA sequencing and analysis. Transgenic and WT tobacco plants infected with *B. cinerea* suspension spraying as described above, with three biological replicates at each time point, were used for RNA sequencing (RNA-seq) and its subsequent analysis. The third mature leaves from 1-month-old tobacco seedlings were sampled at 0.5 and 3 hpi for total RNA extraction, from which 1.5 µg of RNA per sample was used to construct the RNA libraries. Deep sequencing was performed on an Illumina Hi-Seq 2500 platform at Biomarker Technologies Corporation (Beijing, China). After any adaptor sequences were removed and the low-quality sequences were filtered out, the remaining clean reads from each sample were mapped onto the tobacco genome Ntab-K326 (<https://solgenomics.net>) by using the TopHat2 tool and its default parameters (Kim et al. 2013). The fragments per kilobase of transcript per million mapped reads method was selected to calculate the normalized expression level data of each library. Differentially expressed genes (DEGs) were identified by the package DESeq2 as those with a fold change ≥ 2 and a false discovery rate (FDR) ≤ 0.05. Gene Ontology (GO) enrichment was implemented by the Goseq R package (Li et al. 2018), whereas the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to analyze pathway enrichment (Wei et al. 2019). All analyses were completed on the BMK Cloud (<http://www.biocloud.net>; Biomarker Technologies Corporation).

Analysis of broad-spectrum resistance. Seed germination rates of WT and transgenic tobacco plants under different concentrations of salt stress (0, 150, and 250 mM of NaCl) were counted at 0 to 15 days posttreatment. Likewise, growth assays of seedlings were compared between WT and transgenic lines under the same salinity treatments (0, 150, and 250 mM of NaCl). For

this, seeds were sown on MS medium for 7 days, after which the ensuing seedlings were moved to MS medium containing different concentrations of NaCl for 7 days. The 4-week-old seedlings were then moved into one-half-strength Hoagland culture medium differing in NaCl concentration (see above) for the growth assays.

Finally, for the leaf mold resistance analysis, 1-month-old seedlings of transgenic and WT tobacco were infected with *Cladosporium fulvum* (provided by the Northeast Agricultural University Tomato Research Institute) through suspension (10^7 spores/ml) spraying and the phenotype was recorded at 15 dpi (Xue et al. 2017). This was performed as described for the whole-plant *B. cinerea* inoculation assay above.

Statistical analyses. All data are presented as the mean $\pm$ standard deviation. Statistical analysis was carried out with one-way analysis of variance (ANOVA) in SPSS software (SPSS, Chicago, IL). Separations were performed using Duncan's multiple range tests. Differences at $P < 0.05$ were considered statistically significant and are represented by different letters. All experiments were repeated three times.

RESULTS

***atpA* cloning and sequence analysis.** A 1,524-bp full-length cDNA of *atpA*, which encoded a peptide of 507 amino acids, was successfully amplified. There was one single nucleotide mutation (T674→C674) compared with the chloroplast genomic sequence; this mutation was a single amino acid change (Val 225→Ala 225). The amplified sequence was deposited in GenBank under accession number BankIt2317821 *atpA* MT134327.

Overexpression of *atpA* enhances plant resistance to *B. cinerea*. To further analyze the function of *atpA* in response to

B. cinerea, the full-length *atpA* sequence was cloned into the plant expression vector pCAMh+p driven by the 35S-CaMV promoter and transformed into tobacco. These 31 lines were screened out by kanamycin-selection medium, PCR, and qRT-PCR assays to obtain the T₀ generation of transgenic plants in total. Three *atpA* overexpression lines (OE17, OE21, and OE30) with higher expression levels of *atpA* were selected for further analysis (Supplementary Fig. S2).

