Quantitative transcriptional analysis of the cellulolytic genes in the basidiomycete *Phanerochaete chrysosporium*

担子菌*Phanerochaete chrysosporium*におけるセルロース分解関連
酵素遺伝子の発現応答に関する定量的解析

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Chapter 1

Introduction

1.1 Properties and structure of cellulose

Cellulose, a major structural carbohydrate of plant cell wall consisting 50% of its components, is the most abundant carbon resources on earth as produced by photosynthesis as 10^9 t/ year at least (Coughlan, 1985). Cellulose is one of the most important biomaterial and widely used in paper and fibre industry, architectural material, medical devices, and so on. In recent years, cellulose is expected to serve as one of the alternative feedstock for biofuel to fossil fuel in aspect of its reproductivity and sustainability.

The nomenclature of cellulose was proposed by Payen (1838), who first recognized that cellulose and starch were isomeric products. The structure of cellulose is a linear polymer of β-1, 4-linked anhydrous D-glucose residues. The configuration of glucose moieties is the chair form, which is the lowest energy conformation of β-D-glucopyranose. Cellulose chain possesses twofold screw axes and intra- and inter-molecular bonds hold the chain firmly together (Gardener & Blackwell, 1974) and forming crystalline structure, cellulose microfibril. The repeating unit of cellulose is cellobiose, rather than glucose (Blackwell, 1982) (Fig. 1.1). The size of the microfibril is 4 to 20 nm, although it is varied and depends on its origin. In native cellulose microfibril, each cellulose chain is oriented in parallel direction, as called cellulose I (Atalla & Van der Hart, 1984, Van der Hart & Atalla, 1984, Pizzi & Eaton, 1985, Pizzi & Eaton, 1985). It was elucidated that two different forms of chain orientation, called I_α and I_β, exist in native cellulose microfibril. They are different in their intermolecular bonding patterns, revealed by solid-state ^{13}C-NMR studies (Van der Hart & Atalla, 1984). Recently, diffraction analysis of microfibril containing the pure crystalline form allowed establishment of two simple unit cells: a triclinic one-chain unit cell (cellulose I_α) and

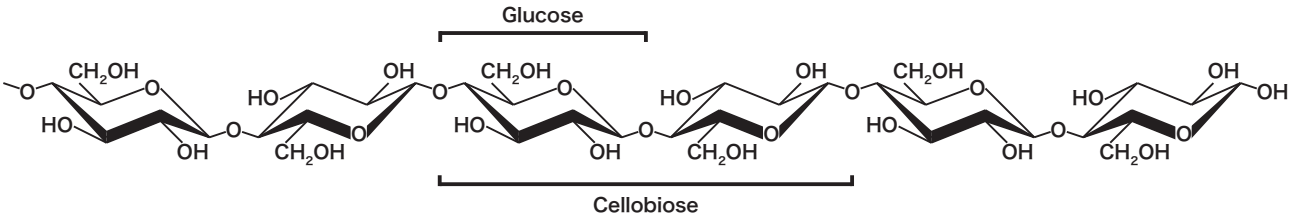


Fig. 1.1. Structure of cellulose.

a monoclinic two-chain unit cell (cellulose I_β), indicating that native cellulose is a complex of cellulose I_α and I_β crystalls (Sugiyama*, et al.*, 1991). More recently, synchrotron X-ray and neutron fiber diffraction analysis revealed the existence of two co-existing hydrogen bonding patterns in I_α and I_β, suggesting a hydrogen-bonding disorder (Nishiyama*, et al.*, 2002, Nishiyama*, et al.*, 2003).

Although cellulose forms highly crystalline structure, it usually contains amorphous region in addition to crystalline region. Previously, it is generally considered that amorphous cellulose is a less ordered section on arrangement of the molecular chains. However, Verlhac et al. suggested that disorganized structure of cellulose was found mostly in the surface of cotton cellulose microfibril, compared to highly crystalline cellulose from alga *Valonia macrophysa* or bacterial cellulose (Verlhac*, et al.*, 1990). O'sullivan also suggested that the content of the amorphous material in various celluloses was approximately equal to the percentage of the cellulose chains on the surface of the crystals (O'sullivan, 1997).

1.2 Biodegradation of cellulose

1.2.1 Concepts for the enzymatic hydrolysis of cellulose

Almost half of the biomass synthesized by photosynthetic fixation of carbon dioxide is made up of cellulose. Hoppe-Seyler (1883) and de Bary (1886) first reported that cellulose is decomposed in nature, and the term "cellulase" was first coined by Pringsheim in 1912. Degradation of cellulose is mainly caused by microorganisms and, due to their abundance, microbial degradation of cellulose is one of the most important processes in nature. Cellulolytic microorganisms commonly produce extracellular hydrolytic enzymes for cellulose degradation.

In 1950, Reese et al. (Reese*, et al.*, 1950) first reported the systematic analysis on the mechanism of cellulose degradation by cellulases and introduced now classic "C_1, C_x" hypothesis (Fig. 1.2A). This theory postulated that crystalline cellulose were first rendered susceptible to

hydrolysis by the C_1-component. The C_1-component was suggested to be a non-hydrolytic chain-separating enzyme. The separation of the cellulose chains was suggested to take place by a splitting of the hydrogen bonds. Cellulose modified in this way is then hydrolyzed by the C_x-enzyme fraction and by the β-glucosidase. King (1965) found that, although glucose was released from cellulose by a crude enzyme mixture, the amount of glucose decreased as the purification of the so-called C_1-component proceeded. The enzyme responsible for the production of cellulose was purified and named exo-glucanase, which was called cellobiohydrolase later (Halliwell*, et al.*, 1972, Wood & McCrae, 1972). Since this enzyme showed synergistic activity with C_x-cellulase (endo-glucanase) (Streamer*, et al.*, 1975, Wood & McCrae, 1978, Fagerstam & Pettersson, 1980, Henrissat*, et al.*, 1985, Uzcategui*, et al.*, 1991, Henriksson*, et al.*, 1999), endo-exo mechanism (Fig. 1.2B), a novel mechanism for cellulose degradation, was proposed. In this theory, the amorphous regions of cellulose, which are easily degraded, first took place by the endoglucanase followed by a successive degradation of crystalline areas in a synergistic action of endoglucanase, cellobiohydrolase and β-glucosidase. The order of attack to cellulose is different between C_1-C_x and endo-exo mechanism, i.e., C_1-cellulase is the first enzyme in former model but endoglucanase comes first in latter one.

As detailed structures of cellulases were revealed, C_1-C_x and endo-exo mechanisms have been used only for the entry mode of cellulose chain into the catalytic center. In 1980s, four cellulase genes were cloned from well studied cellulolytic mold *Hypocrea jecorina* (anamorph; *Trichoderma reesei*) and a remarkable sequence homology has been detected between the N- or C-terminal regions of these cellulases (Shoemaker*, et al.*, 1983, Penttila*, et al.*, 1986, Teeri*, et al.*, 1987). The striking similarity of these regions in these cellulases suggest that they likely have a functional significance. On the basis of the disulfide bonding pattern observed in a cellobiohydrolase from *Hypocrea jecorina* (anamorph; *Trichoderma reesei*), the enzyme was postulated to be comprised of at least two protein domains (Bhikhabhai & Pettersson, 1984), and

studies initiated soon after involving limited proteolysis showed this to be the case (Van Tilbeurgh, *et al.*, 1986). Subsequent investigations of the second cellobiohydrolase from this fungus (Tomme, *et al.*, 1988) and two endoglucanases from bacteria *Cellulomonas fimi* suggested that these structural features may be common among the cellulolytic enzymes (Langsford, *et al.*, 1987, Gilkes, *et al.*, 1988). These domains are known as a catalytic domain and a noncatalytic cellulose-binding domain (CBD), which was renamed as carbohydrate binding module (CBM) for more inclusive terminology. In the case of exo-type enzyme (cellobiohydrolase; CBH), cellulose chain enters to the catalytic domain from either the reducing or non-reducing ends (named CBHI or CBHII, respectively), whereas endo-type enzyme (endoglucanase; EG) randomly catches cellulose chain at the middle of that. In 1990s, the concept of processive and non-processive mechanisms was introduced to illustrate the behavior of catalytic domain (Henrissat, 1998). This concept explains the

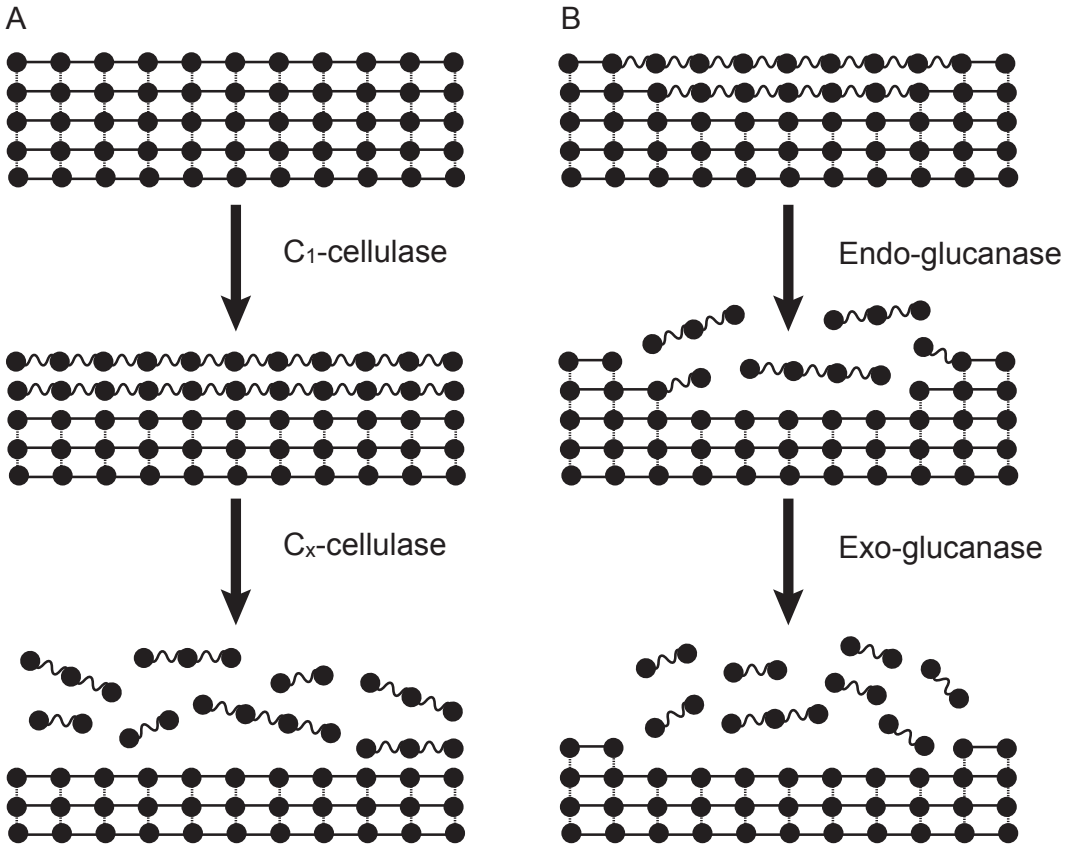


Fig. 1.2. C_1-C_x and Endo-Exo mechanism for enzymatic hydrolysis of cellulose.

action of cellulases following the catalytic reaction, i.e. a processive enzyme slides to the next cellobiose unit while attached to the cellulose chain, whereas a non-processive enzyme detaches from the chain after the hydrolysis of a β-1,4-glucosidic bond. In this concept, cellobiohydrolases and endoglucanases are classified according to their processivity. The three-dimensional structure of the catalytic site of CBHI of *H. jecorina* has been reported to be of "tunnel-type" whereas that of endoglucanase (EGI) was found to be an open groove rather than a tunnel (Divne*, et al.*, 1994, Davies & Henrissat, 1995), indicating the higher processivity of CBHI than EGI. Thus, CBH is an essential enzyme for the deconstruction of crystalline part of cellulose, according to its processivity.

1.2.2 Cellulolytic enzymes in fungi

The cellulolytic enzymes have been defined as the enzymes hydrolyzing cellulose. To date, this definition was modified because, in addition to the hydrolytic enzymes, oxidative enzymes also participate in cellulose degradation (Eriksson & Wood, 1985). The term, "cellulase" is generally used for the enzyme which hydrolyze glucan chains in cellulose polymer to cellodextrin or finally glucose.

Table 1.1. The number of fungal species in GH family 7 database on CAZy[a]

Phylum	Number of the recorded species (% of the total no.)				
	Number of identified Cel7 enzyme[b]				Total
	1	2	3	≧4	
Ascomycota	33 (49)	26 (38)	7 (10)	2 (3)	68 (100)
Basidiomycota	9 (50)	2 (11)	0 (0)	7 (39)	18 (100)

[a] Different strains of a species were counted as the same species.

[b] The number include hypothetical proteins and fragmented sequences of enzymes.

Many microorganisms including fungi and bacteria had been found to produce cellulolytic enzymes, thereby ultimately yielding soluble sugars small enough to pass through the microbial cell, because cellulose is a water-insoluble polymer, which cannot traverse the cell membrane. Fungi producing the necessary enzymes for a cell-free degradation of crystalline cellulose is normally classified into the Ascomycete and Deuteromycete groups or to the Basidiomycetes (Eriksson*, et al.*, 1990). Several fungi are considered in cellulase studies as summarized in Eriksson et al. (1990).

Cellulases were classified into glycoside hydrolase (GH) families, based on the amino acid sequence and possible secondary structure predicted by hydrophobic cluster analysis (Henrissat, 1991, Henrissat & Bairoch, 1996, Henrissat & Davies, 1997). According to this classification, fungal cellulases have been found in several GH families, including GH family 5, 6, 7, 9, 12, 45, 61 and 74 to date. Detailed information is available in Carbohydrate Active enZymes (CAZy) database (http://www.cazy.org/index.html). Recently, in addition to the classification of glycoside hydrolases, fungal oxidative enzymes are also classified based on their sequence and structure and integrated into the Fungal Oxidative Lignin enzymes (FOLy) database (Levasseur*, et al.*, 2008) (http:// foly.esil.univ-mrs.fr./index.html). Although this database mainly dealt with oxidative enzymes which related to lignin degradation, a cellulolytic oxidase, such as cellobiose dehydrogenase (CDH) (Ander*, et al.*, 1990, Eriksson*, et al.*, 1993), is included.

1.2.3 Multiplicity of GH family 7 cellulases in fungi

A plenty of cellulases are identified in ascomycota and basidiomycota organisms and their nucleic or amino acid sequences are deposited in CAZy database. According to the GH family 7 databases, which include fungal CBHs and EGs, multiple enzymes have been identified from a single fungus. In this database, multiplicity of the cellulases (Cel7s) are observed in about a half of deposited ascomycota and basidiomycota at least (Table 1.1). However, the tendency of the

Table 1.2. CAZy entries of fungal GH7 enzymes (number of entries ≧ 3)

Organism	Entries	Function
Basidiomycota		
Chlorophyllum molybdites	6	six CBH fragments
Clitocybe nuda	5	five CBH fragments
Infundibulicybe gibba	9	nine CBH fragments
Irpex lacteus	4	four CBHs
Phanerochaete chrysosporium	6	four CBHs and two CBH fragments
Pleurotus sp.	4	four CBHs
Xylaria polymorpha	7	seven CBH fragments
Ascomycota		
Aspergillus oryzae	3	two CBHs and one EG
Chrysosporium lucknowense	3	two CBHs and one EG
Emericella nidulans	3	two CBHs and one EG
Humicola grisea	3	two CBHs and one EG
Magnaporthe grisea	5	five hypothetical proteins
Penicillium oxalicum	3	one CBH and two CBH fragments
Podospora anserina	6	four CBHs and two EGs
Sarcoscypha occidentalis	3	three CBH fragments
Scytalidium thermophilum	3	three CBHs

multiplicity is different between ascomycota and basidiomycota. In ascomycota, most of species, which have multiple Cel7s, have two Cel7 enzymes and only minor portion of them have three or more Cel7s. In contrast, about 80% of basidiomycota species harboring multiple Cel7s were confirmed to have four or more Cel7 enzymes or corresponding genes. Although not all of genomes of these species have been sequenced and the number of the Cel7s are still provisional, it is suggested that the basidiomycota species have Cel7s with higher multiplicity than ascomycota

species. The list of fungal species, which have three or more Cel7s, are shown in Table 1.2. In contrast to GH family 7, the information about the multiplicity of GH family 6 cellulases (Cel6s) seems to be somewhat limited. According to GH family 6 database, only seven ascomycota and two basidiomycota displayed the multiplicity of Cel6s in each individual organism. It is difficult to identify the tendency of the multiplicity of Cel6s in cellulolytic fungi unless further investigation of Cel6 enzymes is performed in more variety of cellulolytic organisms.

1.3 Concepts for the regulation of cellulolytic enzyme production.

In the previous studies of the cellulolytic enzyme production in several microorganisms, it has been generally observed that the production of these enzymes occurred in the presence of cellulose and were synthesized at low rate or not at all in the presence of glucose (details are described below). The latter phenomenon is known as carbon catabolite repression. The term "carbon catabolite repression" is widely used for negative regulation of a variety of protein production or gene expression, not only for cellulolytic enzymes, provoked by the presence of glucose or other sugars, such as galactose or maltose. The molecular mechanism of how the presence of gluocose trigger a down-regulation of target protein synthesis is well characterized in some bacterial species, *Escherichia coli* or *Salmonella typhimurium*, for example (Deutscher*, et al.*, 2006, Deutscher, 2008). In contrast to bacteria, however, the knowledge about the mechanism of carbon catabolite repression in eukaryotic organisms is still limited. *Saccharomyces cerevisiae* is one of the most intensively studied eukaryote about its carbon catabolite repression, but a entire picture of the mechanism including signal transduction system or intermediary elements has not been illustrated. The detail of yeast catabolite repression was reviewed by Gancedo (Gancedo, 1992, Gancedo, 1998). In this thesis, the term "carbon catabolite repression" or simply "repression" is used to represent a phenomenon that protein production or gene expression down-regulated by the presence of glucose or another carbon source mentioned in each case. Along with the

consumption of glucose in a media, a relief of carbon catabolite repression occurs. This is termed as "derepression". In *S. cerevisiae*, several intermediary proteins, such as Snf1 complex (Ciriacy, 1977, Zimmermann*, et al.*, 1977, Carlson*, et al.*, 1981), are known to be necessary for derepression. Since derepression means a phenomenon resulted from a relief of carbon catabolite repression, it must be distinguished from so called "basal expression", which does not assume an association of repression. Indeed, basal expression, not derepression, of some cellulases are required for the total production of another cellulases in *Hypocrea jecorina* (see below). In addition to carbon catabolite repression, derepression and basal expression, the term "induction" is generally used for up-regulation of protein production or gene transcription triggered by the presence of specific sugars or any other compounds, which are called "inducer". The previous studies about inducer and induction of cellulolytic enzymes are described below.

