Investigation on growth and development of 42 kDa chitinase transgenic peanuts (Arachis hypogaea L.) cultivar L14 under in vivo condition

Hoa Phung Thi Bich1,2, Tue Nguyen Hoang1, Phuong Hoang Lan1, Huy Nguyen Xuan1 and Loc Nguyen Hoang2*

1. University of Education, Hue University, 34 Le Loi St., Hue 530000, VIETNAM
2. University of Sciences, Hue University, 77 Nguyen Hue St., Hue 530000, VIETNAM
3. Department of Science, Technology and International Relations, Hue University, 03 Le Loi St., Hue 530000, VIETNAM

*nhloc@hueuni.edu.vn

Abstract
The purpose of this study was to assess the growth and development of chitinase transgenic peanuts against phytopathogenic fungi including lines WTA-2 containing the Chi42 gene, S1A-15 containing the syncodChi42-1 gene and S2A-12 containing the syncodChi42-2 gene. The present results show that the peanut lines containing two optimized genes derived from the Chi42 wild-type gene of Trichoderma asperellum SH16, S1A-15 (syncodChi42-1) and S2A-12 (syncodChi42-2) seemed to grow stronger and produce a higher number of mature pods, weight of 100 pods and weight of 100 seeds than line WAT-2 with the Chi42 gene and non-transgenic control.

Moreover, lines S1A-15 and S2A-12 also resulted in higher seed quality in terms of lipid and protein content than in comparison to the WTA-2 line and the control. These findings suggest that chitinase transgenic peanuts containing one of two synthetic genes in peanut roots could be a promising candidate for peanut production against phytopathogenic fungi.

Keywords: Arachis hypogaea, Chi42, 42 kDa chitinase, growth and development, transgenic peanut.

Introduction
Peanut (Arachis hypogaea L.) is one of the most important oil crops in the world and is widely cultivated in the tropical and subtropical regions.19 Peanuts are a rich source of oil (40-60%), protein (10-20%), carbohydrates, vitamins, minerals, antioxidants, monounsaturated fatty acids and a source of medicinally important compounds.3,14 In Vietnam, peanut is one of the most important oil seed crops, having a total area of 160 thousand ha, a yield of 2.5 metric tons per ha and a production of 400 thousand metric tons in 2021.34 However, peanut yields, particularly on farmers’ fields, are still low which is largely due to biotic and abiotic stresses and phytopathogenic fungi.

In recent years, root and stem rot caused by Sclerotium rolfsii have become a great threat to peanut production and are destructive worldwide soil-borne diseases. S. rolfsii mainly damages the stem base of peanut and causes the whole plant to wither and die resulting in a reduction of peanut production by 10-80%.8,35,36 Chitinases can enhance the plant’s defense system as they act on chitin, a major component of the cell wall of phytopathogenic fungi and render the fungi inactive without any negative impact on the plants. Along with strengthening plant defense mechanisms, chitinases also improve plant growth and yield.20

In a previous study, we successfully transferred genes encoding 42 kDa chitinase into peanut cultivar L14. Subsequent analysis showed that chitinase-carrying peanuts had strong antifungal activity against S. rolfsii under in vitro and in vivo conditions.16,33

In this work, we report some results of the growth, yield and quality characteristics of the chitinase transgenic peanut lines mentioned above, including S1A-15, S2A-12 and WTA-3, grown under in vivo conditions. These are the lines that exhibited the strongest expression of 42 kDa chitinase and the highest antifungal activity in our previous work.

Material and Methods
Plant materials: Chitinase transgenic peanuts from previous work including lines S1A-15 (for syncod Chi42-1) and lines S2A-12, (for syncod Chi42-2) and WTA-2 (for Chi42) were used in this study (Fig. 1). Syncod Chi42-1 (NCBI accession number: MT083802) and syncod Chi42-2 (MT083803) are optimized sequences for plant expression of the wild-type Chi42 gene (HM191683) from Trichoderma asperellum SH16.22,26 All these three genes were regulated by the same Asy root-specific promoter and encoded the same 42 kDa chitinase.