The *B. cinerea*-treated WT tobacco plants showed severe symptoms, in the form of pronounced lesion development, which included obvious watery circular lesions with a yellowish and obscure boundary, at 7 dpi. No obvious symptoms or smaller range of browning around the leaf wound sites were observed in the transgenic plants (Fig. 1A). The WT leaves showed yellowing and appeared withered at 30 dpi. Necrotic lesions were smaller in the leaves of OE17, OE21, and OE30 than in those of WT (Fig. 1B), suggesting that the spread of necrotic lesions in OE17, OE21, and OE30 was controlled by *atpA* overexpression.

In the whole-plant inoculation assay, gray mold disease symptoms were significantly lower in all *atpA*-overexpressing tobacco plants compared with the WT. The disease incidence was 80, 18.33, 20, and 23.33% in WT, OE17, OE21, and OE30 tobacco, respectively (Fig. 1C), with corresponding disease index values of 54.17, 5, 6.25, and 7.5% (Fig. 1D). Overexpression of *atpA* significantly reduced both the disease index and the disease incidence in tobacco plants infected with *B. cinerea*.

Overexpression of *atpA* stabilized chloroplast structure under *B. cinerea* infection. To clarify whether the ultrastructure of chloroplasts is affected by overexpression of *atpA* under *B. cinerea* inoculation, infected leaves from the WT and transgenic tobacco seedlings were examined under an electron microscope for their green tissues around the necrotic lesions at infected sites. In the

Fig. 1. Necrotic lesion area in leaves at **A**, 7 days postinoculation (dpi) and **B**, 30 dpi and **C**, disease incidence and **D**, disease index for gray mold disease after inoculations with *Botrytis cinerea* in wild-type (WT) and transgenic lines (OE17, OE21, and OE30) of tobacco plants. Error bars represent the standard deviation for three independent replicates. Significant differences between the transgenic lines and WT were analyzed by analysis of variance. Different letters indicate significant differences compared with the WT at $P < 0.05$.

absence of *B. cinerea* infection, the chloroplasts appeared elongated and regularly located along the cell wall. However, the chloroplasts were evidently swollen and more seriously damaged in the WT than transgenic tobacco plants at 15 dpi. In the WT samples, chloroplasts were approximately circular in shape, with the grana lamellae and stroma lamellae of thylakoid distributed loosely, with less lamella and disintegration in the chloroplasts. In addition, large osmiophilic plastoglobules had accumulated and membrane systems were degraded. These results indicated that *atpA* overexpression stabilized the chloroplast structure under *B. cinerea* infection (Fig. 2).

Effect of infection on plant physiological parameters. To assess the potential effect of *atpA* overexpression on *B. cinerea* resistance, tobacco plant chlorophyll content, ROS levels, signal molecules, and enzyme activity were analyzed in leaves of the WT and OE17, OE21, and OE30. Chlorophyll *a* contents were higher in OE17, OE21, and OE30 than in the WT during 12 to 48 hpi. Chlorophyll *b* content was ascendant and higher in the WT than in OE17, OE21, or OE30 within 6 hpi. Yet chlorophyll (*b*) content increased rapidly in transgenic plants at 12 hpi and peaked at 48 hpi, at which time it surpassed that in the WT (Supplementary Fig. S3; Supplementary Table S2).

CAT activity increased before 3 hpi in all tobacco plants, but especially in the transgenic lines. At 12 hpi, CAT activity in OE17, OE21, and OE30 exceeded that in the WT (Fig. 3A). SOD activity decreased in all tobacco plants at 0.5 hpi, but then exhibited a sharp increase at 3 hpi. A slight decrease was observed in transgenic lines at 12 hpi, followed by an increase to a level higher than that in the WT at 24, 48, and 96 hpi (Fig. 3B). Likewise, in all tobacco, POD activity first decreased but then increased by 96 hpi. In the transgenic lines, POD activity began to increase at 72 hpi and reached maximum values at 96 hpi to levels 2.54-, 3.51-, and 2.54-fold higher than in the WT (Fig. 3C). PAL activity increased at 3 hpi in all three transgenic lines to levels slightly higher than that in the WT; the latter's PAL activity declined to a greater extent at 12 hpi, whereas PAL activity was higher in the transgenic lines, and significantly so at 6 and 96 hpi (Fig. 3D; Supplementary Table S3).

