RS4-1

Antifungal Agent Development

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In order to develope a novel antifungal agent against the plant pathogenic fungi, we studied on the purification and characterization of fungicidal and fungistatic substances produced from antifungal bacteria. In addition, we also cloned and characterized a mitogen-activated protein kinase (MAPK) as a new inhibition target of fungal life including growth, differentiation, pathogenesis, stress signal translation and soon from *Colletotrichum gloeosporioides* causing pepper anthracnose.

Chapter 1 : Studies on the purification and characterization of fungicidal and fungistatic substances produced from antifungal microbes

In this study, the antifungal bacteria sixteen were isolated from various environment located in Chung-cheong area, Korea. These isolates were identified the genera *Bacillus* sp, *Pseudomonas* sp. *Alcaligenes* sp., and *Serratia* sp. through morphological, physiological and biochemical analysis. Especially, strain KL1114, KL1143 and KL1367 identified as *Bacillus subtilis* var. *amyloliquefaciens,* and strains KL1326, KL1314 identified as *Pseudomonas aurantiaca* have never been reported internationally. Considering antifungal (AF) spectrum, the strain KL1114 showed the broad range of AF activities on number of pathogenic fungi. Therefore, strain KL1114 was selected with the strong candidate of antifungal bacteria for every purpose and usage related with our research goal (Figure 1).

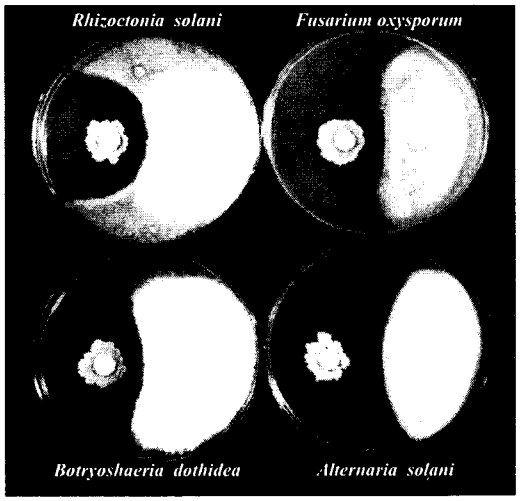

Figure 1. Antifungal activity of KL1114 against plant pathogenic fungi.

B. subtilis var. *amyloliquefaciens* KL1114 producing antifungal substances were produced two types of compounds and polypeptides. A result of analyzed antifungal compound by TLC, HPLC, FAM-MS, and NMR appeared Rf value which has an each other different value (KLMBF-1~3). KLMBF-1 (Mw. 530), one of the compounds isolated from the antifungal bacteria KL1114 has never been reported internationally. Therefore, it has a high possibility of novel antifungal compound. Antifungal polypeptide, other antifungal substances were identified with polypeptide, which have five kinds (LMAFP 1~5) with low molecular weight of about 1058 Da and high molecular weight of 41 kDa. In case of the low molecular polypeptide, a result of SDS-PAGE, PrepRPC HR, UV/Maldi-Mass spectrum and physical and chemical analysis, it seems to associate with the cyclic lipoproein produced by *B. subtilis*. And in case of polypeptide (41 kDa) with high molecular weight, it was confirmed with flagellin protein which has never been reported about antifungal activity up to now. Especially, whole gene encoding 41kDa polypeptide has been cloned and over-expressed by molecular biological techniques and domain with antifungal activity was confirmed also. Mutants were constructed by EMS treatment or γ-ray radiation on purpose of AF-(antifungal activity negative) and AF^{+}(enhanced antifungal activities) mutants. A result of TLC and bioassay analysis, AF-mutants seems to be deleted antifungal synthesis gene by EMS treatment and mutants with enhanced antifungal activities by γ-ray radiation was selected.

Optimal conditions for the productivity of antifungal substances were analyzed under various environmental conditions (carbon source, nitrogen source, phosphate concentration, pH, temperature, amino acids, vitamins, plant sources). Growth rates were different according to carbon and nitrogen source, antifungal substances production yield were not different, however. Productivities of antifungal material according to phosphate are proportional to concentration; the higher in high concentration and the lower in low concentration. And productivity of antifungal substances is was generally high in the range of 30~35℃ at pH7 and in case of adding vitamin B1, lysine to medium it was enhanced. Moreover, bio-degradability upon agricultural chemicals and organic substances by AF bacteria was strikingly effective.