1.4 Regulation of cellulolytic enzyme production in the ascomycete *Hypocrea jecorina.*

1.4.1 Carbon source dependent-regulation of cellulase in *H. jecorina.*

Several ascomycetes were considered for their cellulolytic system, and among them, *Hypocrea jecorina* (anamorph, *Trichoderma reesei*) were most intensively investigated in a viewpoint of cellulase production. This fungus produces two CBHs belonging to the GH families 7 and 6 (Cel7A and Cel6A, or CBHI and CBHII) and at least five EGs (Cel7B, Cel5A, Cel12A, Cel61A and Cel45A or EGI, EGII, EGIII, EGIV and EGV) when cellulosic biomass is utilized as a substrate (Shoemaker*, et al.*, 1983, Vinzant*, et al.*, 2001). These enzymes act cooperatively or synergistically to hydrolyze cellulose (Henrissat*, et al.*, 1985).

In the 1950s, carbon source dependent production of cellulases were investigated using various sugars (Reese & Levinson, 1952, Mandels & Reese, 1957) from the aspect how the induction of cellulases occur even though cellulose is insoluble. In these results, an efficient

induction of cellulase production was observed when the fungus was cultivated on native cellulose, which is derived from various organisms, and lactose. However, cellobiose, the soluble products of cellulose degradation, did not act as an inducer for cellulase production. Later, Mandels and Reese (1960) demonstrated that the low enzyme yields obtained in cellobiose cultures are resulting from rapid growth of fungus, and emphasized that the natural inducers of cellulases are cellobiose and cellooligosaccharides produced from cellulose. Vaheri et al. (1979) showed that the high yield production of cellulases were induced by continuous feeding of cellobiose to a batch culture. In the previous report, a little amount of cellulase activity was detected in glucose culture (Mandels & Reese, 1957). Further research revealed that cellulase induction on glucose has been shown to be due to a trace impurity of reagent glucose (Mandels & Reese, 1959). This comtaminated compound was later identified as sophorose (2-0-β-D-glucopsyranosyl-D-glucose), which act as by far the strongest inducer among known inducers for cellulase production in *H. jecorina* (Mandels*, et al.*, 1962), whereas glucose itself rather repressed the cellulase formation (Nisizawa*, et al.*, 1972). It was found that, in addition to glucose, several disaccharides, such as cellobiose, laminaribiose, sophorose and gentiobiose, were formed in the reaction mixture of cellobiose with the cell extract, which showed only BGL activity, derived from *H. jecorina* grown on glycerol as a sole carbon source (Vaheri*, et al.*, 1979). This finding suggest that BGL possess transglycosilation activity to form sophorose from cellobiose. Sternberg and Mandels (1979, 1980) repoted that the inducing activity of sophorose is related to its concentration. However, there are no report of purified BGL which can convert cellooligosaccharides to sophorose. Besides sophorose, one of the component found in cellulose degradation products, cellobiono-1,5-lactone, was shown to clearly induce the cellulase formation (Iyayi*, et al.*, 1989, Szakmary*, et al.*, 1991).

1.4.2 Regulatory mechanism of cellulase production in *H. jecorina*.

Since the natural substrate cellulose is insoluble, several studies were performed to determine how an insoluble polymer, which cannot traverse the cell wall, would initiate cellulase production. Most of these research emphasized that soluble inducers were produced from cellulose by means of cellulases, which is produced as a low basal level. El-Gogary et al. (1989) revealed that no Cel7A mRNA was detected when antibodies to the major members of the celulolytic system (Cel7A, Cel7B, Cel6A, Cel5A and BGL) were present in the culture medium prior to the addition of cellulose. Messner and Kubicek (1991) performed Northern analysis of Cel7A and Cel6A transcripts under several carbon sources and indicated that the control of these enzyme production operates mainly at the transcriptional level. The analysis of CBH gene expression using the cellulase deleted mutants strongly suggested that a basal expression of Cel6A and Cel5A are indispensable to form the inducer for CBHs from cellulose (Seiboth*, et al.*, 1992, Seiboth*, et al.*, 1997). Moreover, antisense mRNA for Cel6A, Cel7B and Cel5A transcripts inhibit the expression of Cel7A gene transcripts in cellulose-containing culture, but not in the medium with sophorose

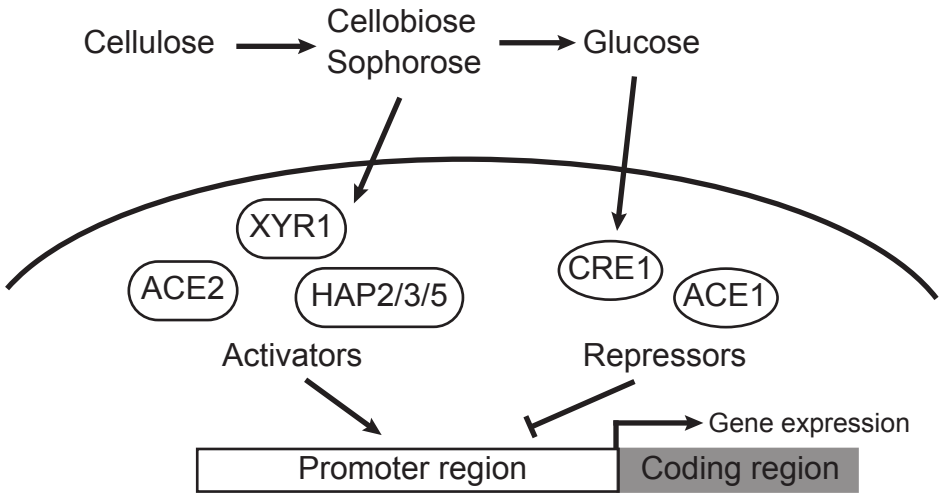


FIg. 1.3. Schematic representation of the transcription factors and their function in cellulolytic gene expression.

(Carle-Urioste*, et al.*, 1997). This results indicated a critical requirement for basal expression of the cellulase system for induction of synthesis of its own transcripts by cellulose.

Cellulase production of *H. jecorina* regulated at the transcriptional level implies tight regulation of the respective promoters. In deed, three positive transcriptional activators (XYR1, ACE2 and the HAP2/3/5 complex) as well as two repressors (ACE1 and CRE1) have been demonstrated to be involved in this regulation (Fig. 1.3). XYR1 (Xylanase regulator 1) was found as an orthologue of the xlnR (xylanase regulator) gene of *Aspergillus niger* (Van Peij*, et al.*, 1998) and is known as a general and main activator of cellulase and hemicellulase gene expression (Stricker*, et al.*, 2006). ACE2 (activator of cellulase expression 2) also identified as a transcriptional activator of *cel7A* and *cel6A* gene expression (Aro*, et al.*, 2001). Deletion of the gene encoding ACE2 led to lowered induction kinetics of cellulase mRNAs and reduced cellulase activity by 30 to 70%. In addition, Zeilinger et al. (1998, 2001) found HAP2/3/5 protein complex as an activator of *cel6A* gene transcription dependent on a CCAAT box (Zeilinger*, et al.*, 1998, Zeilinger*, et al.*, 2001), which is a popular motif for transcription factor binding in eukaryotic gene (Mantovani, 1998). CRE1 (catabolite-responsive element 1) is a key protein for carbon catabolite repression caused by glucose (Ilmén*, et al.*, 1996). This repressor protein was isolated as an equivalent of *Aspergillus nidulans* CreA (Dowzer & Kelly, 1991). CRE1/CreA function have been well characterized in many ascomycetous fungi (reviewed in (Aro*, et al.*, 2005). In addition to CRE1, another repressor protein ACE1 was isolated from *H. jecorina* (Saloheimo*, et al.*, 2000, Aro*, et al.*, 2003). CRE1 and ACE1 have been reported to repress the expression of not only cellulase genes but also the gene encoding the activator XYR1 (Mach-Aigner*, et al.*, 2008).

1.5 Cellulose degradation of the basidiomycete *Phanerochaete chrysosporium*

1.5.1 Cellulolytic enzymes in *P. chrysosporium*

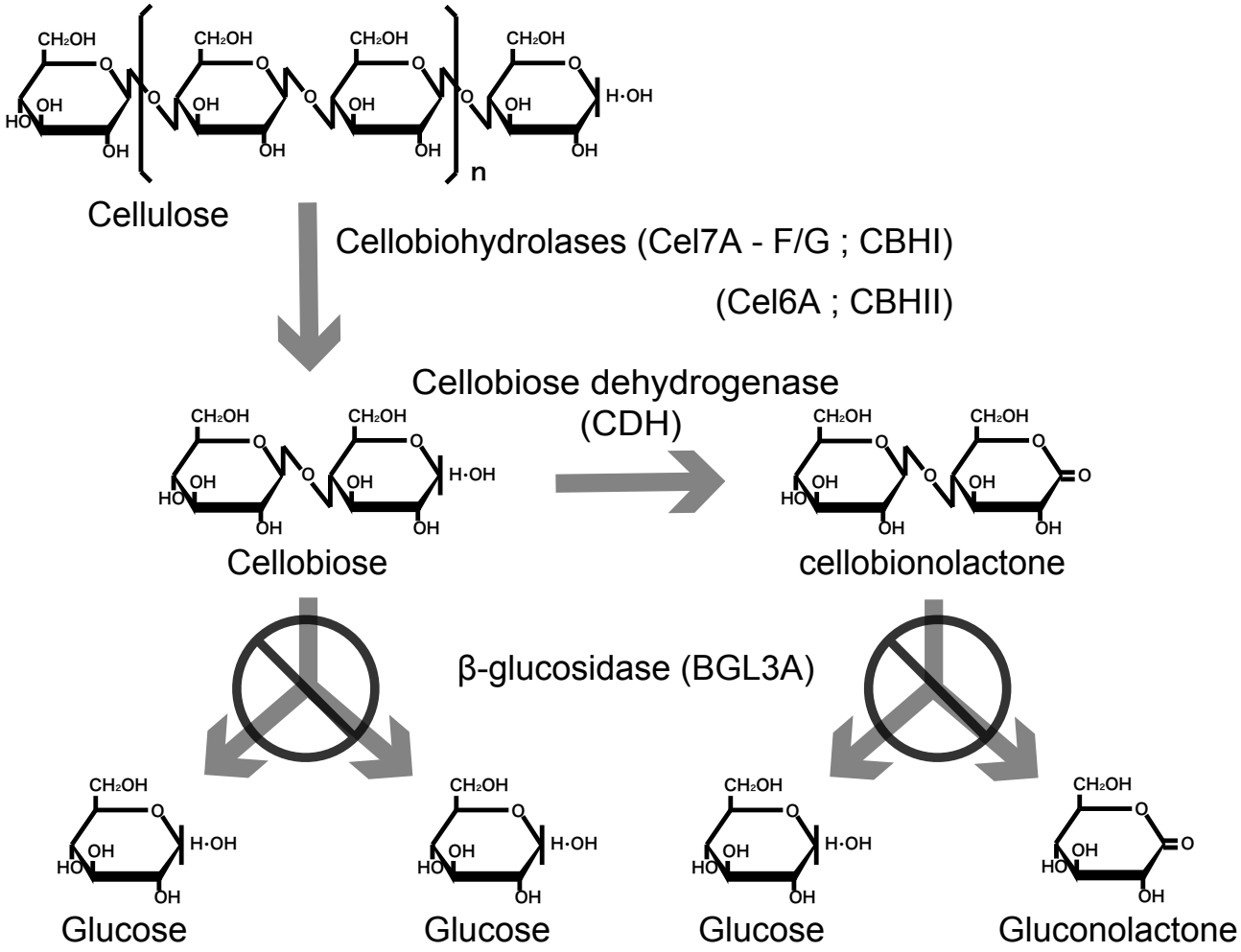
The basidiomycete *Phanerochaete chrysosporium* (anamorph *Sporotrichum pulverulentum*) (Burdsall & Eslyn, 1974) is the most investigated fungus from the viewpoint of lignocellulose degradation. Eriksson and Pettersson (1975) purified exo- and endo-glucanases from the culture filterates of this fungus (Eriksson & Pettersson, 1975, Eriksson & Pettersson, 1975). In the further research, it was appeared that the culture filtrate of *P. chrysosporium* grown in the presence of cellulose contains CBHI and CBHII belonging to GH family 7 and 6 (Uzcategui*, et al.*, 1991), and four EGs classified in GH families 5 and 12 (Uzcategui*, et al.*, 1991, Henriksson*, et al.*, 1999). In 2004, the total genome sequence of *P. chrysosporium* was sequenced (Martinez*, et al.*, 2004) and a number of genes encoding cellulolytic enzymes were predicted. The genome analysis revealed the existence of many genes encoding homologues of *H. jecorina* EGIV (GH family 61), and the cDNA encoding on of these genes has been cloned from a cellulolytic culture of this fungus (Vanden Wymelenberg*, et al.*, 2002). Recently, a gene encoding the homologue of *H. jecorina* EGV (GH family 45) was identified from this fungus. The corresponding enzyme (Cel45A) was heterologously expressed and characterized that Cel45A act in concert with Cel6A, indicating that this enzyme work synergistically with another cellulolytic enzymes (Igarashi*, et al.*, 2008). Thus, homologues of all *H. jecorina* cellulases have been identified in *P. chrysosporium*.

In addition to these hydrolases, *P. chrysosporium* also produces cellobiose dehydrogenase (CDH), an oxidase participated in cellulose degradation. First, cellobiose:quinone oxidoreductase (CBQ) and cellobiose oxidase (CBO) were separately purified from the culture fluids of *P. chrysosporium* (Westermark & Eriksson, 1974, Westermark & Eriksson, 1974, Westermark & Eriksson, 1975, Ayers*, et al.*, 1978). Later, CBQ was shown as a breakdown product of CBO (Wood & Wood, 1992, Habu*, et al.*, 1993, Raices*, et al.*, 2002), and is now called CDH (Bao*, et al.*, 1993).

A single gene coding CDH was found in the genome sequence of *P. chrysosporium* (Martinez*, et al.*, 2004), whereas no CDH was found in the total genome sequence of *H. jecorina* (Martinez*, et al.*, 2008).

1.5.2 The mechanism of cellulose degradation by *P. chrysosporium*

In the cellulolytic system of *P. chrysosporium*, CBHI, CBHII and EGs catalyze the hydrolysis of crystalline cellulose to cellobiose and cellooligosaccharides (Streamer*, et al.*, 1975, Uzcategui*, et al.*, 1991, Uzcategui*, et al.*, 1991) (Scheme 1.1). In addition, CDH oxidizes these products to the corresponding lactones (Ayers*, et al.*, 1978, Bao*, et al.*, 1993, Henriksson*, et al.*, 1998). Igarashi et al. revealed the function of CDH in cellulose degradation that CDH enhances CBHI activity by relieving product inhibition (Igarashi*, et al.*, 1998). Cellobiose, cellooligosaccharides and corresponding lactones have been considered to be further hydrolyzed to glucose and gluconolactone by extracellular β-glucosidase (BGL3A; EC 3.2.1.21; (Deshpande*, et al.*, 1978, Smith & Gold, 1979). In the previous report, however, it was showed that the Km value of cellobiose for BGL3A is considerably higher than that for CDH, and BGL3A does not hydrolyze cellobionolactone, suggesting a kinetic disadvantage of BGL3A under cellulolytic conditions (Lymar*, et al.*, 1995, Igarashi*, et al.*, 2003). In addition, it is reported that BGL3A is functionally a glucan 1,3-β-glucosidase (Igarashi*, et al.*, 2003), playing an important role in fungal cell wall metabolism rather than cellulose degradation. Moreover, transcription of *cdh* was promoted by cellobiose, whereas that of bgl3A was repressed, suggesting that BGL3A is not involved in the extracellular cellulose degradation system of this fungus (Yoshida*, et al.*, 2004). *P. chrysosporium* is predicted to have only one extracellular BGL in its genome, thus, it is predicted that the product of CBH, EG, and CDH were assimilated by the fungal cell as a form of cellobiose, cellooligosaccharides and their lactones, and then hydrolyzed by intracellular β-glucosidases (BGL). Two genes encoding homologous proteins of BGL belonging to GH family 1 (BGL1A and



Scheme 1.1 Cellulose degradation by *P. chrysosporium*.

BGL1B) were cloned from *P. chrysosporium* and characterized as intracellular BGLs (Tsukada*, et al.*, 2006). The transcription of both *bgl1A* and *1B* were observed in cellobiose containing culture, while only that of *bgl1B* repressed by glucose, suggesting that BGL1B may at least be responsible for cellobiose metabolism.

1.5.3 Sequences and structures of Cel7s in *P. chrysosporium*

P. chrysosporium produces multicomponent extracellular cellulases (Eriksson & Pettersson, 1975, Eriksson & Pettersson, 1975, Eriksson, 1978), as described above. Sims et al. (1988) identified a CBHI gene using a DNA fragment of cbhI from *H. jecorina* as a probe (Sims*, et al.*, 1988). In the further genetic analysis of *P. chrysosporium*, six genes encoding CBHI (*cel7A-F*) were identified in the genome (Covert*, et al.*, 1992, Vanden Wymelenberg*, et al.*, 1993, Sims*, et al.*,

1994), while only one gene for CBHII (*cel6A*) were identified (Tempelaars, 1994), and this was confirmed by the total genome sequencing of this fungus (Martinez*, et al.*, 2004). The analysis of genomic organization showed a cluster of *cel7A*, *cel7B* and *cel7C*, in which *cel7A* and *cel7B* are separated by only 750 bp and are located 14 kb upstream of *cel7C* (Covert*, et al.*, 1992, Covert*, et al.*, 1992). Recently, an additional gene was found and named *cel7G*; this is a duplication of *cel7F*, because the nucleic acid sequence is exactly the same, suggesting that this fungus carries a total of seven genes possibly encoding Cel7s (Vanden Wymelenberg*, et al.*, 2006). These *cel7* genes have a region encoding CBM, but it is absent from *cel7A* (Covert*, et al.*, 1992). The nucleic acid and corresponding amino acid sequences of *cel7* genes show a high level of similarity. As showin in Table 1.3, the nucleic acid sequence identities between *cel7A- cel7F/G* catalytic domains and CBMs are about 70–80%. The protein characteristics of Cel7 isozymes have not been investigated, except for two major isozymes (Cel7C and D) (Eriksson & Pettersson, 1975, Streamer*, et al.*, 1975, Uzcategui*, et al.*, 1991, Igarashi*, et al.*, 1998), mainly because the minor Cel7 isozymes are extremely difficult to separate from each other. Thus, enzymatic characteristics of another Cel7s are remain unclear.