F1F0-ATPase activity was higher in transgenic lines during the whole-plant infection period than in the WT. Compared with the WT, early activity of F1F0-ATPase in the transgenic lines increased considerably over time yet it decreased markedly at 12 hpi, although it was still higher (Fig. 3E). F1F0-ATPase activity of the transgenic lines reached its maximum value at 3 hpi to levels 1.93-, 1.82-, and 1.57-fold higher than the WT. Significantly increased ATP content was observed at 0.5 hpi, which peaked at 3 hpi to levels 3.70- to 4.41-fold greater than in the WT. ATP content began to decline at 6 hpi in the WT, becoming 8.00- to 12.00-fold lower than that in the transgenic lines. ATP content decreased in transgenic lines at 12 hpi and began to increase at 24 hpi, so that it exceeded that of the WT as time elapsed (Fig. 3F).

H₂O₂ content in the transgenic lines began to increase at 0.5 hpi and peaked at 3 hpi, whereas it declined in the WT during the postinoculation period. The greatest difference in H₂O₂ content between the transgenic lines and the WT was observed at 3 hpi when the transgenic lines/WT ratio of 3.35 to 4.37:1 was maximal. H₂O₂ content increased in the WT thereafter at 6 and 12 hpi but remained lower than that in the transgenic tobacco (Supplementary Fig. S4A; Supplementary Table S4). The NO content of WT decreased sharply at 0.5 hpi to a level significantly lower than that of the transgenic lines. At 12 hpi, however, the NO level increased markedly in transgenic lines and was maintained at relatively high levels until it sharply declined at 24 and 48 hpi. The NO level increased rapidly again in transgenic lines at 72 hpi, whereas the NO level in the WT was still strongly inhibited (Supplementary Fig. S4B). O₂ content increased steeply in transgenic plants at 48 hpi, attaining levels 2.5- to 3.21-fold higher than in the WT. Furthermore, after 48 hpi, O₂ content in OE17, OE21, and OE30 lines continued to exceed that in the WT (Supplementary Fig. S4C).

Effects on Ca²⁺ and H⁺ flux in cells of the mesophyll. NMT was used to investigate the dynamic change of Ca²⁺ and H⁺ flux in mesophyll cells in living tobacco leaves (third from the top of the plant). Ca²⁺ was characterized by slight efflux under normal conditions, with no sharp distinction between WT and transgenic tobacco plants. A faster decline in Ca²⁺ efflux in transgenic tobacco was observed compared with the WT at 0.5 hpi,

Fig. 2. Transmission electron micrographs of chloroplast in the mesophyll cells of wild-type (WT) and transgenic tobacco plants infected with *Botrytis cinerea*. Magnification ×30,000. CW = cell wall, PM = plasma membrane, Chl = chloroplast, and OS = osmiophilic granule.

whereas total Ca^{2+} efflux was significantly greater in the transgenic lines of tobacco than in the WT at 3 hpi (Fig. 4A, B, and C). For H^+ efflux, it was slightly higher in the WT than in transgenic tobacco under normal growing conditions. After *B. cinerea* infection, the content of H^+ efflux in transgenic plants slightly increased at 0.5 hpi, yet no significant differences were detected among the tobacco plants. Evidently, H^+ efflux in transgenic lines remained stable throughout the *B. cinerea* treatment period, whereas a decreasing trend was noticeable in the WT at 3 hpi (Fig. 4D, E, and F).

Transcriptome sequencing and correlation analysis. Given the physiological characteristics of WT and transgenic tobacco plants under *B. cinerea* infection, transcription level changes in tobacco were estimated. The raw sequence data reported in this study were deposited in the Genome Sequence Archive at the Beijing Institute of Genomics of the Chinese Academy of Sciences, under accession number CRA002377 (publicly available at <https://bigd.big.ac.cn/gsa>).