On the basis of the environmental factor, new formulation of the microbial biocides (KL1114 MBF) and microbe-based products has been developed, which is quite different from the established one in their composition. AF strains were screened and selected from this research can be used in the microbial biocides as well as multifunctional bio-controllers in order to remove plant pathogenic fungi and to clarify the polluted environment. Due to their excellent degradation capability for agricultural chemicals and/ or organic substances, they also can be used to improve soil quality environment-friendly, to ferment compost and to clean up the environment (Figure 2).

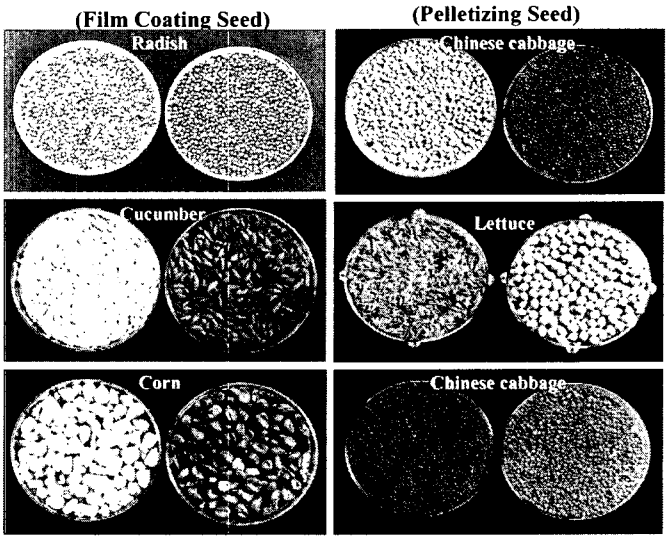

Figure 2. Seeds treated with KL1114MBF.

Chapter 2 : Molecular cloning and characterization of a mitogen-activated protein kinase (MAPK) from *Colletotrichum gloeosporioides* causing pepper anthracnose

Mitogen-activated protein kinases (MAPKs) are a group of protein kinase that execute a variety of important roles in cellular signal transduction pathway such as osmoregluation, cell well biosynthesis, growth, and differentiation. A polymerase chain reaction (PCR) with degenerate primers based on conserved regions of known MAPKs was used to clone the partial MAPK gene from the pepper anthracnose causing pathogen *C. gloeosporioides*.

The part of the partial PCR product was then used to probe a *C. gloeosporioides* pDELTA2 genomic library (contains the *Bam*H I digested genomic DNA fragments) and isolated a clone pK1-72 containing the entire MAPK gene with the 5'- and 3'-flanking sequences. Sequencing revealed the presense of an open reading frame (ORF) of 1068 bp nucleotides encoding a putative protein 355 amino acids. Three potential introns were identified within the ORF by comparing the sequencing of the *C. gloeosporioides* genomic DNA and the cDNA clone.

MAP kinase of *C. gleosporioides* is named as a *CGK*1 and is registered with the accession number: AB047033 in the GenBank. In addition, it expressed 43 kDa of protein. There is 85 to 95 percentage of homology between *PMK1* gene of *M. grisea* MAPK and *CMK1* gene of *C. lagenarium*. *CGK1* has MAP kinase, which has dual specificity on all MAPKs and well-conserved order of tyrosine and threonine is on the TXY (phosphorylation site). *C. gleosporioides* is known as the fungi, causing Pepper Anthracnose. Conidia of *C. gleosporioides* on the surface of host plant can germinate to a germ tube. After germ tube transformed to an infection structure called appressorium (differentiated form) and it penetrates to host plant tissue cell.

When concentrated wax extracted from a host plant pepper put to coat on the hydrophilic cover glass, conidia of *C. gleosporioides* could form an appressorium. However, when cAMP is added on knockout mutant which is deleted *PMK*1 gene of *M. grisea*, appresssorium was developed in *M. grisea*. In case of *C. gleosporioides,* cAMP did not affect the formation and differentiation of appressorium. These results show that appressorium formation on *C. gloeosporioides* is only affected by MAPK signal transduction pathway through *CGK*1 without PKA pathway control. Apparently, the result is contrary to the proved theory in the past that appressorium formation of *M. grisea* need MAPK cascade pathway with signal transduction pathway of cAMP-dependent protein kinase (PKA). Therefore, we produced mutants by targeting with obtained *CGK*1 gene, because MARK signal transduction pathway is important on fungal phytopathogenesis.