Table 1.3 Nucleic acid sequence identity between cel7 genes (%)

	cel7A	*cel7B*	*cel7C*	*cel7D*	*cel7E*	*cel7F/G*
cel7B	67		66	66	63	64
cel7C	73	73		93	82	81
cel7D	71	71	83		81	81
cel7E	70	69	81	81		91
cel7F/G	72	71	84	82	83	

The numbers with the white background indicate catalytic domain sequence identities, and those with the gray background indicate CBM sequence identities.

Three-dimensional structure of Cel7D was determined by Munoz et al. (Munoz*, et al.*, 2001). They also performed homology modeling of another Cel7 enzymes based on the structure of

Cel7D and suggested that Cel7A and Cel7B possibly have endoglucanase like characters compared to Cel7D, because of the amino acid alteration or deletion in tunnel residues around their substrate binding site. In the case of Cel7C, Cel7E and Cel7F/G, however, the difference which affect on substrate binding of these enzymes, were not identified in the comparison with Cel7D, although some amino acid alterations were found in the binding site tunnel of each.

1.5.4 Transcriptinal regulation of *cel7* genes in *P. chrysosporium*

The first transcriptional analysis of a CBHI in *P. chrysosporium* was performed by Sims et al. (1988) by Northern analysis and reported that the transcription was occurred in the presence of cellulose but not in glucose. Thereafter, several transcription analysis was performed by means of polymerase chain reaction (PCR) technique. Covert et al. (1992a) found cluster of three genes encoding CBHI (*cel7A*, *cel7B* and *cel7C*) by means of restriction mapping and sequence analysis of cosmid clones. In this paper, competitive RT-PCR analysis was performed for these genes and revealed that *cel7C* transcripts were abundant in cellulose culture as 1000-fold of *cel7A* and *cel7B* but its transcription was repressed by the presence of glucose. The transcription of *cel7C* was also observed to derepressed when glucose was consumed, whereas *cel7A* and *cel7B* transcription was not subject to glucose repression. Later, they detected another CBHI genes (*cel7D*, celE and *cel7*F) by Southern blot and sequence analysis (Covert *et al*. 1992b). Transcriptional analysis of *cel7* genes were performed by Vanden Wymelenberg et al (1993) and suggested that *cel7D* is most abundantly transcribed among *cel7* genes in the presence of 0.4% cellulose supplemented with 0.1% cellobiose. Substrate dependence of regulation of *cel7* genes were characterized using some natural carbon sources. It was observed that *cel7C* and *cel7D* are coordinately regulated in a substrate dependent manner under Avicel, ball-milled straw and glucose (Tempelaars*, et al.*, 1994, Birch*, et al.*, 1995, Broda*, et al.*, 1995, Broda*, et al.*, 1996). In a culture with low concentration of glucose, derepression of *cel7C* was observed earlier than that of *cel7D* (Broda *et al*. 1995). Lamar et al.

(1995) detected higher amount of *cel7A* transcripts in *P. chrysosporium*-colonized soil culture than in submerged culture containing cellulose (Lamar*, et al.*, 1995). In the case of the cultivation using aspen wood chip, transcription of *cel7C* and *cel7E* were abundant and that of *cel7A* was also observed, whereas *cel7D* was hardly detected (Vallim*, et al.*, 1998). These observation indicate the complex pattern of *cel7* gene transcription, and also implied the importance of the multiplicity of *cel7* genes, which dose not merely existed as multi copy.

In addition to *cel7* genes, a transcriptional regulation of *cel6A* was also reported previously. Although the expression of *cel6A* occurred under cellulose but repressed by glucose as well as *cel7C* or *cel7D*, the pattern of its expression was indipendent from that of *cel7C* and *cel7D* under several carbon sources (Tempelaars 1994, Broda et al 1995). The expression of *cel6A* was also observed as higher amount than that of *cel7C* in the aspen wood chips colonized by this fungus (Vallim 1998). Although the expression of *cel6A* did not seem to be co-regulated with *cel7C* or *cel7D*, high level of its expression under cellulose or cellulosic substrate indicated that *cel6A* play a role in cellulose degradation.

1.6 Methodology for the quantitative analysis of Cel7 enzymes and corresponding genes

Fungal cellulase mixture consists of a high proportion of enzymes belonging to GH family 7, which typically account for more than 50% of total secreted protein, emphasizing the importance of the investigation of this family for achieving a better understanding of fungal cellulose utilization. In the study of *H. jecorina,* several quantification methods for separating and measuring each Cel7 enzyme have been developed. While various staining methods have been described to detect cellulases after electrophoresis (Nummi*, et al.*, 1980, Bartley*, et al.*, 1984, Biely*, et al.*, 1985, Van Tilbeurgh & Claeyssens, 1985, Biely & Markovics, 1988), the enzyme activity was poorly preserved. In addition to these methods, immunological techniques were also used in the analysis of cellulase components (Fagerstam & Pettersson, 1980, Nummi*, et al.*, 1980, Szakacs-Dobozi &

Halasz, 1986). There have been several reports on quantification of CBHI and EGI in culture fluid using polyclonal and monoclonal antibodies in *Hypocrea jecorina* (Messner*, et al.*, 1988, Mischak*, et al.*, 1989, Kolbe & Kubicek, 1990, Luderer*, et al.*, 1991). In these cases, however, it was difficult to avoid non-specific adsorption of each antibody. Later, the quantitaive analysis of Cel7 production was performed as a measurment of mRNA transcribed from a gene encoding each Cel7 enzyme. Northern hybridization was applied for detection of their mRNA (Ilmen*, et al.*, 1997) and is now a widely used method for the detection and semi-quantification of *cel7* transcripts.

In the case of *Phanerochaete chrysosporium*, on the other hand, the situation regarding quantification of Cel7s is more complicated because this fungus carries a total of seven genes possibly encoding Cel7s (Covert*, et al.*, 1992, Covert*, et al.*, 1992, Vanden Wymelenberg*, et al.*, 2006). Transcription of these Cel7s has been examined by means of Northern analysis (Sims*, et al.*, 1988), RT-PCR (Tempelaars*, et al.*, 1994, Broda*, et al.*, 1995), and competitive RT-PCR (Covert*, et al.*, 1992a, Vanden Wymelenberg*, et al.*, 1993, Lamar*, et al.*, 1995, Vallim*, et al.*, 1998). Recently, expressed sequence tag analysis (Sato*, et al.*, 2009) and microarray analysis (Vanden Wymelenberg*, et al.*, 2009) were performed for the quantification of *cel7* gene transcripts. However, quantitative analysis by real-time PCR has not been performed for these genes, because the *cel7* genes show high levels of sequence identity (at least around 70%), making it difficult to design specific primer sets for real-time PCR amplification (Table 1.3).

1.7 Aim of this research

Since cellulose is a water-insoluble polymer and thus requires decomposition to soluble compounds to be recognized by fungi, the mechanism of the initial recognition and subsequent response of fungi for cellulolytic enzyme production is a key point to understand fungal cellulose degradation. As previously suggested, basal expression and/or carbon catabolite derepression of some cellulolytic genes probably have a important role in cellulose recognition of *P. chrysosporium*.

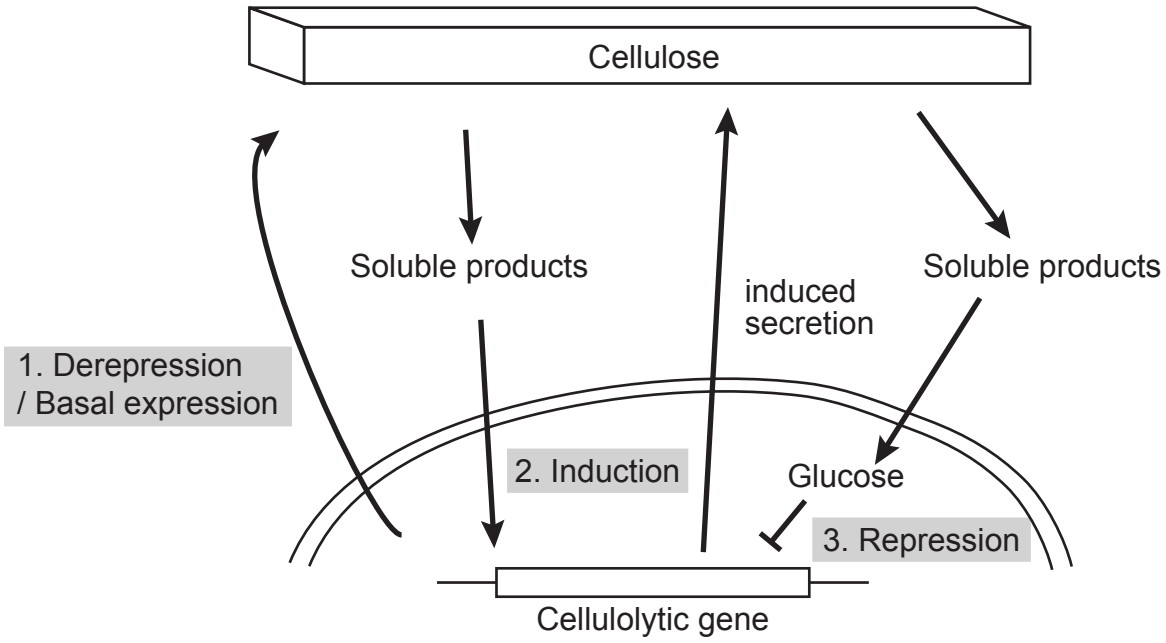
Moreover, some soluble products of initial cellulose degradation may act as inducers for further production of cellulolytic genes, and the increased assimilation of glucose is regarded to repress the enzyme production (Scheme 1.2). However, detailed analysis of protein production or gene expression of a variety of cellulolytic enzymes have not been performed especially for Cel7s, so that the mechanism of cellulose degradation and the significance of multiplicity of cellulolytic enzymes in *P. chrysosporium* is still remain unknown. In this study, the expression of cellulolytic genes under carbon starvation and water-soluble cellooligosaccharides were investigated by real-time RT-PCR analysis, and the transcriptional response of these genes against derepression and induction were evaluated quantitatively.

Since carbon catabolite derepression is considered to occur as a fungal response to carbon starved condition, which cannnot make the fungus grow, the cultivation method of *P. chrysosporium* was developed to analyze the behavior of cellulolytic gene expression under such condition in chapter 2-section 1. In this section, three stages of cultivation was used; a pre-cultivation with glucose, a resting cultivation without any carbon sources, and a main cultivation with glucose or no carbon. The transcript number of *cel6A*, *cel7D*, *cdh* and *bgl3A* were measured by real-time RT-PCR using fluorescent-rabeled DNA probe (TaqMan probe) during the main cultivation, and were compared between the culture containing glucose or no carbon source to determine whether carbon catabolite derepression is occurred in these gene transcription.

It is important to analyze the transcription of another *cel7*s quantitatively in addition to *cel7D* to investigate the multiplicity of *cel7* genes. However, the sequence similarity of *cel7* genes make the specific primer design in real-time PCR difficult. Fluorescent probes, such as TaqMan probe, are generally expensive and thus not practical to use for a number of gene targets, although they may improve the specific detection of target gene transcripts. In chapter 2-section 2, a method was developed to quantify *cel7* gene transcripts individually by means of real-time RT-PCR based on 3'-untranslated regions (3'-UTR), which show little sequence similarity, using SYBR Green I, a

fluorescent molecule which is non-specifically interacting to double-strand DNA. The method developed here was applied to analyze the time courses of the expression of *cel7*s under the condition of carbon stavation to evaluate whether these gene transcription are derepressed or not.

Since cellobiose and cellooligosaccharides are the major products of cellulose hydrolysis and glucose works as a catabolite repressor, it is possible that *P. chrysosporium* regulate the cellulolytic gene expression by accepting such soluble sugars to recognize their environment. However, an inducer of cellulolytic genes have not been detected. In chapter 3, transcription of cellulolytic genes were quantified under glucose, cellobiose, and cellooligosaccharides to investigate the induction of gene transcription using the real-time RT-PCR method developed in chapter 2. Cultivation method was also based on that developed in chapter 2 with some modification as described in chapter 3.



Scheme 1.2. Predicted process of cellulolytic enzyme production in *P. chrysosporium*.

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Chapter 2

Quantitative transcriptional analysis of the genes encoding cellulolytic enzymes in the basidiomycete *Phanerochaete chrysosporium* under carbon starved environment

2.1 Real-time quantitative analysis of carbon catabolite derepression of cellulolytic genes expressed in the basidiomycete *Phanerochaete chrysosporium*

2.1.1 Introduction

The mechanism of initial up-regulation of cellulolytic gene transcription in *Phanerochaete chrysosporium* during cellulose-degrading cultivation is essential for cellulose utilization by the fungus, because cellulose is an insoluble solid substrate and might not be recognizable directly. The production of cellulose-degrading enzymes of *P. chrysosporium* is considered to be regulated by induction, repression, and derepression of transcription of the corresponding genes, and among them, derepression is considered as a key regulation at initial stage of cellulose degradation. Besides derepression, constitutively conducted basal expression could be an another key as well as the case of *H. jecorina*.

In this section, it is demonstrated that carbon catabolite derepression plays a significant role for the initial transcription of the cellulolytic genes. The cultivation method was developed, which is able to assess the cellulolytic gene expression in carbon starved environment, because the fungus cannot grow in a cultivation without any carbon source. Transcription of the cellulolytic genes was analyzed by means of real-time quantitative RT-PCR. Moreover, expression of two cellobiohydrolase genes (*cel6A* and *cel7D*) was quantitatively analyzed in terms of the rate of increase of transcript numbers and the lag-time of derepression in the presence or absence of glucose.

2.1.2 Materials and Methods

Fungal strain and culture conditions

Phanerochaete chrysosporium strain K-3 (Johnsrud and Eriksson, 1985) was cultivated in 400 mL of modified Kremer and Wood medium (Kremer and Wood, 1992), which did not contain urea or $MnSO_4$, for pre-culture. The medium contained, per liter: 2.2 g 2,2-dimethylsuccinic acid, 0.26 g $(NH_4)_2HPO_4$, 3.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 74 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 5 mg $MnSO_4 \cdot 7H_2O$, 5 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $CoCl_2 \cdot 6H_2O$, 0.1 mg thiamine · HCl. Glucose (100 mM) was added to the medium as a sole carbon source. The inoculated medium was maintained at 37˚C and shaken at 150 rpm for pre-grown. After 3 days of cultivation, the mycelia were harvested, washed with the medium containing no carbon source and transferred to 400 mL of same medium without any carbon source (as a resting culture). After 6 h of cultivation, 5000 μM, 500 μM or 50 μM glucose was added to the medium and cultivated another 6 h (as a main culture).

Measurement of extracellular glucose concentration

A 5 mL aliquot of the main culture was harvested every hour, and the supernatant was boiled for 5 min to inactivate extracellular enzymes, then the glucose concentration was measured using Glucose CII-Test Wako (Wako, Japan) as follows: 50 μL of medium was mixed with 950 μL of coloring reagent, and the mixture was incubated for 10 min at 30˚C. Glucose concentration was calculated from the absorbance using a value of $\varepsilon_{505} = 317\ M^{-1}\ cm^{-1}$.

Quantification of cellulolytic gene transcripts

The mycelia harvested from the main culture were immediately frozen in liquid nitrogen and stored at -80˚C to extract mRNA. Total RNA was extracted from collected mycelia using an E.Z.N.A. Fungal RNA Kit (Omega Bio-tek, USA) and mRNA was purified with an Oligotex-

dT30<super>mRNA Purification Kit (Takara Bio, Japan). First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Japan), 5'-RACE cDNA synthesis primer of a SMART RACE cDNA Amplification Kit (Clontech, USA) and 3'-RACE Adapter primer (Invitrogen, USA), and was used as the template. PCR was performed using TaKaRa Ex Taq (Takara Bio, Japan) with primer sets for *cel6A* (Accession No.: S76141), *cel7D* (Accession No.: L22656), *cdh* (Accession No.: X88897), *bgl3A* (Accession No.: AB081121) and actin gene (*act*, Accession No.: AB115328), as listed in Table 2.1.1. Real-time quantitative PCRs were performed in an ABIPRISM™ 7700 Sequence Detection System (PE Applied Biosystems, USA), as follows: 5 µl of template solution was mixed with 20 µl of solution containing 12.5 µl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen, USA), 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 0.5 µl of 10 µM TaqMan probe, and 6 µl of sterile distilled water. The mixtures were initially incubated at 50 °C for 2 min and at 95 °C for another 2 min, followed by amplification for up to 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The transcript number of actin gene was quantified as an internal standard. Primers and TaqMan probes for quantification of each transcript were designed using Primer Express™ version1.0 (PE Applied Biosystems, USA), and are listed in Table 2.1.1.

Table 2.1.1 Primers and TaqMan probes for RT-PCR and real-time quantitative RT-PCR

Primers for RT-PCR			
	cel6A	F	ATTGTTGCTCAGATTCAACAGTTC
		R	CAGCGTCCGGCAGAG
	cel7D	F	CGAGGCTAATGTCGGCAA
		R	TTAGTAGCACTGCGAGTAGTAAGGG
	cdh	F	TCGGTCAACGGAGCTCTCT
		R	CGCCAGAAGTTGAGTTTGG
	bgl3A	F	CAACTCCGGAACAACCTACTTC
		R	CGTTGCCTGCTTCCTGAC
	act	F	GCCGTGTTCCCATCCAT
		R	CACTTGTAGATGGAGTTGTAGGTCGT
Primers for real-time RT-PCR			
	cel6A	F	GGAGAGTTCAGCATTGCCAAC
		R	GCGGACATCAGGGAACTGTT
	cel7D	F	ACTGGTTACTGCGACTCGCA
		R	AGTTGCCGACATTAGCCTCG
	cdh	F	GCAGGTGCTTCTCCCAAACTC
		R	GGGCATAACGGGTAAAGCC
	bgl3A	F	GAGCAAGAGCATTTTCGGGA
		R	TCCGTAGAGTTCATGCTCCGT
	act	F	ATGTGCAAGGCTGGCTTTG
		R	GGCGACCAACGATGGATG
TaqMan probe[a]			
	cel6A		CGGACAAGCCAACTACGAGAACTACATCGA
	cel7D		TGCCCGAAGGACATCAAGTTCATTAACG
	cdh		TGGCGCGCATACTCTGGTTCGG
	bgl3A		TCCAGTTCGTCCAACGTCGATGACAG
	act		ACGATGCTCCCCGTGCCGTGT

[a]At its 5’ end, the probe contains 6FAM (6-carboxyfluorescein), the emission spectrum of which is quenched by TAMRA (6-carboxy-N,N,N’,N’-tetramethylrhodamine) at its 3’ end.