The bar charts in Figure 5 convey the number of up- and downregulated DEGs found between *atpA* overexpression lines and the WT at different time points after *B. cinerea* infection. Far more downregulated and upregulated DEGs were detected at 0.5 hpi (3,961 and 3,463, respectively) than at 0 hpi (2,084 and 1,441, respectively). The number of upregulated DEGs at 3 hpi decreased

to 1,612, whereas the downregulated DEGs decreased to 2,408 (Fig. 5A). Two-way ANOVAs were used for robust statistical analysis of the RNA-seq data for the factors of genotype (WT versus *atpA* overexpression) and treatment (control versus *B. cinerea* inoculation). Overall, 5,421 genes were significantly affected by *B. cinerea* inoculation (having significant differential expression between *B. cinerea*-inoculated and inoculated WT plants; $\text{FDR} \leq 0.05$); 3,530 were significantly affected by *atpA* overexpression, with 9,855 genes found significantly influenced by the interaction between treatment and genotype (i.e., had significant differential expression between the *B. cinerea*-inoculated WT and *B. cinerea*-inoculated *atpA*-overexpressing plants; $\text{FDR} \leq 0.05$; Fig. 5B). Hierarchical clustering was then carried out to discern the differences and similarities among the *B. cinerea*-responsive DEGs at various time points in the WT and *atpA* overexpression tobacco, in a visualized way (Supplementary Fig. S5). The gene expression profiles between the WT and transgenic lines differed significantly among the time points during *B. cinerea* infection.

All unigenes were assessed in the GO database. The results indicated that most transcripts were enriched in the cellular process (GO: 0009987), metabolic process (GO: 0008152), single-organism process (GO: 0044699), response to a stimulus (GO: 0050896) within the biological process category, cell part (GO: 0044464), cell (GO: 0005623), organelle (GO: 0043226) within the

Fig. 3. Effects of *Botrytis cinerea* on the activity of **A**, catalase (CAT), **B**, superoxide dismutase (SOD), **C**, peroxidase (POD), **D**, phenylalanine ammonia lyase (PAL), and **E**, F1F0-ATPase and **F**, the ATP content of wild-type (WT) and transgenic lines (OE17, OE21, and OE30) of tobacco. Error bars represent the standard deviation for three independent replicates. Significant differences between the transgenic lines and WT were analyzed by analysis of variance. Different letters indicate significant differences compared with the WT at $P < 0.05$.

cellular component category, and binding (GO: 0005488) and catalytic activity (GO: 0003824) within the molecular function category (Fig. 6A and B; Supplementary Table S5). More functional terms were found for biological process, however.

We carried out the KEGG annotations separately at 0, 0.5, and 3 hpi (Fig. 6C, D, and E; Supplementary Tables S6, S7, and S8). At

0.5 hpi, the top 20 KEGG enrichments clearly indicated that the DEGs were enriched in the following: circadian rhythm-plant (ko04712), pentose phosphate pathway (ko00030), terpenoid backbone biosynthesis (ko00900), carbon metabolism (ko01200), glutathione metabolism (ko00480), glyoxylate and dicarboxylate metabolism (ko00630), carotenoid biosynthesis (ko00906),

Fig. 4. Transient net **A, B, and C**, Ca^{2+} and **D, E, and F**, H^{+} flux responses to *Botrytis cinerea* for various times in mesophyll cells of wild-type (WT) and transgenic lines (OE17 and OE30) of tobacco. In A and D, the tobacco plants were treated with buffer (mock-inoculated treatment). In B and E, they were treated with *B. cinerea* for 0.5 h postinoculation (hpi). In C and F, they were treated with *B. cinerea* for 3 hpi. Steady-state flux profiles of Ca^{2+} and H^{+} were examined by continuous flux recordings (about 10 min). Positive values represent the Ca^{2+} , H^{+} efflux.

