Induction of systemic resistance and tolerance against biotic and abiotic stress in Chinese cabbage by cyclic peptides producing *Bacillus vallismortis* strain BS07M

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Plant Growth-Promoting Rhizobacteria

Naturally occurring root-colonizing bacteria that benefit plants by growth promotion and biological control.

1 : Movement from seed to root
2 : Spread to root system
3 : Maintenance of population
PGPR-defense priming

Plant pathogens, Insect, Virus

PGPR

Abitic stress

Interaction with other microbes (Rhizobium)

Antibiotics
HCN
CLPs

Secondary metabolites

Enzymes
Siderophore
Small peptides
EPS
VOCs
Biofilm
PGPR against biotic & abiotic stress

- Biotic stress tolerance
- Abiotic stress tolerance

- PGPR
  - Antibiotics
  - Enzymes
  - Siderophores
  - VOCs
  - CLPs
  - Small peptides
  - Secondary metabolites
  - Phytohormones
  - Antioxidants
  - ABA, ROS
  - IST

- Pathogen
  - Improved Plant Strength & Immunity

- Plant
  - Defense priming
  - Growth Promotion
  - Root colonization
  - Competition
  - EPS

- Improved Plant Strength & Immunity
- Abiotic stress tolerance
Why Bacillus strains have great potential as biological control agents in environmental friendly plant health management?

• *Bacillus* spp. are essentially ubiquitous in agricultural systems.

• Important physiological traits:
  – formation of stress–resistance endospore
  – production of peptide antibiotics
  – production of small peptides elicitor for ISR
  – peptide signal molecules
  – extracellular enzymes etc.
Fig. Bacillus producing metabolites (Rainer Borriss 2010)
A rhizobacterium, BS07M was obtained from rhizosphere of Chili-pepper from Korea and was identified as *Bacillus vallismortis* by 16s RNA pattern.

- Bio screening of ISR activity
- Defense mechanisms
- Dipeptide Isolation
- Field application
Fig. Phylogenetic dendrogram constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships between strain *Bacillus vallismortis* strain BS07M and related *Bacillus* species. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points and the species names followed the GeneBank accession numbers. Bar, 1 substitutions per 100 nucleotide positions. *Paenibacillus polymyxa* DSM 36T (AJ320493) was used as an outgroup.
Bacillus vallismortis vs. closely related Bacillus sp.

- *B. vallismortis* is a close relative to other *Bacillus* sp. like *B. amyloliquefaciens* and *B. subtilis*

- It is distinguished from *B. subtilis* by differences in whole-cell fatty acid compositions, DNA sequences and levels of reassociation of genomic DNA (Roberts et al., 1996)

- The *gryA* sequences, code for DNA gyrase subunit A, can be used for accurate identification of *B. vallismortis* and related taxa including *B. subtilis, B. amyloliquefaciens, B. mojavensis, B. atrophaeus* and *B. licheniformis* (Earl et al., 2006; Chun and Bae, 2000)

- *B. vallismortis* has not been much studied as a PGPR. However, our studies have shown it to be a very potential strain for growth promotion as well as disease control
Fig. Antifungal activity against major plant fungal pathogens by dual culture with *Bacillus vallismortis* BS07M

**Antibiosis**

- *Phytophthora capsici*
- *Altanaria altanata*
- *Fusarium oxysporum*
- *Sclerotinia sclerotiorum*
- *Colletotrichum acutatum*

**IAA formation**

- Check
- EXTN-1
- B17
- BS07M

Fig. IAA formation by treatment of *Bacillus vallismortis* BS07M
Epidermal cell strengthening

Fig. Penetration sites on the leaves of cucumber plants inoculated with *Colletotrichum orbiculare* 3 days later. Non-treated control plants (A) and pre-inoculated plants with BS07M (B). All bars = 20 μm. Abb.: a, appressorium; e, epidermal cell; hr, hypersensitive reaction; ih, intercellular hypha; s, spore
Defence gene expression

**Fig. Elicitation of defense-related gene expression in Arabidopsis thaliana mutant lines**

(lane 1: Col-0, lane 2: etr3-5, lane 3: Nah-G and lane 4: npr-1) after treatment with Bacillus vallismortis strain BS07. The expression of PR genes (PR-1a and PDF1.2) analyzed by RT-PCR was examined after 24 hours inoculation with BS07 on A. thaliana mutants.