2.1.3 Results

Expression levels of *cel6A*, *cel7D*, *cdh* and *bgl3A* in cultures containing 5000 μM, 500 μM and 50 μM glucose (G5000, G500 and G50 respectively) or without glucose (G0) after main cultivation for 6 h were compared by RT-PCR, as shown in Fig. 2.1.1a. In the cases of *cel6A* and *cel7D*, G500, G50 and G0 resulted in stronger band intensities of transcripts than G5000. In the case of *cdh*, G0 resulted in moderate expression and only weak signals were observed under other conditions. Weak expression of *bgl3A* was observed almost independently of glucose concentration.

Distinct differences of expression levels were observed among the genes, depending upon the glucose concentration at the start of the main cultivation. The copy numbers of transcripts at the start (0 h) and after 6 h of the main cultivation were quantified to assess gene expression level, by means of real-time quantitative PCR. Fig. 2.1.1b shows the copy numbers of *cel6A*, *cel7D*, *cdh* and *bgl3A* mRNAs from 0 h and 6 h. In the cases of *cel6A*, *cel7D* and *cdh*, a higher concentration of glucose led to a smaller increase of the number of transcripts. For example, the transcript number of *cel6A* after 6 h was 210-fold higher than that at 0 h in G0, whereas it was 61-fold higher in G50 and 80-fold higher in G500, but only 3.3-fold higher in G5000. The transcript number of *cel7D* at 6 h was 310-fold higher than that at 0 h in G0, but only 0.97-fold in G5000. In the case of *cdh*, the corresponding values were 21-fold in G0 and 3.1-fold in G5000. On the other hand, there was no apparent relationship between transcript number and added glucose concentration in the case of *bgl3A*, the increases were 2.0-, 1.4-, 1.6- and 1.9-fold in G0, G50, G500 and G5000, respectively.

The time course of extracellular glucose concentration in the main culture medium is shown in Fig. 2.1.2. In G5000, the amount of remaining glucose was 2800 μM after cultivation for 6 h. In contrast, glucose in G500 and G50 was completely consumed after 4 h and 2 h, respectively. In G0 culture, 18 μM glucose was detected at the start of the cultivation as a contamination from pre-grown culture. However, these amount of glucose was totally consumed within 1h of the main

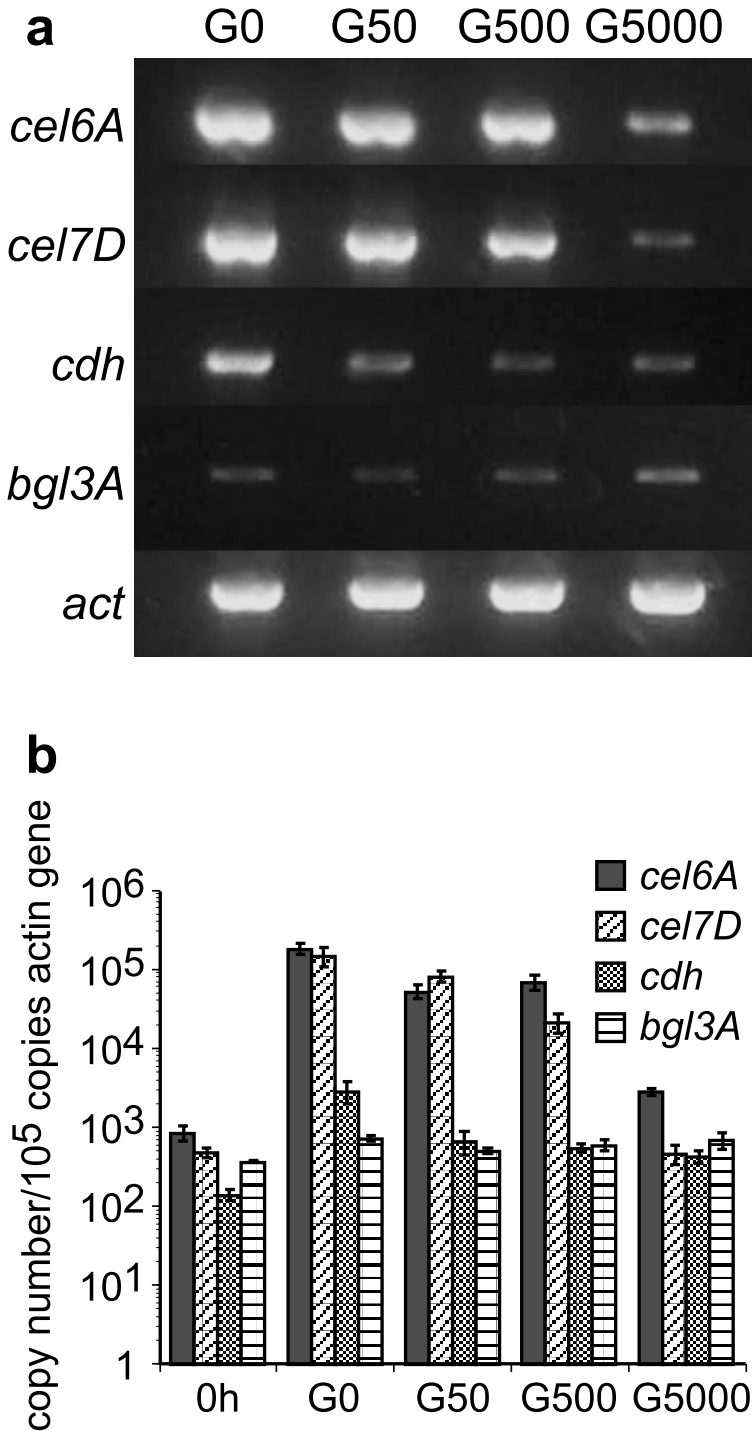

Fig. 2.1.1. Gene expression of *cel6A*, *cel7D*, *cdh* and *bgl3A* monitored by electrophoresis (**a**) and quantitative RT-PCR (**b**). **a** RT-PCR was performed using cDNAs from 6 h culture. Expression of the actin gene was analyzed as a control to compare expression levels between different culture conditions. **b** cDNAs immediately before the start of main cultivation (0 h) and after cultivation for 6 h under the indicated condition (G0, G50, G500 and G5000) were used. Copy number was normalized by the copy number of actin transcripts from the same sample. The vertical axis indicates the copy number of each gene per 10^5 copies of actin transcript. Each error bar shows the standard error in triplicate tests for each sample.

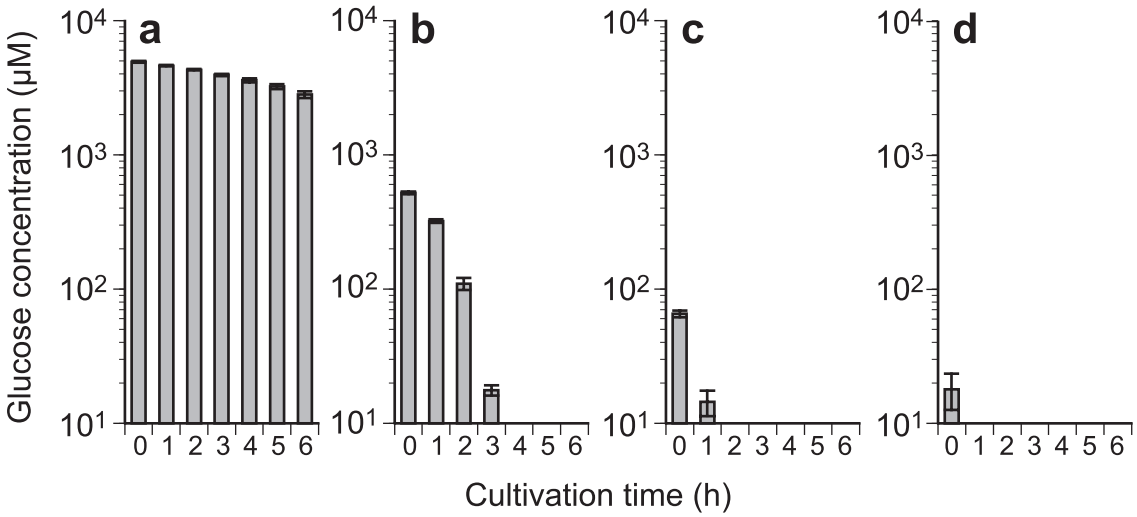

Fig. 2.1.2. Time course of extracellular glucose concentration in the main culture medium. Glucose 5000 μM (**a**), 500 μM (**b**), 50 μM (**c**) or 0 μM (**d**) was added at the start of the cultivation. Glucose, which remained from the resting culture, was detected at the start point (0 h). Each error bar shows the standard error in triplicate tests for each sample.

cultivation. Therefore, G0 culture could be taken as a control experiment without any glucose in the present study. The glucose consumption was associated with high expression levels of *cel6A* and *cel7D* in the cells in G0, G50 and G500, while the expression of that genes were repressed in G5000 (Fig. 2.1.1).

Since the transcripts number of *cel6A*, *cel7D* and *cdh* were greatly increased in G0 or G50, the time course of transcript levels was analyzed under these two conditions to examine the activation of gene expression. As shown in Fig. 2.1.3, the copy numbers of *cel6A*, *cel7D* and *cdh* transcripts increased exponentially after 2 h. This time point is consistent with the time when glucose was totally consumed from the G50 medium. Similar exponential increases were observed in both G0 and G50, though the transcript numbers were larger in G0 than G50 at all time points. In contrast, the copy number of *bgl3A* transcripts showed no time dependence, and no obvious difference of transcript numbers was observed between G0 and G50.

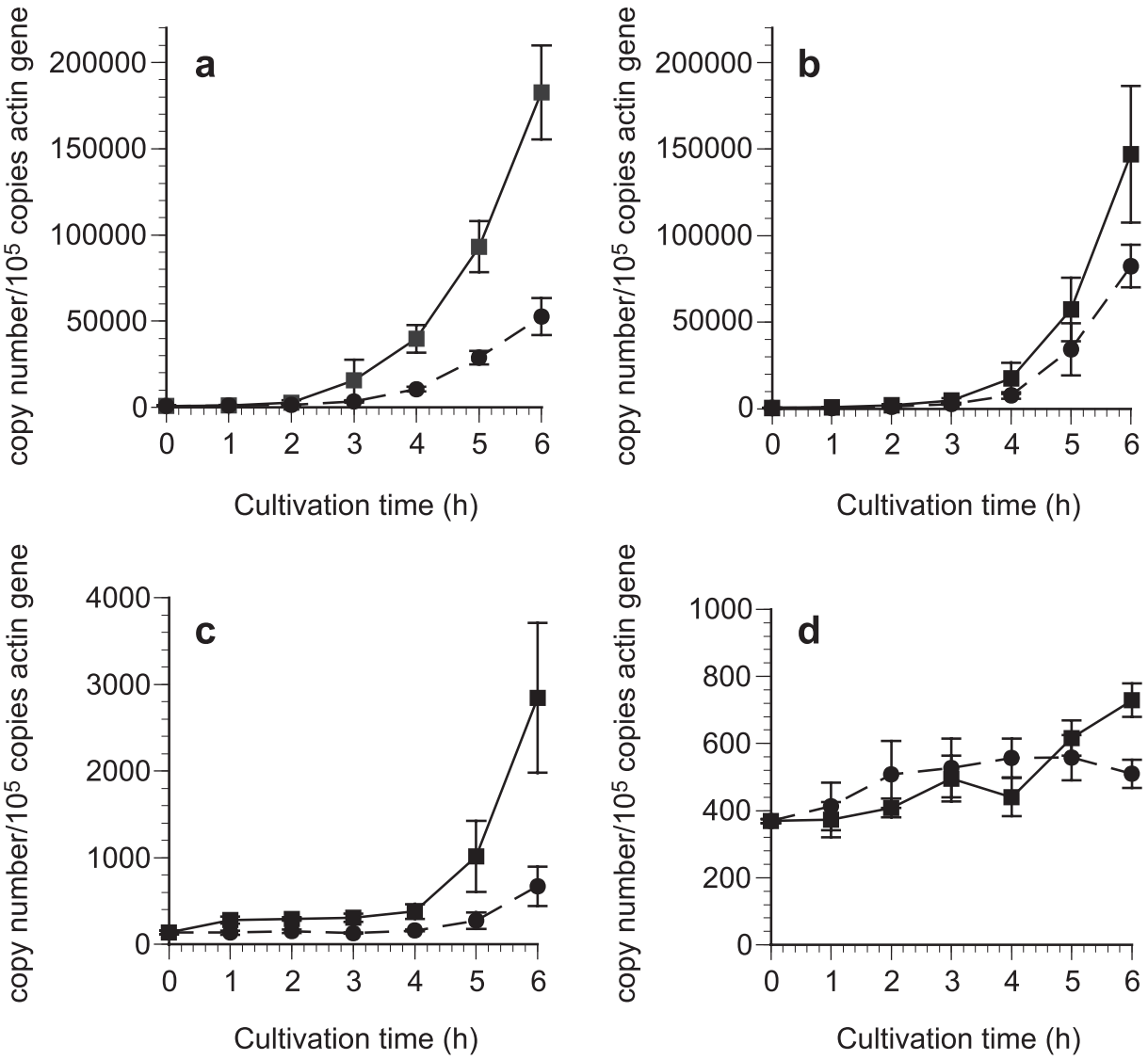

Fig. 2.1.3. Time course of *cel6A* (**a**), *cel7D* (**b**), *cdh* (**c**) and *bgl3A* (**d**) transcripts measured by quantitative RT-PCR. Transcript numbers of each gene in G0 (■) and G50 (●) were normalized as described in Fig. 2.1.1b. Each error bar shows the standard error in triplicate tests for each sample.

The transcript numbers of *cel6A* and *cel7D* in G0 and G50 (Fig. 2.1.3) were re-plotted with an exponential y-axis (Fig. 2.1.4), and the parameters of the resulting plots ($y=a*10^{bx}$) are listed in Table 2.1.2. The rates of increase of transcripts of *cel6A* and *cel7D* were not affected by the glucose concentration of the culture, and were 2.1 times/h and 2.7 times/h, respectively. Moreover, the distance between plots for G0 and G50, which indicates the time-lag of gene derepression

caused by addition of 50 μM glucose, was 1.6 h for *cel6A* and 0.6 h for *cel7D*, indicating that the responses of *cel6A* and *cel7D* transcription to derepression are different.

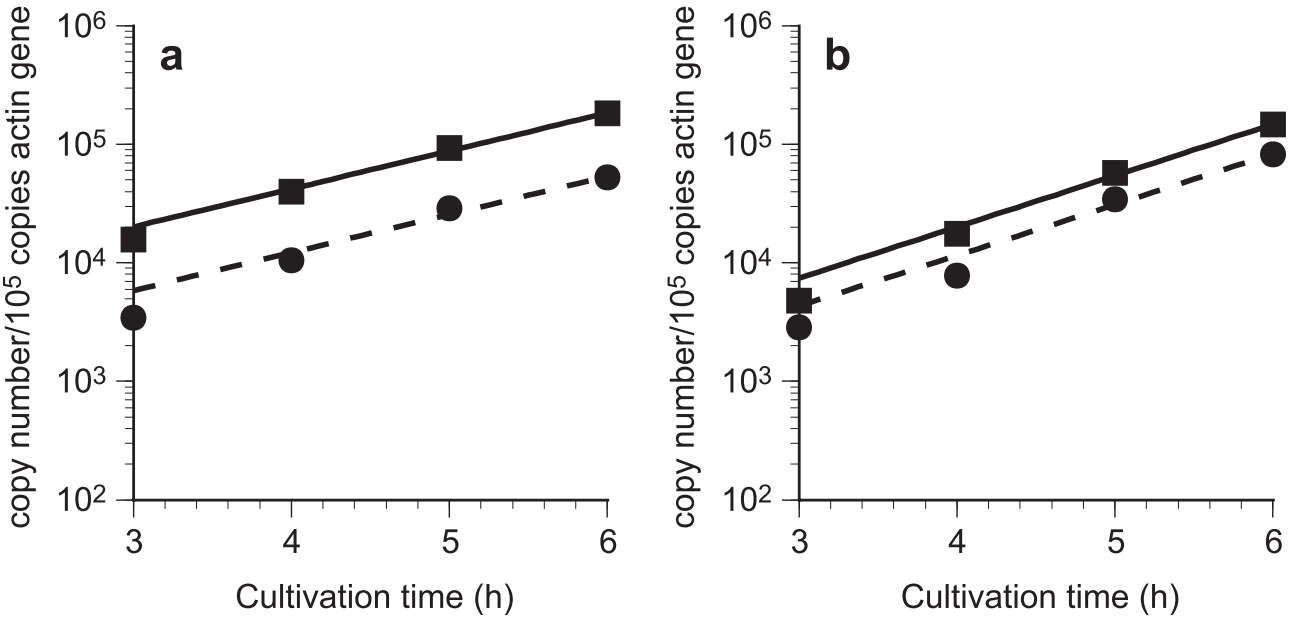

Fig. 2.1.4. Time course of *cel6A* (**a**) and *cel7D* (**b**) transcripts during exponential increase phase (3-6 h). Transcript numbers of each gene in G0 (■) and G50 (●) (Fig. 2.1.3) were re-plotted with an exponential y-axis.

Table 2.1.2 Parameters of regression curve ($y=a*10^{bx}$)

		a	b	slope (copy no./hour)[a]	time for 10^4 copies (hour)
cel6A	G0	2200±500	0.32±0.02	2.1	2.1
	G50	640±330	0.32±0.04	2.1	3.7
cel7D	G0	370±110	0.43±0.02	2.7	3.3
	G50	220±130	0.43±0.04	2.7	3.9

[a]The slope of each curve was calculated as 10^b.

2.1.4 Discussion

According to the previous studies, it has been suggested that BGL3A is not involved in the extracellular cellulose degradation system of this fungus (Lymar, *et al.,* 1995, Igarashi, *et al.,* 2003, Yoshida, *et al.,* 2004). In this chapter, we found *bgl3A* expression is essentially independent of glucose concentration, indicating that *bgl3A* is regulated in a different manner from the other three cellulolytic genes, and may not act in concert with the other cellulose-degrading enzymes.