In vitro transgenic peanuts and wild-type control (about 6.8 cm high with 8-10 roots of 4-8 cm long) were grown in 30 (D)×35 cm (H) plastic pots and placed in a net house with an ambient temperature of 25-27°C and 70% humidity. The planting soil contained a mix of alluvial soil and organic fertilizer (Fertilizer Growel 3-3-3, Behn Meyer Agricore Co. Ltd., Vietnam) in a ratio of 8:2. Watering was carried out every 2 days from growing to harvesting on day 145.

Characteristics of plant growth and development: Traits such as plant height, number of compound leaves, number of branches, numbers of flowers, the ratio of effective flowers, number of mature pods, the weight of 100 pods and the weight of 100 seeds at different growth and development stages during peanut cultivation were observed and measured as described by Grodzinzki and Grodzinzki.13
Figure 1: 42 kDa chitinase transgenic peanuts. (A) in vitro peanut, (B) peanut with 4 compound leaves grown in a pot, (C) flowering peanut

Quantification of chlorophyll: Chlorophyll content was determined by the spectrophotometry method according to Arnon.\(^1\) 10 mg of peanut leaves (3rd leaf from top) were ground in 0.5 mL of 100% acetone, the resulting extract was then added to 1 mL of 80% acetone. After centrifuging the extract at 12,000 rpm for 15 min at 4°C, the supernatant was recovered and diluted to 2 mL with 80% acetone. The absorbance of the diluted extract was measured at 645 and 663 nm and chlorophyll content was calculated using the following formulas:

\[
\text{Chl(a)} = (12.7 \times A_{663} - 2.69 \times A_{645}) \times \frac{V}{Fw} \\
\text{Chl(b)} = (22.9 \times A_{645} - 4.68 \times A_{663}) \times \frac{V}{Fw} \\
\text{Total chlorophyll: Chlo(a+b) = Chl(a) + Chl(b)}
\]

where V is volume of extract and Fw is fresh weight of leaves.

Quantification of lipid: Lipid from peanut seeds was extracted through a Soxhlet apparatus as described by Grassby et al.\(^12\) Two grams of dried peanut powder were extracted with 100 mL of petroleum ether overnight. The solvent was then removed using an evaporator rotary. The lipid residues in the flask were recovered after drying at 105°C for 2 h. Lipid content (%) was calculated as the following formula:\(^10\)

\[
\text{Lipid(%) = } \frac{a - b}{a} \times 100
\]

where a is the sample weight before extraction (g) and b is the sample weight after extraction (g).

Quantification of reducing sugar: Reducing sugars were measured according to the method of Miller\(^27\) using 3,5-dinitrosalicylic acid (DNS) reagent. One gram of dry peanut powder was dispersed in 10 mL of ddH\(_2\)O. The mixture was boiled for 10 min, then cooled and centrifuged at 10,000 rpm for 5 min at room temperature. The supernatant was recovered for reducing sugar determination according to the following steps: 0.1 mL of supernatant was added to 0.9 mL of ddH\(_2\)O, then mixed with 3 mL of DNS and boiled for 10 min. The absorbance of the reaction solution was determined at 550 nm and the reducing sugar content was calculated based on a standard curve of glucose.

Determination of total soluble protein content: Two grams of dried peanut seeds were ground in liquid nitrogen and dissolved in 10 mL of phosphate buffer (pH 7). The mixture was centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was then precipitated with 70% acetone. Centrifugation was carried out at 10,000 rpm for 5 min at 4°C and the precipitate was naturally dried, then dissolved in phosphate buffer. Total soluble protein content was determined according to Bradford’s method\(^5\) by measuring the absorbance of the protein extract on a spectrophotometer at 595 nm and calculating based on the standard curve of bovine serum albumin.

Statistical analysis: All experiments were performed in triplicate. One-way ANOVA (Duncan’s test) was performed with SPSS 20 software to evaluate the statistical significance of the differences between the groups at p value of 0.05.