C. gleosporioides hyphae comprising traditional light gray or light green color of circle shape turned to light orange or white color of hyphae, since formation rate of conidia decreased dramatically in case of mutants. It proves the contrary of the decrease of a number of conidia and it results in the decrease of infectivity on the pepper. So to speak, the deletion of *CGK*1 gene leads several changes as follows; ① the decrease of conidia, ② the decrease of pathogenicity, ③ changes of pigmentation, ④ no formation of appressorium. However, there is no difference in the growth rate of hyphae and in vitro morphogenesis between wild type and *CGK1* deletion mutant of *C. gleosporioides* (Figure 3).

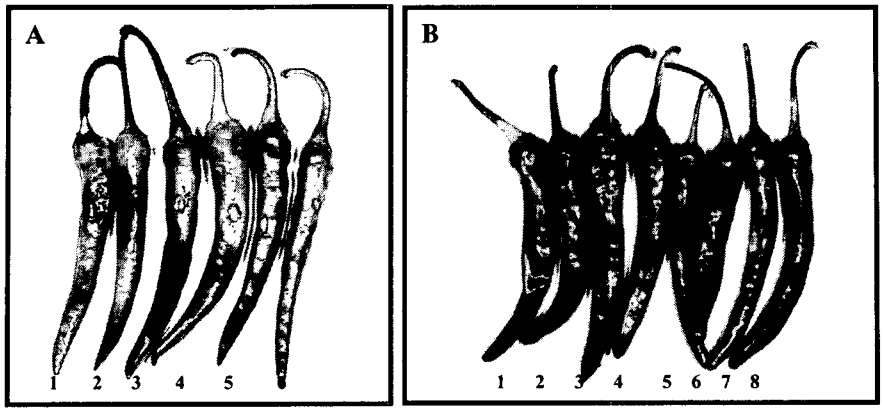

Figure 3. Pathogenicity test of anthracnose symptoms caused by *C. gloeosporioides*. MAP kinase (*CGK*1) mutant CG43, CG48 and wild type strain were also inoculated as control. Green (A) and red (B) pepper fruits unwounded or wounded by pin-pricking 7 days after inoculation. 1 and 2, *C. gloeosporioides* wild type; 3 and 4, *CGK*1 mutant CG48; 5~8, *CGK*1 mutant CG43.

It is proved that there were significant differences from wild type in a protein profile by 2-D qualitatively and quantitatively. In addition, the expression patterns of several isozymes and exo-enzymes were considerably distinguished between wild type and *CGK*1 mutants.

Therefore, produced mutants are expected to use as a model to apply complementation test of *CGK*1 gene. Although complementation test for *CGK*1 gene performed on *fus*3 deleted yeast, arrest of cell cycle by pheromone signal on G1 period was not happened on budding yeast.

This means that CGK1 is not a family of FUS3 or KSS1 involved in mating or invasion/filamentation growth of yeast as well. In order to check whether CGK1 MAPK might be functioned like ERK MAPK (extracellular signal-regulated kinase MAPK) or not, immunoblotting assays were carried out. As the result, wild type expressed the chemiluminescent signal on anti-Elk-1, anti-phosphorylated Elk-1 (Ser383) but *CGK*1 deletion mutants did not express. It is very strong evidence that CGK1 MAPK can play a role like an ERK MAPK immunologically at least. Translational control of eukaryotic cell is done by both elf4F and p70 S6 kinase, which are relating with recognition of mRNA 5' cap and phosphorylation of ribosomal protein, respectively.

In addition to the result that CGK1 should be a type of ERK through immunological approach, from another immunoblotting experiments, using an anti-phospho-4E-BP1 (Ser65) and anti-phospho-4E-BP1 (Thr70).

We can find out that wild type can express the immuno-signal upon an anti-phospho-4E-BP1 (Thr70) but *CGK*1 deletion mutants cannot. In case of immunoblatting against an anti-phospho-4E-BP1 (Ser65), there is no chemiluminescent signal from both wild type and *CGK*1 mutants. Presumably, *CGK*1 may affect the phosphorylation of 4E-BP1 (Thr70) indirectly, considering that 4E-BP1 and p70S6 kinase, the components involving in translational initiation complex that were controlled by ERK MAPK pathway. ERK MAPK family is chiefly in charge of cell growth and differentiation. Anyhow, *CGK*1 may be a type of ERK MAPK family from the viewpoint of cellular function relating with growth and differentiation (especially appressorium differentiation). And also since appressorium morphogenesis is the key for invasion process

and phytopathogenesis, CGK1 function can be expanded in many aspects.

These valuable results can be applied not only to isolate genes for MAPK signal transduction pathway but also not to cause infection of pathogenic organ fungi. In sum, the results of the research can be used to investigate new medicine targetting MAPK signal transduction pathway and will be very helpful to develope a variety of innovative medicines in the near future.

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