In filamentous fungi, glucose generally serves as a repressor of cellulolytic genes and a small amount of gene expression is provoked when the glucose concentration falls (Broda, *et al.*, 1995, Ilmen, *et al.,* 1997). In some cases, however, lower expression level of *cbh* (*cel7A* and *cel7B*) has been detected constitutively (Covert, *et al*., 1992), although the actual number of transcripts may be small compared to those of *cel7C* or *cel7D*. The presence of glucose in the culture or environment may be recognized as concentration or as intake flux by fungi, and it was reported that concentration is more influential than flux for yeast (Meijer, *et al.,* 1998). However, the relationship between the glucose concentration and the levels of cellulase gene expression were not investigated quantitatively. Accordingly, the change of glucose concentration with cultivation time was monitored and the correlation with cellulolytic gene expression was analyzed to examine the effect of derepression. Moreover, transcript numbers of *cel6A* and *cel7D* were analyzed and compared with the derepression rate of the two genes and the lag time at two glucose concentrations (G0 and G50). The time course analysis of cellulolytic gene expression was performed during the main culture, which was started after 6 h of the resting cultivation (without any carbon source). The reason for the necessity of this resting cultivation is that glucose, which is contained in pre-grown culture, was seemed to be remained in or out of the fungal cells even after washing the mycelia. Indeed, the increases of cellulolytic gene transcripts did not occur during the resting cultivation and were observed after 2 h in the main cultivation. This is indicating that glucose was not completely

washed out and glucose repression may still be effective throughout resting cultivation. The Remaining glucose appears to be consumed totally by the fungus during main cultivation and then carbon catabolite derepression occurred. In the previous study, it has reported that the copy number of *cdh* transcripts showed no significant difference between the culture with 2% glucose and no carbon (Yoshida, *et al*., 2004). In the present study, however, it was observed that the number of *cdh* transcripts was clearly increased during the cultivation with no carbon source. The contradiction between these two investigations could be because of the resting cultivation. In the previous report, glucose from the pre-grown culture or produced by degradation of hyphae was carried over into the transferred medium, so that carbon catabolite repression may continue. In contrast, since the resting cultivation avoid the undesirable influence of glucose contamination, expression of cellulolytic genes were not repressed and derepressed by a relief of carbon catabolite repression. This fact suggest the importance of the resting culture to eliminate the effects of pre-culture conditions.

The derepression rates of *cel6A* and *cel7D* were specific to each gene, and were not influenced by the glucose concentration in the culture medium. The lag times indicated that the response of *cel6A* was slower than that of *cel7D*. Since gene transcription is controlled by the promoter regions, the quantitative analysis of derepression rate and lag time of gene expression could be able to illustrate the character of the promoter activity. It is well known that cellulase production and cellulolytic gene expression in filamentous fungi are controlled by the induction and repression of the relevant genes. Several oligosaccharides have been proposed as candidate inducers in fungi. For example, sophorose induces cellulase expression in *H. jecorina* (Mandels, *et al.,* 1962), gentiobiose induces cellulase of *Penicillium purpurogenum* (Kurasawa, *et al.,* 1992), cellobiose probably act as an inducer in several fungi (Eberhart, *et al.,* 1977, Eriksson, 1978, Canevascini, *et al.,* 1979, Rho, *et al.,* 1982) and cellopentaose is recently reported as a probable inducer for cellobiohydrolases and endoglucanases in *Polyporus arcularius* (Ohnishi, *et al*., 2007a, b). In this study, however, we observed a drastic increase of cellulolytic gene expression in the

absence of inducers. Gene expression of cellulolytic enzymes is regulated by several transcriptional factors in *Hypocrea jecorina* and *Aspergillus* spp. (Schmoll and Kubicek, 2003, Aro, *et al.,* 2005), and these are also thought to regulate in basidiomycete *Trametes versicolor* (Stapleton and Dobson, 2003). As far as we know, however, there has been no report on the identification of cellulolytic gene regulators in basidiomycetes. Since glucose repression of cellulolytic genes in basidiomycetes seems to be similar to that of ascomycetes, it is expected that a repressor protein, such as CREA of *Aspergillus nidulans* (Dowzer and Kelly, 1989, Dowzer and Kelly, 1991, Cubero and Scazzocchio, 1994), regulates in basidiomycetes. Two possibilities are suggested for the mechanism of derepression, release of repressor protein or activation without releasing the repressor, though it is impossible to conclude the true mechanism from the results obtained in the present study. Since basal expression indicates the constitutive transcription of genes, the up-regulation of cellulolytic genes observed here is considered as derepression rather than basal expression. Although the molecular mechanism of the release of the repression is still unknown, the presented results clearly indicate that carbon catabolite derepression plays a key role in cellulolytic gene expression, as well as induction or repression. Moreover, these results suggested that the similar quantitative analysis of another *cel7* gene expression is critical to understand the significance of their multiplicity in carbon starved condition.

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2.2 Quantitative transcriptional analysis of the genes encoding glycoside hydrolase family 7 cellulase isozymes in the basidiomycete *Phanerochaete chrysosporium*

2.2.1 Introduction

According to the results obtained in chapter 2.1, carbon catabolite derepression is considered to be a key regulation in cellulolytic gene expression, as well as induction or repression. Moreover, these results suggested that the quantitative transcription analysis of all *cel7* genes is essential to understand the significance of their multiplicity in carbon starved condition. However, conventional techniques, such as Northern analysis or competitive RT-PCR, were not sufficient for the quantification of the transcription of *cel7* genes. Althogh expressed sequence tag analysis (Sato, *et al*., 2009) and microarray analysis (Vanden Wymelenberg, *et al*., 2009) were performed for the quantification of *cel7*s recently, real-time RT-PCR have not been applied.

In this section, a method to quantify *cel7* gene transcripts individually in *P. chrysosporium* was developed by employing real-time RT-PCR with specific primer sets designed based upon the sequences of the 3' untranslated regions (3'-UTR) of these genes, which show little similarity. This method was applied to analyze the time-courses of expression of *cel7*s under the conditions of carbon starvation to evaluate the regulation by derepression.

2.2.2 Materials and methods

Fungal strain and culture conditions

Phanerochaete chrysosporium strain K-3 (Johnsrud & Eriksson, 1985) was cultivated in 400 mL of modified Kremer and Wood medium (Kremer & Wood, 1992) for pre-culture and resting culture, as described previously (Suzuki*, et al.*, 2008). After 6 h of resting cultivation, mycelia were cultivated for another 6 h without any carbon source (main culture). A 5 mL aliquot of the main culture was harvested every hour for cDNA preparation from collected mycelia. cDNA was constructed as previously described (Suzuki*, et al.*, 2008) and used as the template for real-time quantitative RT-PCR.

Identification of 3'-UTR sequences

The cDNA prepared from the cellulose-grown mycelia was used as the PCR template for 3' rapid amplification of cDNA ends (RACE). The 3'-UTRs of *cel7A*, *cel7B*, *cel7C*, *cel7D*, *cel7E*, and *cel7F/G* genes (GenBank accession nos. **X54411** for both *cel7A* and *cel7B*, **Z22528**, **L22656**, **Z11727** and **Z11729**) were amplified using abridged universal amplification primer (Invitrogen) and gene-specific primers designed based on the total genomic sequences, as listed in Table 2.2.1. The reaction was performed for 35 cycles of denaturation at 98 ˚C for 10 s, annealing at 60 ˚C for 30 s, and extension at 68 ˚C for 30 s using KOD-plus (version 2, Toyobo). Approximately 900 bp of the downstream region of *cel7A*, *cel7C*, *cel7D*, and *cel7F/G* genes was amplified from total genomic DNA of *P. chrysosporium* using the gene-specific primer sets (Table 2.2.1). The reaction was performed for 30 cycles of denaturation at 98 ˚C for 10 s, annealing at 60 ˚C for 30 s, and extension at 68 ˚C for 60 s. All PCR products were cloned in pCR4Blunt-TOPO by using a Zero Blunt TOPO PCR cloning kit (Invitrogen) and *E. coli* JM109 and were sequenced by using DTCS Quick Start Master Mix with DNA sequencer CEQ 8800 (Beckman Coulter).

Verification of the specificity of the primer sets for real-time quantitative RT-PCR

PCR was performed by using the *cel7A-F/G* primer sets for real-time RT-PCR to verify their specificity. The template DNA of this PCR was pCR4Blunt-TOPO containing 3'-RACE products of *cel7A*, *cel7C*, *cel7D*, *cel7E*, and *cel7F/G* and the same vector containing *cel7B* gene ORF. The reaction was performed for 15 cycles of denaturation at 95 ˚C for 30 s, annealing at 60 ˚C for 30 s, and extension at 72 ˚C for 20 s using Takara Ex Taq (Takara Bio). RT-PCR was also performed using cDNA from 2-day-old cellulose-grown mycelia to confirm specific amplification with each primer set. The reaction was performed for 30 cycles of denaturation at 94 ˚C for 30 s, annealing at 60 ˚C for 30 s, and extension at 72 ˚C for 20 s. The RT-PCR products were sequenced as described above. Dissociation curve analysis was performed in a Mx3000P Real-time QPCR System (Stratagene) as follows: 5 μL of template solution was mixed with 20 μL of solution containing 12.5 μL of Brilliant II SYBR Green QPCR Master Mix (Stratagene), 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, 0.375 μL of ROX reference dye solution, and 6.125 μL of sterile distilled water. The mixtures were initially incubated at 95 ˚C for 15 m, followed by amplification for up to 40 cycles of 95 ˚C for 20 s and 60 ˚C for 30 s. After thermal cycling, reaction mixtures were heated from 60 ˚C to 94 ˚C for 20 min at a constant rate and the fluorescence of SYBR Green I was measured continuously during heating. Fluorescence data was analyzed using MxPro Ver.4.0 software (Stratagene) to plot the dissociation curves. The template cDNA was prepared from 2- to 5-day-old cellulose-grown culture for triplicate tests.

Quantification of *cel7* gene transcripts

Real-time quantitative PCRs were performed in a Mx3000P Real-time QPCR System as described for dissociation curve analysis. For each standard curve, the dilution series of pCR4Blunt-TOPO vectors described above was used as templates. The transcript number of the actin gene was

quantified as an internal standard using the following primers, actin-F 5'-GCATGTGCAAGGCTGGCTTTG-3', actin-R 5'-AGGGCGACCAACGATGGATG-3'. Fluorescence intensity analysis were performed with MxPro ver. 4.0 software.

2.2.3 Results

Identification of 3'-UTR of *cel7* genes

Design of specific primer sets for PCR amplification of *cel7* genes faced two difficulties, i.e., the high similarity of *cel7* gene sequences and genetic polymorphism because *P. chrysosporium* K-3 is a heterokaryotic strain. As shown in Table 1.3, the nucleic acid sequence identities between *cel7A-cel7F/G* catalytic domains and carbohydrate-binding modules (CBMs) are about 70-80%. In fact, appropriate sequences for the specific primer sets for SYBR Green I mediated real-time PCR amplification were not found in the coding regions of *cel7* genes. Therefore, the 3'-UTR sequences of *cel7* genes were required to evaluate the possibility of using them for specific primer design. The 3'-RACE method was applied for the identification of 3'-UTRs by using gene-specific primers (as shown in Table 2.2.1) and universal amplification primer. As a result, 3'-UTRs of *cel7A*, *cel7C*, *cel7D*, *cel7E*, and *cel7F/G* were successfully amplified and sequenced. However, the 3'-UTR of *cel7B* was not amplified in 3'-RACE, because of its low expression level. To detect the genetic polymorphism of each gene, approximately 900 bp of the genomic sequences downstream of the respective stop codons was amplified using specifically designed primers (Table 2.2.1) and then sequenced. The genomic PCR was performed for *cel7A*, *cel7C*, *cel7D*, and *cel7F/G,* since the genetic polymorphism in *cel7E* 3'-UTR had already been detected by 3'-RACE analysis. As a result, polymorphisms were detected in *cel7A*, *cel7D*, and *cel7F/G*, but no nucleic acid alteration was found in *cel7C*. We then compared the sequence identities of 3'-UTRs between *cel7* genes (except *cel7B*), as well as catalytic domains and CBMs, and found that they showed less than 10% identity. This result suggested that it is possible to design specific primer sets based on the 3'-UTR region of *cel7A*, *C*, *D*, *E*, and *F*/*G*. For *cel7B*, the sequence of its CBM showed lower similarity than that of other *cel7* genes (Table 1.3) and therefore we considered that a specific primer set could be designed based on its CBM and flanking linker region.

Table 2.2.1 Gene-specific primers for 3'-RACE and genomic PCR

Primers for 3'-RACE	
cel7A	GTTCAGGCCTGCGTGAATATTG
cel7B	ACTATTCTCAGTGTATCTAATGCTG
cel7C	AGTGCTACTAAGTTACGGAATGGAT
cel7D	TGCTACTAAGAGGCGCTTGGACAT
cel7E	TCGCAGTGCTACTAGATGGTGTAC
cel7F/G	TACTCCCAGTGCTACTAAGTCGC
Primers for genomic PCR[a]	
cel7A	ATGTATCCACCGCCAGTTCACG
cel7C	GATGGATACTCTGTTGGAAGGAC
cel7D	ACCGACGATGGCTACAACATCG
cel7F/G	CGCTTTGCACAAACACATCAAGC

[a]The listed primers for genomic PCR match the reverse sequence. The primers for 3'-RACE were used as the forward primers for each genomic PCR.

Primer design for specific amplification of *cel7* genes

The primer sets were designed to amplify *cel7* genes specifically in real-time PCR by using their 3'-UTR sequences, except in the case of *cel7B*, for which the linker region and CBM were used. Fig. 2.2.1a shows the structures of the 3'-ends of *cel7* genes and the positions of the primer sets. The designed primer sequences are listed in Table 2.2.2. To verify the specificity of each primer set, PCR was performed by using these primer sets and the template vectors harboring each target sequence reciprocally (Fig. 2.2.1b). When the primer set for *cel7A* was used, only the vector harboring *cel7A* 3'-RACE product was amplified. This indicated that the *cel7A* primer set did not cause non-specific amplification of other *cel7* targets. The other five primer sets also did not amplify other *cel7* targets and only amplified their own target sequences. Furthermore, RT-PCR was performed using cDNA prepared from 2-day-old cellulose-grown culture as a template. Fig. 2.2.2a shows the RT-PCR products generated with the primer sets for *cel7A* - *cel7F/G*. A single

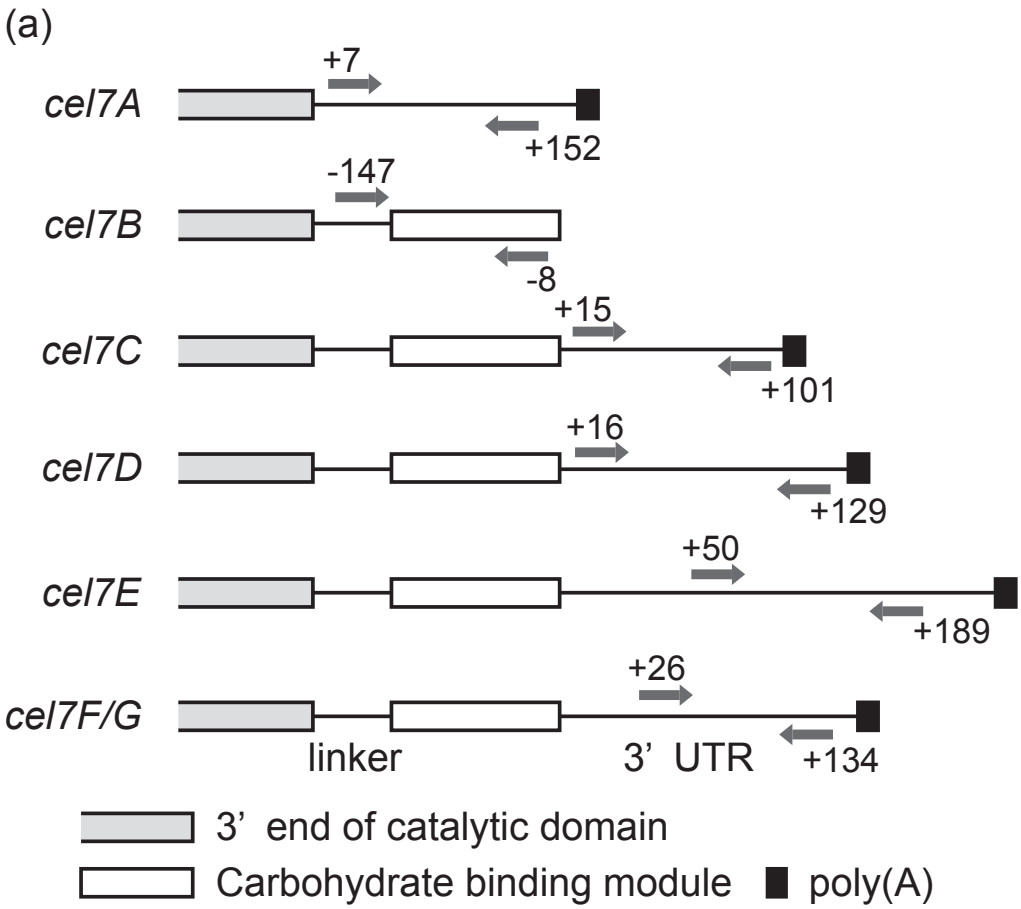
Table 2.2.2 Gene-specific primers for real-time quantitative RT-PCR

cel7A		
	F	GTGTGTAACTTAGATTCGTGGTGT
	R	TTCGATACCATGGGCTGACGAC
cel7B		
	F	GGATCCGCACCTGCATCTTCA
	R	CACTGAGAATAGTAGATATTGAGAG
cel7C		
	F	GCGGGGGTTGGATTTGTTGCAC
	R	AGGAACAAAGAAATATGATTTGAGGT
cel7D		
	F	TGCAGCCTCGAAAATAGCAGACT
	R	GTATGTGTGCATTTCACGGATCG
cel7E		
	F	TTGGCGCAGACGGGATTTTCC
	R	CACGCAAACTACCCCTTTCCA
cel7F/G		
	F	AGGGTGTTTCTTCTCTTTTTGGCT
	R	GTATATCTTTCTGCTGATTCTACAG

band was observed in each lane at an appropriate location, indicating that the primer sets for *cel7* genes successfully amplified their target genes from the cDNA sample. These RT-PCR products were sequenced and confirmed to be the specific products. Moreover, dissociation curve analysis of *cel7* RT-PCR products was performed to ascertain whether minor non-specific products were accumulated (Fig. 2.2.2b). All products showed a single peak in the dissociation curve, indicating that no non-specific product was amplified. Consequently, it was established that these primer sets specifically amplify *cel7* genes without generating any significant amount of non-specific products.

