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Induction of systemic resistance against tobacco mosaic virus by Ningnanmycin in tobacco

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ABSTRACT

Ningnanmycin (NNM) is an antiviral agent firstly isolated from *Strepcomces noursei var·xichangensisn*. Studies have shown that NNM promotes PAL, POD and SOD activity and possesses antiviral activity against tobacco mosaic virus (TMV). In this study, our results demonstrated that NNM inhibited the polymerization process of TMV coat protein (TMV-CP) *in vitro* and promoted the systemic accumulation of pathogenesis-related proteins (PRs), which are the markers of systemic acquired resistance (SAR). An non-expressor, pathogenesis-related genes 1 (NPR1) that regulates SAR and induces systemic resistance (ISR), increased. In addition, the Jaz3 expression increase showed that NNM also induced ISR. Based on the results of this work and earlier reports, it is suggesting that NNM induces tobacco systemic resistance against TMV via activating multiple plant defense signaling pathways.

1. Introduction

Tobacco mosaic virus (TMV) is one of the most well-studied plant viruses and often considered as plant cancer. TMV infects more than 400 plant species and seriously threats the quality and yield of tobacco, which has been a challenging problem to control in the agricultural sector.

Previous reports demonstrate that the inhibition of photosynthetic activity results from the association of viral CP with chloroplast membranes or the photosystem II (PS II) complex [1,2] and inhibition of photosynthetic electron transport through PS II [3]. Similarly, the α and β subunits of the ATP synthase and the 33-kDa protein of the oxygen evolving complex were reduced in the TMV-infected leaves [4]. In order to combat TMV, extensive research work had been done and a lot of methods were presented [5–7]. The application of traditional pesticides with high residue level and negative impact on the environment has not proved effective to enhance productivity. Natural products from microorganism have been proved to be a rich resource [8–10].

Ningnanmycin (NNM) is cytosine nucleoside type antibiotic and isolated from fermentation broth of *Strepcomces noursei var·xichangensisn*. It is a pollution-free microbial pesticide characterized by broad spectrum, high-efficiency, low toxicity and less-persistent and enhances resistance in host plants by destruction of CP [11]. It have also been shown that NNM generally exhibits antimicrobial activity against TMV [12–14] and promotes PAL, POD and SOD activity which results in the markedly enhancement of plant resistance against TMV [15]. However, the antiviral activity of NNM and the underlying mechanisms in plant resistance are not well understood. Thus, the present study is to investigate the mechanism of NNM against TMV and discuss its possible role as an inducer of acidic PR proteins, SAR and ISR in the defense of plants against pathogen infection.

2. Materials and methods

2.1. Plant material, TMV-inoculation and NNM treatment

Tobacco (*Nicotiana tobacum* L.) plants were allowed to grow in the greenhouse under a 16 h photoperiod (100 μE m^{-2} s^{-1} of photosynthetically active radiation) at 22 ± 1 °C. Plants were watered on alternate days and once a week they were supplied with half-strength Hoagland's nutrient solution (2 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, and many trace elements, pH 7.0). When a tobacco plant grew to the five- to six-leaf stage, they were divided into nine groups. Each group consisted of three replications. The inoculum was prepared from infected top leaves, ground in a mortar containing 0.1 M phosphate buffer pH 7.2 and carborundum. The inoculum was rubbed onto the leaves of

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tobacco. TMV strain PV230 was used as virus materials. The leaves were harvested after 10 days post inoculation for further studies when the wounding effects were negligible.

2.2. Determination of the pigment content

Briefly, 1 g of sample leaf was ground in 20 ml of 80% acetone, centrifuged at 5000 rpm. The residue was repeatedly ground in acetone till to become colorless and the supernatant was collected. The pigment content was calculated by reading the absorbance at 663, 645, and 470 nm.

2.3. Total soluble protein extraction

Total soluble tobacco proteins were isolated by grinding equal weight leaf tissue in liquid nitrogen, which was extracted by using 2× Laemmli buffer. An equal volume (20 μl) of the protein extract (qualitative analysis) of total soluble protein was loaded and the protein was separated by SDS–PAGE using 15% PAGE in the separation gel.

2.4. Preparation of TMV-CP and effect of NNM on TMV-CP in vitro polymerization

Gooding method was used for the purification of the TMV [16] and the CP was purified by the acetic acid method [17]. Effect of NNM on TMV-CP *in vitro* polymerization was operated according to the report method [15], whereas the buffer was 0.05 M potassium phosphate buffer (pH 8) for the TMV-CP and the concentration of the CP was 0.2 mg/ml. Native PAGE was carried out on 15% (w/v) PAGE in the separation gel under non-denaturating condition.