Amplified products were separated by gel electrophoresis and visualized by ethidium bromide staining. As a positive control, plants were treated with 1.0 mM ASA (Amino salicylic acid). The experiment was repeated two times obtaining with the same results.
ISR activity

Fig. ISR by treatment of Iturin A2 & A4 from *B. vallismortis* BS07M

**Iturin 2**
LSD\( (P=33.6) \)  

% disease plant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>BTH</th>
<th>0.1ppm</th>
<th>1.0ppm</th>
<th>10.0ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSD</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

**Iturin 4**
LSD\( (P=37.3) \)

% disease plant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>BTH</th>
<th>0.1ppm</th>
<th>1.0ppm</th>
<th>10.0ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSD</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
</tr>
</tbody>
</table>

Fig. Induced systemic resistance on chili pepper by Iturin A2 from BS07M
ISR activities

Fig. ISR activity against *P. carotovorum* SCC1 and *C. acutatum* by treatment with *B. vallismortis* BS07M
Fig. Cold stress tolerance by spray of *B. vallismortis* BS07M on Chinese cabbage
Cyclic dipeptide Isolation and Biological Activity

Cyclic dipeptide, Diketopiperazine, DKP  Cyclo(AA-AA)
Found in beer, coffee, cocoa, Japanese sake
Distributed in central nervous system, gastrointestinal tract, and body fluids. Peptides, 1995, 16, 151-164

Agricultural Usages

Plant growth regulator comparable to GA3
Isolated from Penicillium brevicompactum
Cyclo(Try-Leu)  JNP, 2005, 68, 237-239

Drought resistance in rice (water stress caused by sodium chloride)
Phytochem. 1990, 29, 35-39

Isolated from marine sponge
Antifouling activity (potent inhibitory effect on the settlement of barnacle larvae),
field-tested in antifouling paints for the ships. Ecofriendly attractive candidate to replace tributyltin oxide
Cyclo(Br-Try-Arg)  JNP, 2008, 71, 330-333

Medicinal Usages

Isolated from Aspergillus fumigatus
Mammalian cell growth inhibition
JMC, 2002, 45, 1559-1562

Collagenase-1 inhibitor (matrix metalloproteases, MMPs)
- rheumatoid arthritis
JMC, 1999, 42, 1348-1357

Isolated from Vibrio anguiarum
Antibiotic against Vibrio anguiarum (marine bacteria)
Cyclo(D-Phe- D-Pro)  JNP, 2003, 66, 11299-1301

Isolated from marine sponge
Antifouling activity (potent inhibitory effect on the settlement of barnacle larvae),
field-tested in antifouling paints for the ships. Ecofriendly attractive candidate to replace tributyltin oxide
Cyclo(Br-Try-Arg)  JNP, 2008, 71, 330-333
Table 1. Specific examples of biological activity exhibited by cyclic dipeptides