glycolysis/gluconeogenesis (ko00010), photosynthesis (ko00195), and porphyrin and chlorophyll metabolism (ko00860) (Fig. 6D; Supplementary Table S4). Later, at 3 hpi, the DEGs were highlighted in protein processing in the endoplasmic reticulum (ko04141), plant hormone signal transduction (ko04075), plant–pathogen interaction (ko04626), circadian rhythm (ko04712), and inositol phosphate metabolism (ko00562) (Fig. 6E; Supplementary Table S5). These results suggested that plants have evolved a range of defense mechanisms that predominate at different stages of fungal infection, and that infection by *B. cinerea* can lead to changes in many metabolic pathways mediated by *atpA*.

Analysis of broad-spectrum resistance/tolerance. To estimate whether overexpression of *atpA* in plants confers salt tolerance in them, both WT and transgenic tobacco were treated with differing concentrations of NaCl. The transgenic lines exhibited a significantly better seed germination rate and seedling growth under salt stress than did the WT (Supplementary Fig. S6). In addition, the WT and transgenic tobacco plants were sprayed with a spore suspension to further investigate the function of *atpA* in response to *Cladosporium fulvum*; in this experiment, the transgenic tobacco showed increased resistance to *Cladosporium fulvum* (Supplementary Fig. S7).

DISCUSSION

AtpA, encoded by *atpA*, is a key protein for energy metabolism and multiple cell processes and was identified as specifically enriched in tomato when induced by *Clonostachys rosea* under *B. cinerea* infection. Hence, *atpA* may play a key role in tomato defense that is primed by *Clonostachys rosea*. In this study, *atpA* was cloned and validated for its function of resistance toward *B. cinerea* infection by heterologous expression of *atpA* in *Nicotiana tabacum*. The isolated *atpA* gene has one single base that differs from the published sequence.

We found that expression of *atpA* greatly suppressed the gray mold disease index and disease incidence compared with the WT. That the visible lesions were significantly reduced in transgenic tobacco is a good morphologic indicator of the capability of *atpA* expression to foster plant resistance to *B. cinerea*. Moreover, our results indicated that characteristic HR-like necrotic lesions at the site of infiltration were evident and the lesions did not develop

beyond the initial invaded area in *atpA*-overexpressing plants at 30 dpi. These results confirmed that *atpA* overexpression reinforces plant resistance to gray mold disease by inhibiting the ability of *B. cinerea* to utilize dead tissue to facilitate its further colonization of hosts. In short, *atpA* overexpression can prevent the fungus from disrupting the host's defense mechanism, an action key to its pathogenicity. Accordingly, corresponding effects of *atpA* overexpression on photosynthetic parameters, antioxidant systems, and gene expression under *B. cinerea* inoculation were demonstrated at the structural, physiological, and molecular levels.

In a previous study, infection by the stripe rust pathogen altered the thylakoid structure, therefore resulting in a reduction of photosystem II (PSII) activity in susceptible wheat plants (Chen et al. 2015). Other work found that *B. cinerea* was blocked in the area of completely destroyed *Mesembryanthemum crystallinum* cells, and no hyphae were found in the vicinity of leaf lesions (Gabara et al. 2012). In our study, *B. cinerea* infection destroyed the structure of chloroplasts, thereby likely lowering PSII activity in the WT. Excessive damage to the structure of plant chloroplasts significantly weakened their photosynthesis and this reduced the available supply of ATP. However, the chloroplast ultrastructure in the cells surrounding the necrotic lesions could be maintained with overexpression of *atpA* to confer resistance by tightly inhibiting the HR-like lesions and the pathogen-induced senescence of leaf tissue.