2.5. Acid PRs extraction

Acid PRs were prepared as described [18]. The leaves were homogenized in the ice-cold acid buffer (71 mM citric acid, 15 mM K_3PO_4, 33 mM β-mercaptoethanol, 2 mM ascorbic acid, pH 2.8). The homogenate was centrifuged at 12,000×*g* at 4 °C for 20 min. The supernatant was dialyzed with the buffer (10 mM Tris–HCl, pH 8.3). Native PAGE was carried out on 15% (w/v) PAGE in the separation gel under non-denaturating condition. After electrophoresis, protein was visualized by Coomassie blue staining for protein patterns analysis, and then photographed. Protein concentration was determined using the Bradford method [19].

2.6. Measurement of β-1,3-glucanase enzyme activities

β-1,3-glucanase activity (EC 3.2.1.39) was determined according to the method described [20]. The assay mixture included 10 ml of enzyme solution, 2.5 ml of laminarin (10 mg/ml) and 37.5 ml of 50 mM sodium acetate buffer (pH 5.0). After incubation at 37 °C for 1 h, 0.15 ml of 3,5-dinitrosalicylic acid was added. The reaction was stopped by heating in boiling water for 5 min. The absorbance was immediately measured at 550 nm. The reducing sugar was calculated from a standard curve obtained from a known concentration of glucose. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 mM of glucose per hour.

2.7. Semi-quantitative RT–PCR analysis

Total RNA was isolated from leaves using plant RNA Kit (OMGEA bio-tek, USA) according to the manufacturer's instructions. A cDNA copy was generated from 1 μg of total RNA using oligo $d_{(T)}18$ primer and M-MLV reverse transcriptase following the standard protocol (TaKaRa Bio Inc., China).The cDNA was amplified by PCR using the following primers:

NtNPR1, forward 5′-GATGTGTGTGTTTGTGTGGACAACGAGT-3′ and reverse 5′ CCATCGGATGTCAGATCAGAAGGTCTAG 3′;

NtJAZ3, forward 5′-ATGGCATCATCGGAGATTGTGG-3′ and reverse 5′-CTAGAATTGCTCAGCTTTCACTGG-3′; actin (tac9), forward 5′-GATGGTGTCAGCCACACTGTC-3′ and reverse 5'-ATGCTGCTAGGAGCCAGTGC-3'.

2.8. Statistical analysis

There were at least three independent replicates for each determination. The significance of differences was determined using analysis of variance (ANOVA) and *P*-value <0.05 was considered statistically significant. Data were expressed as mean values ± SD.

3. Results

3.1. Effect of NNM on the pigment content of tobacco

In order to determine whether NNM has resistance against TMV, we firstly measured the level of photosynthetic pigments. We found that NNM increased the content of pigments that was about 38% and 153% for chlorophyll (Chl) and carotenoids in group 4 compared with the infected group (group 3), respectively. Although all virus-infected plants treated with NNM showed the slight increase of the Chl content, the total carotenoids content in the group 9 increased as well as treated with NNM. This increase was up to 5.9-fold in case of carotenoid compared with infected control group, while the Chl content increased to 32% of the control (Table 1). Our results demonstrated that NNM could increase in Chl and carotenoids content in both the uninfected and infected plants.

3.2. Effect of NNM on the total soluble protein and CP of the tobacco

To further detect whether NNM has systemic protection against TMV and the effect on total soluble protein patterns and CP of TMV-infected and NNM-treatment plants, several leaf samples were collected from the infected plants and their leaves of NNM-treatment plants and then the total soluble protein and CP were analyzed by SDS–PAGE. As shown in Fig. 1, we observed that the CP was inhibited to 73% with NNM post-treatment (group 4) and to 84% with NNM pre-treatment (group 8), compared to the infection group. This data indicated that NNM might be an effective agent to reduce the CP content in both post-treatment and pre-treatment. The level of CP in other groups also decreased. The bands of Rubisco large subunit (Rubisco LSU) and Rubisco small subunit (Rubisco SSU) were confirmed by using antibody against tobacco Rubisco [21]. In the group 2, NNM increased the level of Rubisco LSU and SSU compared to the group 1. In addition, NNM increased the level of Rubisco LSU and SSU in the infected plant treated with NNM (Fig. 2).

3.3. Effect of NNM on TMV-CP in vitro polymerization

The absorbance of CP at 320 nm is directly proportional to the degree of polymerization with itself, and the degree of change of TMV-CP *in vitro* polymerization is also in positive proportion to the temperature according to the report [22]. As shown in Fig. 3, NNM had an effect on inhibiting the polymerization process of TMV-CP *in vitro*. With the increase in temperature, the inhibiting effect was more obvious (Fig. 3A). TMV-CP polymerization was also inhibited by native-PAGE in the presence of NNM (Fig. 3B).