Results
The results of this study showed that chitinase transgenic peanuts seemed to exhibit more substantial growth and development than the control, although fungal infection was not recorded in all investigated plants. Meanwhile, several similar studies on other transgenic crops found insignificant differences in the physiological and biochemical traits of transgenic individuals and wild-type controls.\(^23-25,39\)

Growth of transgenic peanuts: Observations showed that the transgenic peanut lines had higher plant heights than the control at all studied growth and development stages.
The line S2A-12 has the highest height among all, with values of 10.1 cm at five-leaf, 22.0 cm at flowering, 25.2 cm at the end of flowering and 33.0 cm at harvest. Meanwhile, the control was only around 7.2 cm, 14.2 cm, 17.9 cm and 24.1 cm high at the corresponding growth and development phases (Fig. 2).

Also, higher than the wild-type control were the number of branches and compound leaves per transgenic plant (p<0.05). In transgenic lines, the number of compound leaves peaked at the end of flowering stage with 17-18.4 leaves, while the control was only 15.6 leaves (Fig. 3). The number of branches went to the highest levels at 6.2-6.8 in three transgenic lines during the harvest stage, while the control was only 5.6 (Fig. 4). This study also showed that in most cases, the transgenic lines exhibited comparable growth capacity.

**Figure 2:** The height of chitinase transgenic peanut lines grown under *in vivo* conditions. Control: wild-type peanuts. It is time to evaluate the growth of peanuts: five-leaf on day 20, flowering on day 65, end of flowering on day 80 and harvest on day 145. Different letters represent statistically significant differences based on Duncan’s test (p<0.05)

**Figure 3:** Number of compound leaves of chitinase transgenic peanut lines grown under *in vivo* conditions. Control: wild-type peanuts. It is time to evaluate the growth of peanuts: start of flowering on day 60, flowering on day 65, end of flowering on day 80 and harvest on day 145. Different letters represent statistically significant differences based on Duncan’s test (p<0.05)
Chlorophyll content: Chlorophyll, one of factors that has a crucial role in photosynthesis, was investigated at five-leaf, flowering and pod-filling stages. Generally, a significant difference in chlorophyll content was observed between transgenic peanut lines and the control at all three studied stages. The transgenic peanut lines at the flowering stage, which is crucial for determining peanut yield, had the highest Chlo(a+b) content at 1.66-1.83 mg/g while the control was only 1.5 mg/g. The Chlo(a+b) content of two lines, S1A-15 and S2A-12, was similar (p>0.05) and different (p<0.015) from that of line WTA-2 (Fig. 5). The higher chlorophyll content of the transgenic lines at the investigated stages supported the results of their growth.

Peanut yield components: The peanut yield components of the transgenic lines and control are presented in Table 1. The line S2A-12 was found to have the highest number of flowers, ratio of effective flowers, number of mature pods, weight of 100 pods and weight of 100 seeds. Almost all yield components of chitinase transgenic peanuts were higher than those of wild-type controls (p<0.05). In general, the peanut line carrying the Syncod Chi42-2 gene (S2A-12) showed stronger development than the peanut lines carrying the other two chitinase genes, Chi42 (WTA-2) and Syncod Chi42-1 (S1A-15).

Major quality characteristics: Except for the reducing sugar, the protein and lipid content of the chitinase transgenic peanut seeds were higher than the control seeds (Table 2). The highest protein content (26.33 g/100 g) was found in the S2A-12 line, 1.02, 1.08 and 1.18 times higher than the S1A-15 line, WTA-2 line and control respectively. Line S2A-12 also displayed the highest lipid content of 49.15 (g/100 g) among transgenic peanut lines (Table 2). Reducing sugars are key components in the formation and development of peanut flavor. However, the reducing sugar contents of chitinase transgenic peanut lines were similar to control.

Control: wild-type peanuts. It is time to evaluate the growth of peanuts: flowering on day 65 and harvest on day 145. Different letters represent statistically significant differences based on Duncan’s test (p< 0.05).