<table>
<thead>
<tr>
<th>Activity</th>
<th>Cyclic Dipeptide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunomodulation</td>
<td>cyclo(Arg–Lys), cyclo(Asp–Lys)</td>
<td>Blažíčková et al., 1994</td>
</tr>
<tr>
<td>Modulation of Glucose Metabolism</td>
<td>cyclo(His–Pro)</td>
<td>Hwang et al., 2003</td>
</tr>
<tr>
<td>Hormonal Regulation</td>
<td>cyclo(His–Pro)</td>
<td>Fragner et al., 1997</td>
</tr>
<tr>
<td>Anticonvulsant</td>
<td>cyclo(Gly–Phe), cyclo(Ala–Phe)</td>
<td>Szkaradzinska et al., 1994</td>
</tr>
<tr>
<td>Antifungal</td>
<td>cyclo(Phe–Pro)</td>
<td>Ström et al., 2002</td>
</tr>
<tr>
<td></td>
<td>cyclo(Trp–Pro), cyclo(Trp–Trp)</td>
<td>Graz et al., 1999</td>
</tr>
<tr>
<td>Antibacterial</td>
<td>cyclo(Trp–Trp), cyclo(Trp–Pro), cyclo(Phe–Pro),</td>
<td>Graz et al., 1999</td>
</tr>
<tr>
<td></td>
<td>cyclo(Tyr–Pro)</td>
<td></td>
</tr>
<tr>
<td>Inhibition of Cell Division</td>
<td>Dehydro derivatives of cyclo(Phe–Phe)</td>
<td>Kamzalo et al., 2000</td>
</tr>
<tr>
<td>Inhibition of Dietary Fat Intake</td>
<td>cyclo(Asp–Phe)</td>
<td>Lin et al., 1994</td>
</tr>
<tr>
<td>Induction of Differentiation in</td>
<td>cyclo(Phe–Pro), cyclo(Tyr–Pro), cyclo(Pro–Pro),</td>
<td>Graz et al., 2000</td>
</tr>
<tr>
<td>Cancer Cell Lines</td>
<td>cyclo(Pro–Pro), cyclo(Trp–Pro)</td>
<td></td>
</tr>
<tr>
<td>Molecular Recognition by Peptide</td>
<td>cyclo(Gly–Leu), cyclo(Leu–Leu)</td>
<td>Christofaro and</td>
</tr>
<tr>
<td>Hosts†</td>
<td></td>
<td>Chamberlin, 1994</td>
</tr>
</tbody>
</table>

† Enantioselective and Diastereoselective Molecular Recognition of Cyclic Dipeptides by a C$_2$ Macrolactam Host
Preparative HPLC: YMC pac pro C18, 60-80% aqueous MeOH for 120 min, flow 7 mL/min, 220 nm

Fig. Purification of Dipeptides from culture filtrates of *B. vallismortis* BS07M by preparative HPLC
Fig. Identification of dipeptides from strain BS07M by NMR data
<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cold injured plant(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>CON</td>
<td>71.9</td>
</tr>
<tr>
<td>BTH</td>
<td>84.4</td>
</tr>
<tr>
<td>P1(Thr-Pro)</td>
<td>46.9*</td>
</tr>
<tr>
<td>P4(Gln-Pro)</td>
<td>37.5*</td>
</tr>
<tr>
<td>H3(Ala-Pro)</td>
<td>50.0</td>
</tr>
<tr>
<td>Q3(Gly-Pro)</td>
<td>21.9*</td>
</tr>
<tr>
<td>D52(Cys-Pro)</td>
<td>71.9</td>
</tr>
<tr>
<td>MIX(P1, P4, H3, Q3, D52)</td>
<td>68.8</td>
</tr>
<tr>
<td>LSD($p=0.05$)</td>
<td>24.7</td>
</tr>
</tbody>
</table>

R$^1$, R$^6$: H; C1~C6 alkyl
R$^2$, R$^3$, R$^4$, R$^5$: H, hydroxy, mercapto, amino, guadinino, carbamoyl, C1~C6 alkylcarbonyl, C1~C6 alkylthio, tritylthio, acetylamino, phenyl, hydroxyphenyl, indolyl
R$^2$, R$^3$: 5~7 membered ring
R$^4$, R$^5$: 5~7 membered ring with R6
Fig. Soft rot disease control against *P. carotovora* SCC1 on tobacco plant by dipeptide Q3 of *B. vallismortis* BS07M

Fig. Cold resistance by cyclic dipeptide Q3 of *Bacillus vallismortis* BS07M on tobacco plant (1.0°C 24h)
Fig. Evaluation of disease symptom and cold tolerance by synthesized Q3 (cyclic Gly-Pro) in tobacco plants.

(A) Assessment of disease incidence caused by *Pectobacterium carotovara* SCC1. Disease symptom was monitored in Q3 treated tobacco plants.