Furthermore, *atpA*-overexpressing transgenic tobacco plants had higher cpATPase activity and greater ATP content during the early stage of *B. cinerea* infection. Kanazawa et al. (2017) confirmed a decisive role for ATP synthase regulation in maintaining photosynthetic control of electron transfer to prevent photodamage via modulation of the thylakoid proton motive force, whereas Takagi et al. (2017) showed that cpATPase regulates H⁺ efflux activity in response to such changed drivers of photosynthesis. Consistent with those findings, we found that H⁺ efflux was stabilized at a relatively high level at 3 hpi, a result consistent with the high cpATPase levels we observed in transgenic tobacco. It is conceivable that *atpA* plays a crucial role in stabilizing chloroplast ultrastructure and H⁺ efflux and also enhances cpATPase activity, which together sustain sufficient photosynthesis to produce adequate ATP to cope with the *B. cinerea* infection. Buchert et al. (2012) confirmed that a change in ATP synthase activity is able to minimize ROS-promoted photoinhibition. ATP is vital for ensuring resistance to *B. cinerea*.

Fig. 5. Differentially expressed genes (DEGs) in wild-type (WT) and chloroplast ATP synthase CF1 α subunit (*atpA*)-overexpressing (OE) tobacco plants under *Botrytis cinerea* inoculation. **A**, Number of up- and downregulated DEGs at 0, 0.5, and 3 h postinoculation after exposure to *B. cinerea*. **B**, Statistical analysis of the effect on gene expression from the genotype (WT versus *atpA* overexpression) and treatment (control versus *B. cinerea*) factors, by two-way analysis of variance of the RNA sequencing data. The numbers of genes showing a significant change (false discovered rate-adjusted $P < 0.05$) are displayed in the Venn diagram.

Fig. 6. A and B, Comparative Gene Ontology enrichment analysis and **C, D, and E**, Kyoto Encyclopedia of Genes and Genomes pathway analysis at 0, 0.5, and 3 days postinoculation (dpi) of the differentially expressed genes (DEGs). The x-axis indicates the enrichment factor on a scale from 0 to 2.4. Dot color and size indicate the Q value and gene number, respectively, as shown on the keys to the right of the lower panels. OE = overexpressing and WT = wild type.

Fig. 7. The proposed mechanism of plant defense response as mediated by the chloroplast ATP synthase CF1 α subunit (AtpA). Arrows indicate positive regulation. PRR = pattern recognition receptor, ROS = reactive oxygen species, HR = hypersensitive response, SOD = superoxide dismutase, POD = peroxidase, CAT = catalase, and PAL = phenylalanine ammonia lyase.

Moreover, ATP has been reported to function as a signal that can modulate various metabolic functions (Hao et al. 2012); in addition, ATP may be recognized by P2K1, a plasma membrane-localized purinergic receptor, which can elevate cytosolic free Ca^{2+} concentrations (Tanaka et al. 2014), leading to the production of NO or ROS (Demidchik et al. 2009; Song et al. 2006). In our study, both NO content and H_2O_2 content were increased by *atpA* overexpression under *B. cinerea* infection. Higher H_2O_2 content can protect cells of leaf veins from fungal invasion (Gabara et al. 2012), whereas NO is critically involved in fungal physiology and development and fungal plant infection (Zhao et al. 2020). Furthermore, NO accumulation figures prominently in β -amino-butyric acid-induced resistance against *B. cinerea* in tomato plants (Li et al. 2020).

H_2O_2 , NO, and Ca^{2+} are significant signaling molecules in plants that trigger multiple cellular responses that induce the expression of defense-related genes under *B. cinerea* infection. We found that ROS (i.e., O_2^- and H_2O_2) and Ca^{2+} generation increased rapidly in transgenic plants treated with *B. cinerea*, a response that could have fostered the defense-associated HR within a short time. Consistent with our results, an earlier study showed that Ca^{2+} efflux was an essential factor causing the HR of *N. benthamiana* to *Pseudomonas syringae* (Nemchinov et al. 2008).