Quantitative analysis of *cel7* gene expression under carbon starvation

The time-course of transcript levels was analyzed in medium lacking a carbon source to assess the effect of derepression. As shown in Fig. 2.2.3, the time course of expression appeared to



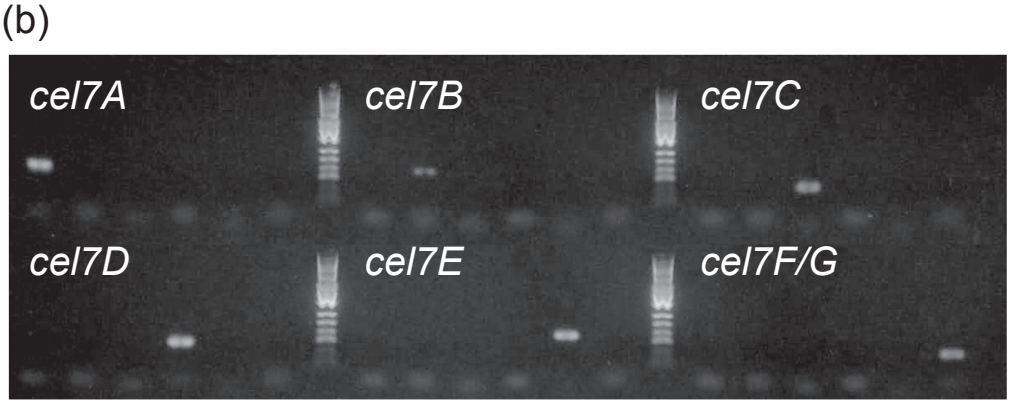


Fig. 2.2.1. (a) The structures of the 3'-ends of *cel7* genes. Arrows represent primer locations. The number presented beside each arrow indicates the position of the 5'-end of each consensus sequence for the primer. Numbers of nucleotides begin with the last nucleotide of the stop codon as -1. The specificity of designed primer sets was validated by PCR (b). The agarose gel shown here is divided six blocks with *cel7* gene names. Each gene name presented on the gel indicates the primer set of the corresponding gene used in that block. There are six lanes in one block, and each lane shows the PCR product using the template vector harboring the target sequence of *cel7A*, *cel7B*, *cel7C*, *cel7D*, *cel7E*, or *cel7F/G*.

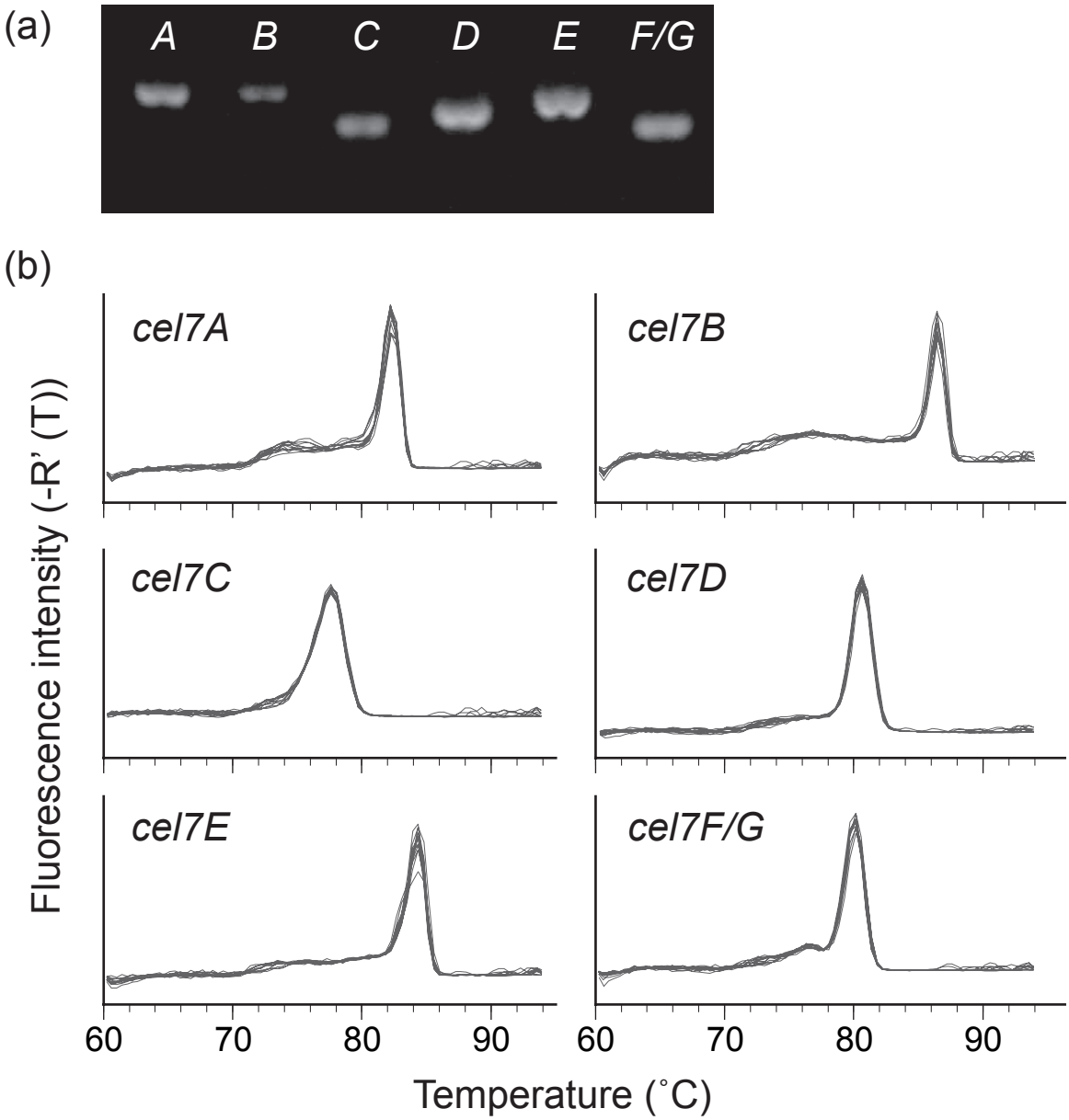


Fig. 2.2.2. Verification of the specificity of our primer sets. (a) RT-PCR amplification of *cel7* genes from cDNA template by using the primer sets designed in this study. Each lane shows the RT-PCR product using the primer set for *cel7A* (A), *cel7B* (B), *cel7C* (C), *cel7D* (D), *cel7E* (E), or *cel7F/G* (F). (b) Dissociation curve analysis of RT-PCR products using SYBR Green I. Decreasing fluorescence intensity of SYBR Green I was measured for each product from 60 ˚C to 94 ˚C. Derivative plots of dissociation curves are indicated.

be different for each *cel7* gene. *cel7D* transcripts showed repressed expression in the early stage of cultivation, followed by an exponential increase after 2 h of cultivation (Fig. 2.2.3d), in agreement with the previous result using the TaqMan probe method (Suzuki*, et al.*, 2008). In the case of *cel7C*, gene expression remained repressed up to 5 h of cultivation (Fig. 2.2.3c). The number of transcripts

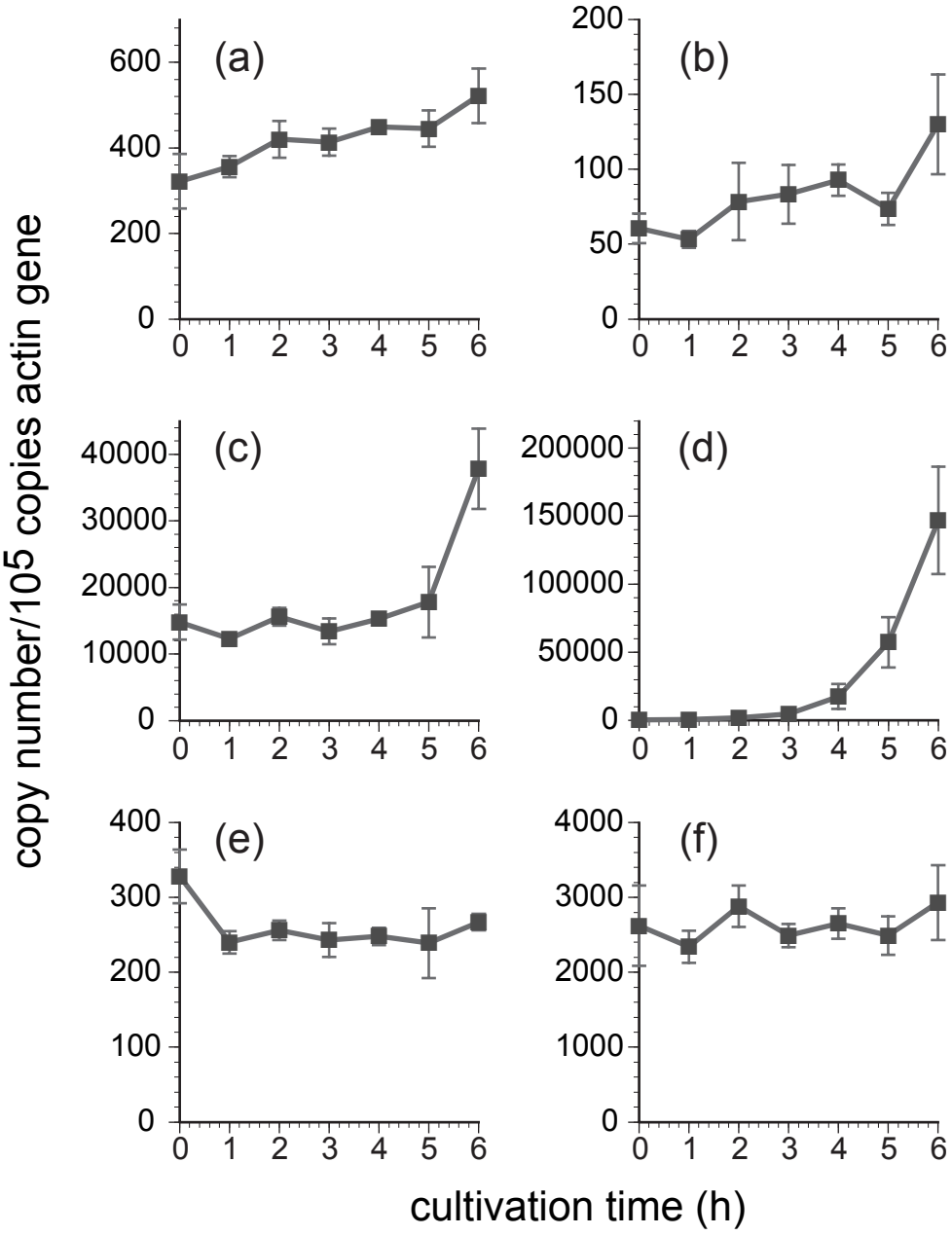


Fig. 2.2.3. Time courses of *cel7A* (a), *cel7B* (b), *cel7C* (c), *cel7D* (d), *cel7E* (e), and *cel7F/G* (f) transcripts measured by quantitative real-time RT-PCR. cDNAs from culture without any carbon source were used. Transcript numbers of each gene were normalized by the copy number of actin transcripts from the same sample. The vertical axis indicates the copy number of each gene per 10^5 copies of actin transcript. Each error bar shows the standard deviation in triplicate tests for each cultivation.

of *cel7C* was 35-fold higher than that of *cel7D* in the repressed stage, in spite of the fact that *cel7D* is known to be the most highly expressed *cbh* gene in cellulose-grown culture (Vanden Wymelenberg*, et al.*, 1993). In contrast, *cel7C* transcripts were 3.5-fold lower than those of *cel7D* at 6 h of cultivation under derepressed conditions. On the other hand, *cel7A*, *cel7B*, *cel7E*, and *cel7F*

showed no clear increase of transcripts during 6 h of cultivation (Fig. 2.2.3a, b, e, f). The numbers of transcripts of *cel7A* and *cel7E* were close to the repressed level of *cel7D* transcripts, and the level of *cel7F* transcripts was 10-fold higher than that of *cel7E*. The number of *cel7B* transcripts showed the lowest level during the entire cultivation.

2.2.4 Discussion

A specific quantification method for expression of *cel7* genes in *P. chrysosporium* was developed in this study. Using this method, even a low-expressed gene transcript, such as that of *cel7B*, could be detected quantitatively and independently. Therefore, this real-time PCR assay is applicable for quantitative evaluation of *cel7* genes under a variety of cultivation conditions and is expected to be a helpful tool for elucidating the cellulose degradation mechanism of *P. chrysosporium*. Moreover, the 3'-UTR-based primer design strategy should be applicable to other cellulolytic fungi, which have multiple cellulases with similar sequences.

In chapter 2.1, the quantitative analysis of derepression of cellulolytic genes in *P. chrysosporium* was described, and the drastic increase of *cel7D* transcripts as derepression provoked the interest in the expression behavior of other *cel7* genes under carbon starved condition. Therefore, we performed real-time RT-PCR amplification of *cel7* genes using the novel primer sets to analyze the gene expression patterns quantitatively during cultivation of *P. chrysosporium* under carbon starvation. However, *cel7G* cannot be distinguished from *cel7F* because they have identical sequences, including the 5'- and 3'-UTR. Thus, the quantity of *cel7F/G* transcript should be regarded as the sum total of *cel7F* and *cel7G*.

The results presented here indicate that *cel7C* is less susceptible than *cel7D* to glucose-mediated repression and derepression. The presented result of *cel7C* derepression is consistent with the previous finding that transcription of *cel7C* is increased in 7-day-old glucose grown culture (Covert et al., 1992b). Moreover, it was suggested that *cel7A*, *cel7B*, *cel7E*, and *cel7F* are not derepressed, at least under these culture conditions, and maybe transcribed constitutively at basal level. Since the transcript number of these genes are comparatively smaller than that of derepressed *cel7C* and *cel7D*, derepression is considered to be further significant for cellulolytic gene expression under carbon starvation. However, the regulatory differences among these genes need further investigation.

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Chapter 3

Quantitative analysis of transcriptional induction of the genes encoding cellulolytic enzymes in the basidiomycete *Phanerochaete chrysosporium*

3.1 Introduction

Although basidiomycetes usually have multiple CBHs and efficiently degrade crystalline cellulose, the information about the induction of CBHs is still limited in contrast to ascomycetes. In chapter 2, the up-regulation of *cel7C* and *cel7D* transcription was reported in *P. chrysosporium* under carbon starved condition using real-time RT-PCR and proposed that carbon catabolite derepression plays an important role, rather than basal expression, in the initial degradation of cellulose. According to the induction model of *H. jecorina*, a soluble inducer for *P. chrysosporium* Cel7s may be produced from cellulose by basal or derepressed level of enzymes. However, any soluble compound, which act as an inducer for the expression of *cel7* genes during cellulose degradation, have not been determined and the transcriptional difference in induction of a variety of *cel7*s are still unclear.

In this chapter, the quantitative analysis of *cel7* gene expression against glucose, cellulose and water-soluble cellooligosaccharides were performed and compared to the transcription level under these sugars to evaluate the induction manner of the *cel7* gene transcription. In addition, the transcription of *cel6A* and *cdh* were quantified and compared to that of *cel7*s.

3.2 Materials and methods

Fungal strain and culture conditions.

P. chrysosporium strain K-3 (Johnsrud & Eriksson, 1985) and modified Kremer and Wood medium (Kremer & Wood, 1992) in composition as described in the previous report (Suzuki*, et al.*, 2008) were used in this study. For the transcription analysis under cellulose and glucose, 1×10^9 liter^{-1} spores of the fungus were inoculated in 400 ml of this medium containing 2% Cellulose (CF11; Whatman, Fairfield, NJ) or 100 mM glucose (Wako Pure Chemical industries, Osaka, Japan) as a sole carbon source. The inoculated media were maintained at 37˚C and shaken at 150 rpm for 5 days. A 5-ml aliquot of the culture was harvested every 24 hours. For the cultivation with cellooligosaccharides, the spores were inoculated in 200 ml of the medium and cultivated in the same condition for pre-grown. After 3 days of cultivation, the mycelia were harvested, washed three times with 100 ml of the same medium containing no carbon source and transferred to 200 ml of fresh medium containing 20 mM glycerol (Wako). After 6 hours of cultivation, 100 μM glucose (Wako), cellobiose, cellotriose, cellotetraose and cellopentaose (Seikagaku Corporation, Tokyo, Japan) were added to the medium respectively and cultivated another 6 hours. A 5-ml aliquot of the culture was harvested every hour.

Measurement of glucose and cellooligosaccharide concentration in the culture supernatant.

The culture supernatant were boiled for 5 min to inactivate the enzymes secreted by the fungus. The concentration of glucose and cellooligosaccharides in the supernatant were measured by high-performance liquid chromatography (HPLC; LC-2000 series; Jasco, Tokyo, Japan), using a corona charged aerosol detector (ESA Biosciences, Chelmsford, MA). The supernatant were filtrated using MultiScreen HTS 96-well Filtration System (Millipore Corporation, Billerica, MA), then separated on Shodex Asahipak NH2P-50 4E (Showa Denko K. K., Kanagawa, Japan) in isocratic elution

(65% acetonitrile, 35% H_2O [vol/vol]). In addition, isocratic elution of 75% acetonitrile-25% H_2O was used to separate cellobiose from other disaccharides. The amount of each sugar was quantified by using glucose (BioUltra; Sigma-Aldrich, St. Louis, MO) and cellooligosaccharides with degree of polymerization (DP) values of 2 to 6 (Seikagaku Corporation) as standards.

Real-time RT-PCR analysis of cellulolytic gene transcripts.