Table 1

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>Number of flowers/plant</th>
<th>Ratio of effective flower (%)</th>
<th>Number of mature pods/plant</th>
<th>Weight of 100 pods (g)</th>
<th>Weight of 100 seeds (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTA-2</td>
<td>25.2&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>31.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>113.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.68&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S1A-15</td>
<td>26.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>115.52&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>36.94&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S2A-12</td>
<td>28.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>119.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.74&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>24.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>107.26&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.76&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Control: wild-type peanuts. Data are expressed as the mean of three replicates. Different letters in a column represent statistically significant differences based on Duncan’s test (p< 0.05).
In the peanut processing industries, seed chemical composition is a crucial criterion to determine technological properties and culinary qualities in order to define their use.\textsuperscript{30,32} Chitinase transgenic peanut seeds showed higher lipid and protein content than control which are two interesting parameters for peanut processing industries.\textsuperscript{2} This data further confirms that chitinase transgenic peanuts can be suitable for use as an additional source of disease resistance in peanut breeding programs without reducing yield and grain quality.

### Discussion

In contrast to our results on the growth and development of transgenic peanuts, Zeng et al\textsuperscript{19} did not find a significant difference in plant height when transferring two genes: \textit{McCHIT1} encoding chitinase and herbicide-resistant PAT, into rice. Overexpression of the chitinase gene \textit{CmCH1} in transgenic soybean also showed no adverse effects on plant growth and development.\textsuperscript{32} However, the study by Chan et al\textsuperscript{7} revealed that overexpression of C-repeat/DRE binding factors (\textit{CBF3}) in \textit{Arabidopsis thaliana} had negative effects on vegetative growth and reproductive development including delayed bolting and flowering and reduced leaf number, rosette diameter, plant height, dry weight and seed yield relative to the parent genotype.

It seems that the influence of foreign genes on the growth and development of recipient plants is a complex issue, so further studies are needed to answer this phenomenon. The results of chlorophyll content in transgenic peanuts are similar to those reported by Hong et al\textsuperscript{17} who showed that \textit{A. thaliana} overexpressing the foreign chitinase gene (\textit{CACh12}) has higher chlorophyll content than non-transgenic controls. Other studies on chitinase heterologous expression in tomato and \textit{A. thaliana} have also provided outcomes similar to ours.\textsuperscript{6,9,31} Several previous studies have also described the beneficial effects of chitinase overexpression on plant phenotypes. Consequently, the current belief seems to be that chitinase genes have a lot of prospects for crop improvement with the aim of sustainable production of grain, fruits, vegetables etc. For example, Gongora et al\textsuperscript{11} reported that transgenic tomato plants overexpressing the endochitinase and chitobiosidase
genes from *Streptomyces albidosflavus* significantly increased the number of flowers and fruit on the plants, resulting in an increase in the yield of fruit. Huang et al.\(^\text{18}\) also showed that wheat lines carrying the class I chitinase (RC24) from rice enhanced grain yield by up to 27–36% compared with non-transgenic controls. Liu et al.\(^\text{21}\) discovered that the *CHITINASE 2 (LeCHI2)* gene in transgenic tobacco and maize does not impair growth and yield under normal cultivation conditions.

The findings of protein content in transgenic peanuts in the present study are in accordance with those of Hassan and Ahmed\(^\text{15}\) and Mora-Escobedo et al.\(^\text{28}\) Despite the statistical significance of the differences between chitinase transgenic peanut lines and control, the variances of their lipid content were minor and within the range of the values reported in the literature for various peanut cultivar.\(^\text{4,9,20}\) Peanut cultivar lipid content is typically influenced by genotype, seed maturity, growth conditions, geographical area, growing season and climate status.\(^\text{28}\)

In the peanut processing industries, seed chemical composition is a crucial criterion to determine technological properties and culinary qualities in order to define their use.\(^\text{23,24}\) Chitinase transgenic peanut seeds showed higher lipid and protein content than control, which are two interesting parameters for peanut processing industries.\(^\text{2}\) This data further confirms that chitinase transgenic peanuts can be suitable for use as an additional source of disease resistance in peanut breeding programs without reducing yield and grain quality.

**Conclusion**

Our findings demonstrated that chitinase transgenic peanuts were more productive and had a more developed vegetative apparatus than the non-transgenic control. The high chitinase expression also improved the chemical content of the seeds by increasing the amount of protein and lipids suitable for peanut processing. In summary, the *Chi42*-derived optimized chitinase genes could be a good candidate gene for peanut production not only against phytopathogenic fungi but also their yield was unaffected.

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