Defense enzymes are related to the biosynthesis of signaling molecules and are important to plant disease resistance. We observed that overexpressing *atpA* could tightly control the HR-like lesion, inhibiting the formation of gray mold, and it significantly enhanced CAT, SOD, POD, and PAL enzyme activity in host tobacco leaves. This result suggests that *atpA* overexpression enables plants to avoid oxidative damages more effectively than can WT counterparts.

The coregulation of carbohydrate metabolism and photosynthesis with defense mechanisms is a general plant response to an invading pathogen (Gabara et al. 2012). Fungal pathogen infection is closely correlated with changes in metabolic pathways in host plant tissues such as photosynthesis. The leaf organ, as a “source” tissue, functions to synthesize energy-rich molecules via photosynthesis for the transport of carbon (Xue et al. 2018). It is noteworthy that *atpA* overexpression promoted the expression of genes related to carbon metabolism, photosynthesis, the pentose phosphate pathway, and both porphyrin and chlorophyll metabolism, all of which belong to sucrose synthesis at 0.5 hpi; at 3 hpi, the DEGs were involved in protein processing, hormone signal transduction, plant–pathogen interaction, and metabolism. These results suggest that *atpA* can regulate the genes involved in those pathways central for synthesizing more energy and sources early in the fungal infection process (at 0.5 hpi), after which it regulates the genes related to disease resistance reactions and signal transduction (at 3 hpi). We hypothesize that the resistance response mediated by AtpA is rapid and strong during the initial stage of infection, a critical stage for *B. cinerea* development. Other research has claimed that the cells surrounding an invaded area of leaf are maintained by a continuing supply of the tricarboxylic acid (TCA) cycle, conferring resistance by both tightly controlling the defense-associated HR and slowing pathogen-induced senescence (Seifi et al. 2013). In addition, C:N metabolism in the plant host plays an important role in its resistance response against necrotrophic pathogens (Seifi et al. 2013). Consistent with this observation, we found that those genes involved in the TCA cycle and carbon metabolism were substantially upregulated in transgenic tobacco plants at 0.5 hpi.

Thus far, most studies have focused on pathogen-resistant genes or proteins. Moreover, few researchers have examined the effects of pathogenic infection on proteins related to vital photosynthetic parameters (Chen et al. 2015; Yang et al. 2016). This study is the first to find that AtpA may have critical biological functions in *B. cinerea*, in *Cladosporium fulvum*, and even in how plants may respond to NaCl stress. The results are generally consistent with a

previous study claiming that overabundant AtpA may increase the synthesis of ATP to satisfy greater requirements of energy for persistent salt resistance (Asrar et al. 2017). Our study further supports two assertions about the process conferring plant resistance: it is complex, and one likely regulated by the interaction of multiple pathways (Fig. 7) (Gong et al. 2017; Mouekouba et al. 2014; Wang et al. 2019). Taken together, our results lead us to presume that both PTI and ETI are involved in AtpA-mediated resistance to *B. cinerea*, a view requiring more analysis in our future work. This study nonetheless provides a starting point to clarify the molecular mechanism of broad-spectrum resistance of AtpA. Whether the *atpA* gene is generalizable to other species and field settings merits careful evaluations in the near future. This study has taken valuable steps toward elucidating the complex, defense mechanism responses to biotic and salt stress in plants.

Conclusion. Our work revealed that overexpression of *atpA* positively regulates plant resistance to *B. cinerea*, as inferred by a balanced ROS, reduced oxidative damage, and increased photosynthesis efficiency compared with WT tobacco plants. Overexpression of the critical *atpA* gene could modulate greater expression of genes related to plant–pathogen interaction, as well as photosynthetic-related and metabolism genes, during the early stages of *B. cinerea* infection. In addition, *atpA* overexpression simultaneously improved both salt-stress tolerance and resistance of plants to *Cladosporium fulvum*, suggesting that it is capable of inducing broad-spectrum defense.

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