(B) Q3 treated plants resulted in improvement of cold tolerance. Asterisks indicate the significant difference compared to control ($P = 0.05$).
Fig. Cucumber protection against *Pectobacterium carotovora* SCC1 by infiltration of a promising cyclic dipeptide Q3
Fig. Suppression of soft rot disease on chinese cabbage by treatment of selected cyclic dipeptides
Fig. Biotic abiotic stress tolerance gene activation by treatment of Bacterial strain BS07M & Dipeptide Q3(cyclic Gly-Pro) on Chinese cabbage

**Heat tolerance**

- **KIN1**, **RD29A**

**Salt tolerance**

- **P5CS**, **RD29A**, **KIN1**

**Soft rot**

- **PR1**, **COR15A**, **RAB12**

**Gene Descriptions**

- **P5CS**: DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE1
- **RD29A**: RESPONSIVE TO DESSICATION 29A
- **KIN1**: SNF1 KINASE HOMOLOG 1
- **RD22**: RESPONSIVE TO DESSICATION 22
- **PR1**: PATHOGENESIS-RELATED GENE 1
- **COR15A**: COLD-REGULATED 15A
- **RAB12**: RESPONSIVE TO DESSICATION 17
Fig. Chinese cabbage plant growth promotion by seed treatment of Cyclic dipeptide Q3 isolated from *B. vallismortis BS07* in field condition
Field Application of PGPR and Bacterial Dipeptide Q3
Field Application of EXTN-1 with Dipeptide Q3
Chinese cabbage (Brassica campestris var. pekinensis) is a major crop in Korea.

Production of Chinese cabbage is severely affected by high temperature with heavy reduction during summer season.

Control of soft rot disease is difficult due to wide range of hosts, survival of the bacteria in plant debris in soil and host susceptibility.

Farmers opt for highland cultivation which gives geological advantage for cool temperature.

However, conventional culture practices and chemicals are either not efficient enough.
PGPR Seed Priming

Application of BS07 (107 cfu/ml) + Dipeptide Q3 (1.0 ppm)
- Seed soaking 2 hours before planting
- Soil drench of BS07+Q3 suspension 3 times at weekly interval
- Transplanting of 40 day-old-seedlings in the field
Fig. Soft rot disease control by combination treatment of BS07M and Q3 (Cyclic Proline- Glycine)

Fig. Abiotic stress resistance to high temperature by treatment of BS07M and Q3 on chinese cabbage (38°C, 12hrs)

Fig. Plant growth promotion by treatment of BS07M with cyclic dipeptide Q3
Fig. Disease suppression of bacterial soft rot against *Pectobacterium carotovora* SCC1 (Kangreung Aug. 8 2013)
Table. Plant growth promotion by combination treatment of BS07M with dipeptide Q3 on chinese cabbage

Fig. Plant growth promotion by combination treatment of BS07M with dipeptide Q3 on chinese cabbage
Commercial Product of Plant Defense Activator

A Promising Plant Dense Activator by RDA

R¹, R⁶: H, C₁~C₆ alkyl
R², R³, R⁴, R⁵: H, hydroxy, mercapto, amino, guadinino, carbamoyl, C₁~C₆ alkylcarbonyl, C₁~C₆ alkylthio, tritylthio, acetylamino, phenyl, hydroxyphenyl, indolyl
R², R³: 5~7 membered ring
R⁴, R⁵: 5~7 membered ring with R⁶

Patent related
1. Korean patent (10-2010-0039xxx): Defense activator
2. Korean patent (10-2010-0068xxx): 
5. PCT patent (PCT/KR2011/005xxx): 

Waiting mass production and registration of the peptide!
Conclusion

● 14 CDPs were isolated from the culture broth of *B. vallismortis* BS07M and their structure were characterized base on their spectral data

● The results indicate an important role of BS07M on plant growth promotion and induced systemic resistance in plants

● Proline-based dipeptides elicit induced systemic resistance of tobacco & chinese cabbage in response to *P. carotovora* SCC1.

● Induced systemic resistance can be positively regulated by Q3 (cyclic Pro-Gly) application in a dose-dependent manner.

● The biotic & abiotic tolarence is significantly increased by treatment of BS07M plusQ3 on plants than in control plants.
Thank you..