The mycelia collected from the culture aliquots were immediately frozen in liquid nitrogen and stored at -80˚C to extract RNA. Frozen fungal mycelia were ground to a fine powder using Multi-Beads Shocker (Yasui Kikai, Osaka, Japan), then total RNA was extracted by using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and treated with RNase-Free DNase Set (Qiagen), according to the manufacturer's instructions. First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan), as described previously (Suzuki 2008). Real-time reverse transcription (RT)-PCRs were performed in an Mx3000P Real-time QPCR System (Stratagene, La Jolla, CA) as follows; 5 μL of template solution was mixed with 20 μL of solution containing 12.5 μL of Brilliant II Fast SYBR Green QPCR Master Mix (Stratagene), 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, 0.375 μL of 6-carboxy-X-rhodamine (ROX) reference dye solution and 6.125 μL of sterile-distilled water. The mixtures were initially incubated at 95 ˚C for 2 min, followed by amplification for up to 40 cycles of 95˚C for 5 sec and 60˚C for 20 sec. After thermal cycling, reaction mixtures were heated from 60˚C to 94˚C for 20 min at the constant rate and the fluorescence of SYBR Green I was measured continuously during heating for the dissociation curve analysis. Fluorescence data was analyzed using MxPro Ver.4.0 software (stratagene). The sequences of oligonucleotide primers for the amplification of the cDNA fragments derived from *cel7A*, *cel7B*, *cel7C*, *cel7D*, *cel7E*, *cel7F/G*, *cel6A* and *cdh* genes (GenBank accession nos. X54411 for both *cel7A* and *cel7B*, Z22528, L22656, Z11727, Z11729, S76141 and X88897) are listed in our previous reports (35, 36). The transcript number of actin gene was quantified as an internal standard by using

the following primers; actin-F (5’-GCATGTGCAAGGCTGGCTTTG-3’) and actin-R (5’-AGGGCGACCAACGATGGATG-3’).

3.3 Results

Gene transcription level of *cel7A* - *cel7F/G*, *cel6A* and *cdh* were quantified in the media containing 2% cellulose and 100mM glucose by means of real-time RT-PCR. In 2-day-old cellulose culture, the number of gene transcripts of *cel7A* - *F/G*, *cel6A* and *cdh* were 2.4×10^3, 1.3×10^3, 7.6×10^5, 5.9×10^4, 7.2×10^3, 1.0×10^5, 7.2×10^3 and 1.1×10^3 copies per 10^5 copies of actin gene transcripts, respectively (Fig. 3.1). The number of transcripts were higher in cellulose culture than in glucose culture for most of genes, although little difference were observed in *cel7A* and *cel7E*. To compare the difference of transcript levels between these cultures in time course, relative level of gene transcripts in cellulose culture were calculated against that in glucose culture for 2 to 5 days of cultivation (Table 1). It was observed that higher level of the transcripts compared to glucose culture were occurred in *cel7B*, *cel7C*, *cel7D* and *cel7F/G* at all time points. That of *cel6A* and *cdh* were observed as well. On the other hand, transcript number of *cel7A* and *cel7E* in cellulose culture were similar amount to that in glucose culture thorough the whole cultivation. In the case of

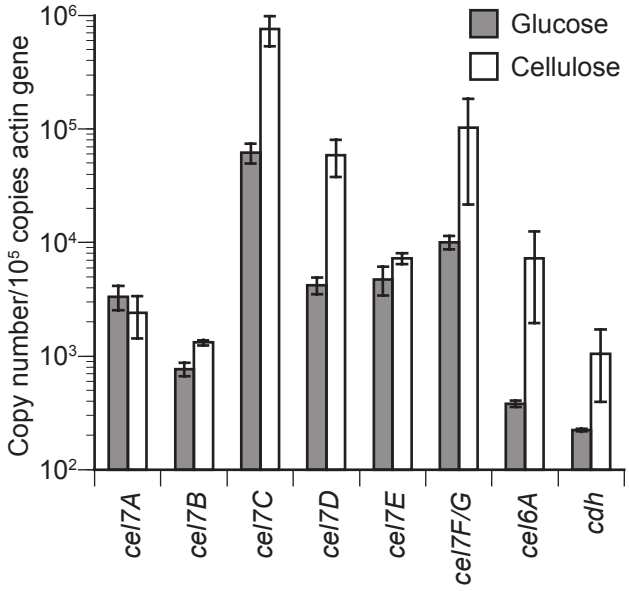

Fig. 3.1. Copy number of *cel7*s, *cel6A* and *cdh* transcripts quantified by real-time PCR in 2-day-old culture supplemented with 100mM glucose or 2% cellulose as a carbon source, respectively. The vertical axis indicates the number of transcripts normalized by 10^5 copies of actin gene transcripts in the same sample. Each error bar shows the standard deviation in triplicate tests.

Table 3.1. Relative level of gene transcripts (cellulose / glucose)

	Day2	Day3	Day4	Day5
cel7A	0.72	0.94	1.3	0.83
cel7B	1.7	2.2	3.5	3.5
cel7C	12	2.9	15	11
cel7D	14	3.1	41	31
cel7E	1.5	0.74	1.6	1.1
cel7F/G	10	4.5	25	7.1
cel6A	19	2.2	29	35
cdh	4.7	2.0	6.5	6.9

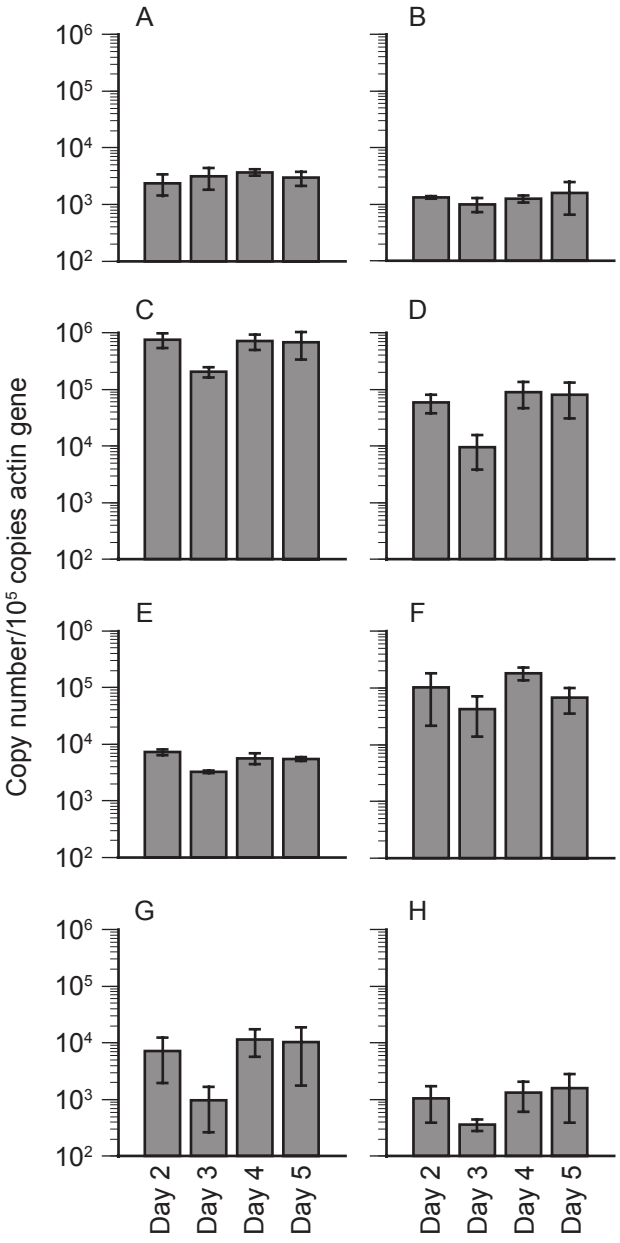

Fig. 3.2. Copy number of *cel7*s, *cel6A* and *cdh* transcripts quantified by real-time PCR during 2 to 5 days of cultivation supplemented with 2% cellulose as a carbon source. Template cDNAs were prepared from the culture every 24 hours. The vertical axis indicates the number of transcripts normalized by 10^5 copies of actin gene transcripts in the same sample. Each error bar shows the standard deviation in triplicate tests.

cellulose culture, the transcript number of *cel7C*, *cel7D*, *cel7E* and *cel7F/G* were decreased from day 2 to day 3 by 73%, 84%, 55% and 59% respectively and then increased from day 3 to 4 for the similar amount of day 2 (Fig. 3.2C, 3.2D, 3.2E and 3.2F). There were no obvious alteration during day 4 and day 5 except *cel7F/G*, which transcription level was decreased again by 63% in this time point. In contrast, apparent daily alteration of transcript number was not observed in *cel7A* and *cel7B* (Fig. 3.2A and 3.2B). The transcripts number of *cel6A* and *cdh* were decreased from day 2 to day 3 by 87% and 66%, respectively (Fig. 3.2G and 3.2H). In the aspect of absolute number, the transcripts of *cel7C* was most abundant and *cel7D* and *cel7F/G* were transcribed at similar level in cellulose culture.

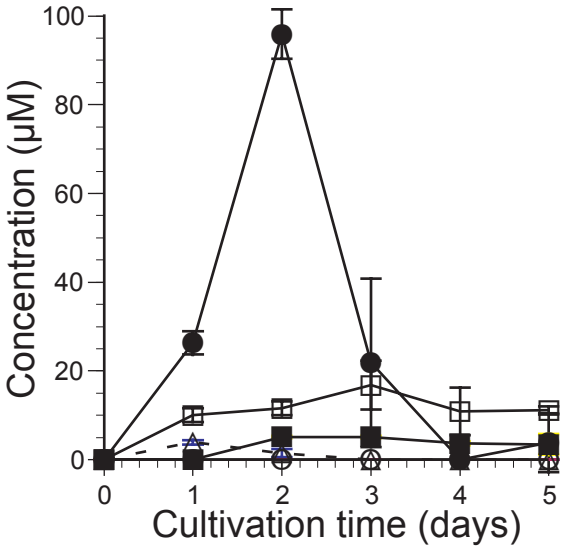

Fig. 3.3. Concentration of glucose and cellooligosaccharides in the extracellular culture fluids from 2% cellulose medium. The concentration was quantified by HPLC every 24 hours for 5 days. Open square, glucose; filled circle, cellobiose; open triangle, cellotriose; filled square, cellotetraose; open circle, cellopentaose. Each error bar shows the standard deviation in triplicate tests.

Accumulation of soluble cellooligosaccharides in the supernatant of cellulose containing culture were monitored and the daily alteration of the concentration of them were quantified by HPLC (Fig. 3.3). In the presented condition, cellobiose was accumulated up to approximately 100 μM in two days and then totally decreased by day 4. Cellotriose was produced about 4 μM in day 1 and then decreased. The production of cellotetraose was observed from day 2 and accumulated as approximately 5 μM in further cultivation. In contrast to these oligosaccharides, accumulation of cellopentaose and cellohexaose were not detected from the culture fluids.

To evaluate the transcript level of cellulolytic genes against soluble cellooligosaccharides, *P. chrysosporium* was cultivated in the culture containing 20 mM glycerol as a carbon source supplemented with 100μM of glucose and cellooligosaccharides with DP value of 2 to 5 respectively. Time course of the concentration of these sugars were quantified using HPLC during six hours of cultivation. In the glucose added culture, the initial concentration of glucose was 120μM (Fig. 3.4A) because 20μM of glucose was remained after the pre-cultivation. Similar amount of glucose was observed in all media tested (Fig. 3.4). In the cellobiose culture, the total amount of cellobiose was consumed within three hours (Fig. 3.4B). In the culture supplemented with DP 3 to 5 oligosaccharides, it was observed that oligosaccharides with lower DP value were synthesized during cultivation. As shown in Fig. 3.4C, cellotriose was totally assimilated or

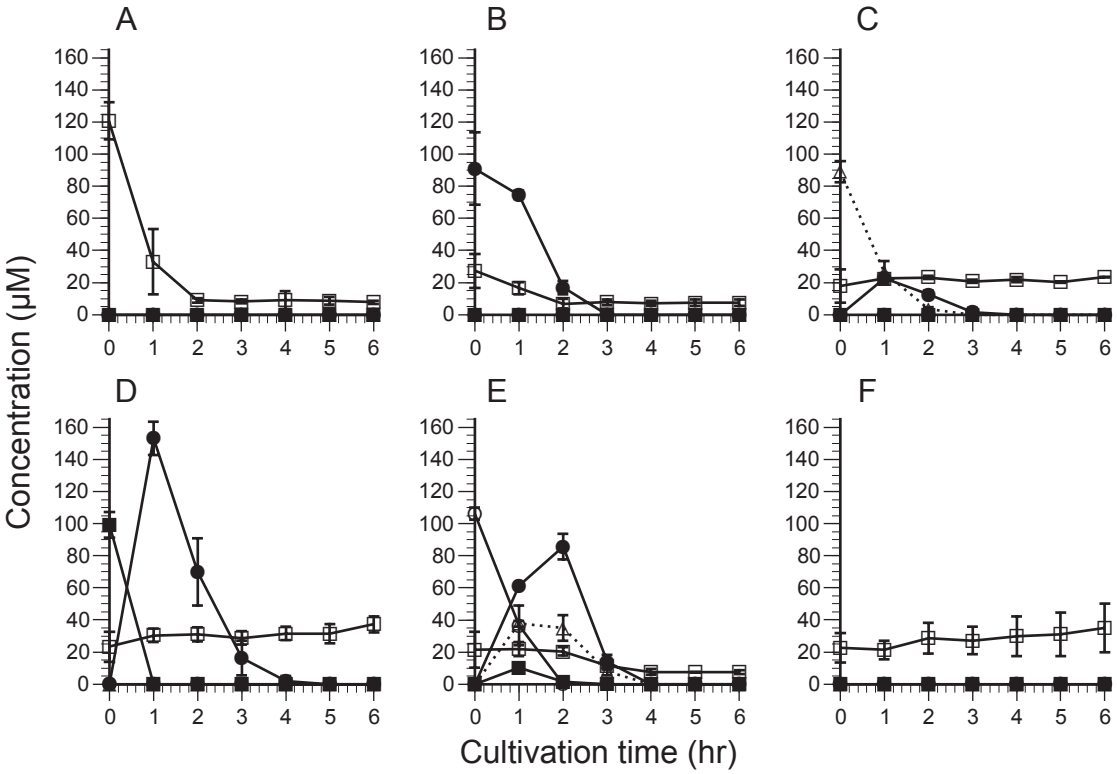

Fig. 3.4. Time course of glucose and cellooligosaccharides concentration in the extracellular culture fluids from 20mM glycerol culture, supplemented with 100μM of glucose (A), cellobiose (B), cellotriose (C), cellotetraose (D), cellopentaose (E), and no addition as a control (F). The concentration was quantified by HPLC for 6 hours after the addition of each sugar. Open square, glucose; filled circle, cellobiose; open triangle, cellotriose; filled square, cellotetraose; open circle, cellopentaose. Each error bar shows the standard deviation in triplicate tests.

hydrolyzed within four hours and 23μM of cellobiose was produced after one hour of cultivation in the cellotriose culture. In the culture with cellotetraose, hydrolysis or assimilation of cellotetraose was rapidly accomplished within one hour and 150μM of cellobiose was produced, whereas no cellotriose was detected (Fig. 3.4D). All of possible hydrolyzed products were detected in the cellopentaose culture (Fig. 3.4E). In the case of the control cultivation without any additives, only glucose was detected as shown in Fig. 3.4F.

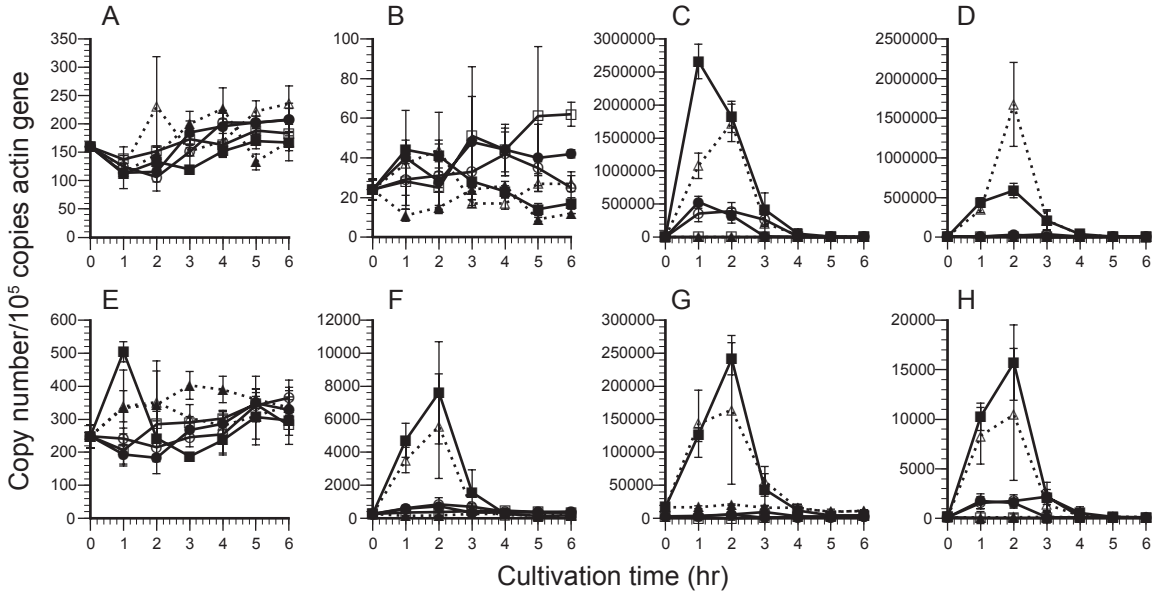

Fig. 3.5. Time course of the copy number of cellulolytic genes during 6 hours of cultivation supplemented with glucose (open square), cellobiose (filled circle), cellotriose (open triangle), cellotetraose (filled square), cellopentaose (open circle), and no addition as a control (filled triangle) respectively. The transcript number of *cel7A* (A), *cel7B* (B), *cel7C* (C), *cel7D* (D), *cel7E* (E), *cel7F* (F), *cel6A* (G), and *cdh* (H) were quantified by real-time PCR, normalize as described in Fig. 3.1. Each error bar shows the standard deviation in triplicate tests.

In the culture containing glucose and each cellooligosaccharide, the transcription of cellulolytic genes were quantified by real-time RT-PCR in time course. Through entire cultivation, no apparent alteration of transcript number was detected for all genes tested in the control culture containing only glycerol, thus it is confirmed that glycerol works as a neutral carbon source for cellulolytic gene expression in this condition. The patterns of the transcription against cellooligosaccharides were different between the genes, as shown in Fig. 3.5. The transcription of

cel7C was apparently up-regulated by the addition of cellotriose and cellotetraose (Fig. 3.5C). The maximum amount of *cel7C* transcripts were obtained after one hour in cellotetraose culture as much as 968-fold of its number at the start of the cultivation. Addition of cellobiose led *cel7C* transcription to increase by 192-fold in one hour, albeit as a lower amount than in cellotriose and cellotetraose culture. In cellopentaose culture, the number of *cel7C* transcripts were increased to a similar amount of cellobiose culture in a slower manner. In the case of *cel7D* (Fig. 3.5D), in contrast, the maximum transcription were occurred in two hours of cellotriose culture and its number was 338-fold of the starting point. Cellotetraose also affected the up-regulation of *cel7D* as much as 119-fold of the initial number. Conversely, addition of cellobiose showed little effect for the transcription of *cel7D*. In addition, cellopentaose hardly up-regulated its transcription. About *cel7F/G* (Fig. 3.5F), the transcription was up-regulated in cellotriose and cellotetraose culture as well and the largest number of transcripts were detected at two hours of cultivation in cellotetraose culture, as 29.6-fold of its starting number. Addition of cellobiose and cellopentaose were positively but slightly affected for *cel7F/G*. The expression of *cel6A* (Fig. 3.5G) and *cdh* (Fig. 3.5H) were both up-regulated by cellotriose and cellotetraose. The number of their transcripts were higher in cellotetraose cult

3.4 Discussion

A number of Cel7 hydrolases have been identified from various fungi and recorded in CAZy database, summarized in Table. 1.1. As for the microorganisms, which are recorded in GH family 7 database on CAZy, 39% of the basidiomycota but only 3% of the ascomycota are reported to have four or more Cel7s, although that include putative enzymes and prediction from gene fragments. Moreover, all of these Cel7s of basidiomycota, which have four or more enzymes, are characterized or predicted as CBHI. Thus, the multiplicity of Cel7 enzymes seems to be a feature of basidiomycota. However, the physiological advantage of the multiplicity of Cel7 enzymes have not been understood to date.

In the study of Cel7 isozymes in *P. chrysosporium*, Uzcategui et al. reported that Cel7C and Cel7D act synergistically in hydrolysis of cellulose (Uzcategui*, et al.*, 1991) and suggested that these two enzymes attack cellulose in a different fashion. The three-dimensional structure of Cel7D was revealed by Munoz et al. and homology modeling was also performed for another Cel7s (Munoz*, et al.*, 2001). The homology models of Cel7s showed several structural differences in tunnel-forming loops of these enzymes, which indicated that Cel7A and Cel7B have endoglucanase-like structure but another four Cel7s are very close to each other and the functional difference could not be predicted. Transcriptional analysis revealed that the expression of *cel7A* and *cel7E* are at higher level but *cel7D* is lower in colonized aspen wood (Vallim*, et al.*, 1998) than in the submerged cellulose culture (Covert*, et al.*, 1992, Vanden Wymelenberg*, et al.*, 1993). According to these results, it is expected that Cel7A, Cel7B and Cel7E have some difference from another Cel7s on the role in cellulose degradation. The results obtained from the presented work strongly support this hypothesis because the transcription of *cel7A*, *cel7B* and *cel7E* were not affected by the addition of cellooligosaccharides, which apparently induced another cellulolytic gene expression. Furthermore, it is possible that Cel7A and Cel7B are not participated in cellulose degradation because the level of their gene expression level were only slightly altered during the cultivation

with cellulose, whereas similar alteration was observed for another genes tested in this work. In addition, the expression of *cel7A* and *cel7E* did not appeared to be subject to glucose repression, so that these gene expression may be concerned with the metabolism of the non-glucan polysaccharide, such as xylan or glucomannan, rather than cellulose. However, the relationship between the function and the structure of Cel7E need further investigation.

In contrast to *cel7A*, *cel7B* and *cel7E*, another three *cel7*s showed dynamic response under cellulose and cellooligosaccharides. Interestingly, transcriptional response of each *cel7* gene against cellooligosaccharides were apparently distinct from each other. In the case of *cel7C*, the induction by cellobiose was clearly observed but 5-fold lower level than that of the addition of cellotetraose. Thus, cellobiose which appeared in one hour of cellotetraose culture probably affected the expression of *cel7C*, but its effect was considered to be a small because the transcript level induced by cellobiose were low level compared to cellotetraose. Therefore, it is suggested that cellotetraose itself have a strong inductive effect for *cel7C* expression. Moreover, cellotriose also have a good effect for induction of *cel7C*, because only small amount of cellobiose was formed in cellotriose culture and it was not sufficient for *cel7C* expression in this condition. The same situation was observed for *cel7F/G* and *cdh*, although the effect of cellobiose for *cel7F/G* was quite poor. On the other hand, the effect of cellobiose were significantly small for *cel7D* and *cel6A*, indicating that these gene expression were not induced by the formation of cellobiose. In the case of *cel7D*, cellotriose is regarded as a better inducer than cellotetraose, by contrast to *cel7C*. The effect of cellopentaose was difficult to evaluate, because that of cellopentaose was considered to be a integrated effect of shorter oligosaccharides formed during the cultivation. At least, cellopentaose itself is not considered to be a good additive for the induction of all five genes, because they were poorly up-regulated in the cellopentaose culture.

As a counterpart of the induction by cellooligosaccharides, carbon catabolite repression is caused by glucose, which is a hydrolysate of cellooligosaccharides produced by β-glucosidase

(BGL). To estimate the effect of repression, time course of the sugar assimilation was calculated as a sum total and commuted to a concentration of glucose residue during the cultivation with cellooligosaccharides (Fig. 3.6). The expression of *cel7C*, for example, was up-regulated by two hours of cultivation in cellobiose, cellotriose or cellotetraose, and at that time point, larger number of glucose molecule was assimilated in cellotriose and cellotetraose culture than in cellobiose culture. In *P. chrysosporium*, hydrolysis of cellooligosaccharide is possibly catalyzed by intracellular BGL (BGL1B) (Tsukada*, et al.*, 2006). k_{cat}/K_m values of BGL1B are much bigger for cellotriose and cellotetraose than for cellobiose (Tsukada, 2009). Therefore, intracellular concentration of glucose is considered to be higher in cellotriose and cellotetraose culture and subject to catabolite repression, although the level of expression is higher in these culture. It is suggested that the lower level of induction by cellobiose was not related to cellobiose catabolism and consequent repression by glucose.

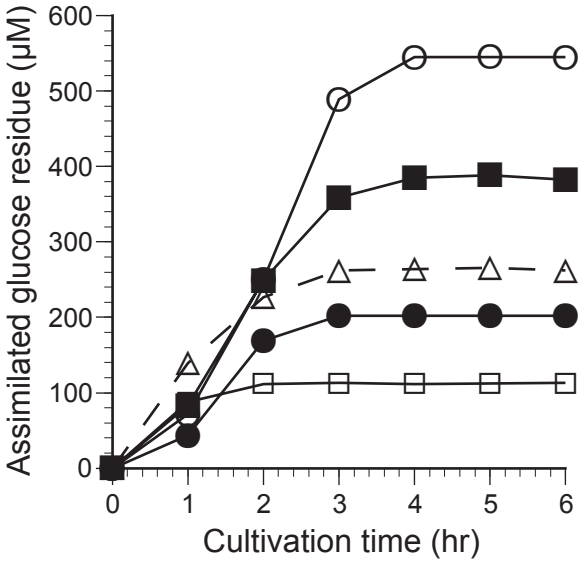

Fig. 3.6. Time course of the sugar assimilation during 6 hours of cultivation supplemented with glucose (open square), cellobiose (filled circle), cellotriose (open triangle), cellotetraose (filled square), and cellopentaose (open circle). The vertical axis indicates the sum total of assimilated glucose residue from the culture, calculated as the product of reduced amount from the initial concentration of the cultivation and corresponding DP value of each oligosaccharide.

Since cellooligosaccharides are the major products of cellulose hydrolysis, it is possible that cellulolytic organisms regulate the cellulase production by accepting water-soluble cellooligosaccharides. In deed, transporting system specific for cellobiose and cellotriose have been identified from the cellulolytic bacteria, *Streptomyces reticuli* (Schlosser & Schrempf, 1996, Schlosser*, et al.*, 1999). Moreover, it was reported that *Clostridium thermocellum* assimilates

cellopentaose preferentially during growth on cellulose (Zhang & Lynd, 2005). In addition, the transcription of endoglucanase genes are induced by cellotriose rather than cellobiose in *Ruminococcus flavefaciens* (Wang*, et al.*, 1993). These findings indicate the presence of selective assimilation and response mechanism for specific cellooligosaccharide in these bacteria. In the case of cellulolytic fungi, however, selective response for cellooligosaccharide have been reported only in the case of the basidiomycete *Polyporus arcularius*, which showed the up-regulation of cellobiohydrolase and endoglucanase gene expression in the culture containing cellopentaose (Ohnishi*, et al.*, 2007, Ohnishi*, et al.*, 2007). The result presented in this chapter showed the intriguing characteristics of the selective induction of cellulolytic gene expression in *P. chrysosporium* by means of cellotriose and cellotetraose. Although the machinery of receptors or transporters for cellooligosaccharides are still remain unknown, the variation of the cellooligosaccharides composition in extracellular environment could be a signal for cellulolytic enzyme production by *P. chrysosporium*.

Recently, genome-wide investigation of transcriptome and secretome of *P. chrysosporium* revealed a complex pattern of production of wood degrading enzymes (Vanden Wymelenberg*, et al.*, 2005, Sato*, et al.*, 2007, Sato*, et al.*, 2009, Vanden Wymelenberg*, et al.*, 2009), and advocated the necessity of detailed investigation for the regulatory mechanism of each enzyme production. The results presented here indicate that cellotriose and cellotetraose are the potential candidates of inducer for some cellulolytic enzymes, whereas cellobiose is regarded as a actual inducer during cellulose degradation. In addition, *cel7* genes of *P. chrysosporium* are not only differentially transcribed in a complex manner, but also not all of Cel7 enzymes seems to be participated in cellulose degradation. However, some of the results were not consist with a previous report using homokaryotic strain RP-78 or BKM-F-1767, in which *cel7D* is the most abundantly transcribed gene among *cel7*s in BKM-F-1767 (Vanden Wymelenberg*, et al.*, 1993) but *cel7C* is in heterokaryotic strain K-3, which is used in this study. Moreover, *cel7E* of strain RP-78 is up-

regulated in cellulose culture compared to glucose culture (Vanden Wymelenberg*, et al.*, 2009), whereas that of strain K-3 showed no difference between these carbon sources. Accordingly, it may suggest that the manner of cellulolytic enzyme production is different between the strains of *P. chrysosporium*.

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Chapter 4

Summary and concluding remarks

In chapter 2, the significance of carbon catabolite derepression for transcription of cellulolytic genes was presented. The cultivation method was developed, which is able to assess the cellulolytic gene expression in carbon starved environment. Transcription of the *cel6A*, *cel7D*, *cdh* and *bgl3A* were analyzed by means of real-time quantitative RT-PCR. As a result, the transcription of *cel6A*, *cel7D* and *cdh* were up-regulated after the consumption of glucose, indicating that carbon catabolite derepression has occurred for these gene transcription. Moreover, expression of two *cel6A* and *cel7D* was quantitatively analyzed in terms of the rate of increase of transcript numbers and the lag-time of derepression in the presence or absence of glucose. These gene transcripts were exponentially increased, and the derepression rates of *cel6A* and *cel7D* were specific to each gene, and were not influenced by the glucose concentration in the culture medium. Furthermore, a real-time PCR method for the detection and quantification of *cel7* genes was established using PCR primer sets designed based on the 3'-UTR region sequences. It was confirmed by agarose gel electrophoresis, sequencing, and dissociation curve analysis of the PCR products that each *cel7* transcript was specifically amplified by the corresponding primers. This method was applied to evaluate the time course expression of *cel7* genes quantitatively in *P. chrysosporium* under conditions of carbon starvation. As a result, the expression of *cel7C* was up-regulated under carbon starved condition, but less susceptible than *cel7D*. In addition, another four *cel7* genes did not obviously respond to the carbon starved condition.

In chapter 3, quantitative transcriptional analysis of cellulolytic genes were performed in *P. chrysosporium*, using the real-time quantitative RT-PCR developed in chapter 2. The behavior of each gene transcription was calculated in the culture containing glucose, cellulose and soluble cellooligosaccharides. The transcript number of *cel7B*, *cel7C*, *cel7D* and *cel7F/G* were at higher level in cellulose culture than in glucose culture at all time point, although that of *cel7A* and *cel7E* were similar amount. The transcript number of *cel7C*, *cel7D*, *cel7E* and *cel7F/G* were decreased from day 2 to day 3 of cellulose culture and then increased from day 3 to 4 for the similar amount of

day 2. The transcription of *cel7C*, *cel7D* and *cel7F/G* were obviously up-regulated by the addition of cellotriose or cellotetraose, whereas the transcript level of another *cel7* genes were not affected. The highest transcript number of *cel7C* and *cel7F/G* was observed in cellotetraose culture whereas that of *cel7D* was occurred in cellotriose culture, as much as 2.7×10^6, 7.6×10^3 and 1.7×10^6 copies per 10^5 of actin gene transcripts. These numbers were higher than that in cellulose culture, except *cel7F/G*. Addition of cellobiose showed an inductive effect for *cel7C*, but *cel7D* and *cel7F/G* were hardly up-regulated by cellobiose. The result presented in chapter 3 showed the intriguing characteristics of the selective induction of cellulolytic gene expression in *P. chrysosporium* by means of cellotriose and cellotetraose. Moreover, it is indicated that the variation of the cellooligosaccharides composition in extracellular environment could be a signal for cellulolytic enzyme production by *P. chrysosporium*.

The transcript number in cellulose and cellooligosaccharide-supplied culture and the maximum number of gene transcripts in carbon starved condition (obtained from the results in chapter 2), which provoke carbon catabolite derepression for *cel7C*, *cel7D*, *cel6A* and *cdh*, were compared (Table 4.1). In the case of *cel7C*, the maximum amount of gene transcripts were higher in cellotriose or cellotetraose culture but lower in carbon starved culture than in cellulose culture. Therefore, induction caused by the presence of cellulose or cellooligosaccharides is considered to be a major factor for *cel7C* transcription rather than derepression. The transcription of *cel7D* is at higher level in both cellotriose and carbon starved culture than in cellulose culture, indicating that the transcription of *cel7D* is regulated both induction and derepression. In the same aspect, the transcription of *cel6A* is strongly regulated by derepression, although it is affected by cellotriose and cellotetraose-mediated induction. The transcription of *cdh* is appeared to be regulated mainly by induction, because the number of transcripts are higher in cellotetraose culture but are similar level in carbon starved condition compared to cellulose culture. In 2-day-old cellulose culture, gene transcription of *cel7C*, *cel7D*, *cel6A* and *cdh* were regarded to be dominantly affected by cellobiose because the maximum number of these gene transcripts in cellobiose culture are close to that in

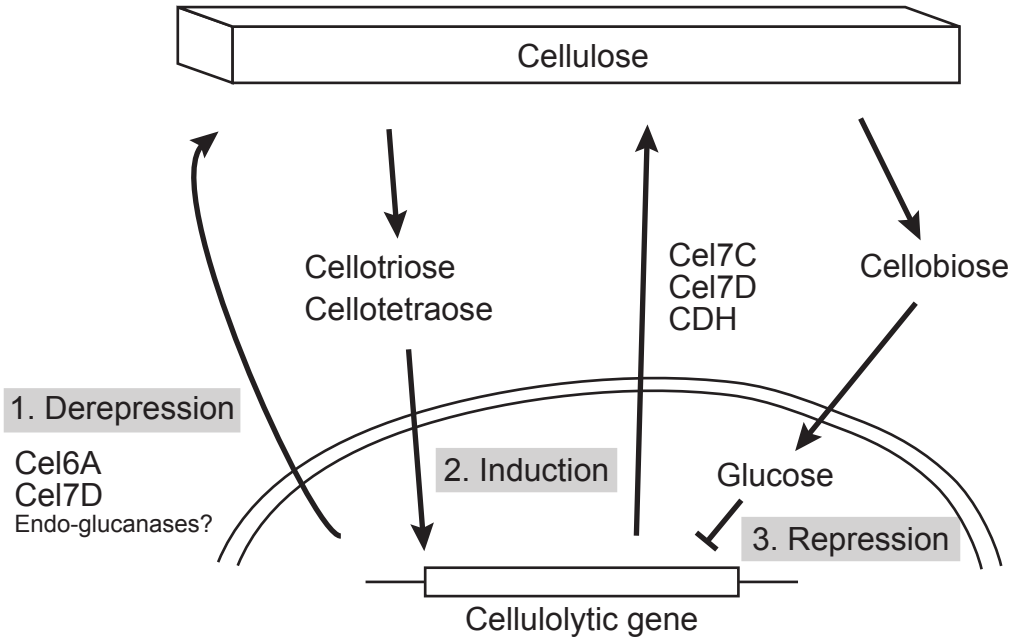
Table 4.1. Maximum value of gene transcripts during cultivation

	Carbon starvation	Cellulose	C2	C3 or C4	Glycerol
cel7A	5.22×10^{2}	3.67×10^{3}	2.07×10^{2}	2.36×10^{2}	2.27×10^{2}
cel7B	1.30×10^{2}	1.58×10^{3}	4.80×10^{1}	4.40×10^{1}	2.60×10^{1}
cel7C	3.78×10^{4}	7.58×10^{5}	5.28×10^{5}	2.66×10^{6}	3.59×10^{3}
cel7D	1.39×10^{5}	9.04×10^{4}	2.86×10^{4}	1.67×10^{6}	5.10×10^{3}
cel7E	3.28×10^{2}	7.23×10^{3}	3.49×10^{2}	5.04×10^{2}	4.02×10^{2}
cel7F/G	2.93×10^{3}	1.83×10^{5}	7.34×10^{2}	7.60×10^{3}	2.81×10^{2}
cel6A	1.83×10^{5}	1.15×10^{4}	6.16×10^{3}	2.41×10^{5}	3.18×10^{3}
cdh	2.85×10^{3}	1.59×10^{3}	1.74×10^{3}	1.57×10^{4}	1.43×10^{2}

cellulose culture. Indeed, cellobiose was accumulated but cellotriose and cellotetraose were produced as only small amount in cellulose culture fluids. In contrast, the expression of *cel7F/G* are different manner from *cel7C* and *cel7D*. The transcript number of *cel7F/G* in the culture with any cellooligosaccharide or carbon starvation were not reached the level of transcription in the cellulose culture. Furthermore, the decrease of the number of transcripts form day 4 to day 5 in cellulose culture was observed only for *cel7F/G*. These results indicate that, although *cel7F/G* seems to be related to cellulose degradation, another soluble compound, not cellooligosaccharides, are concerned with the induction of *cel7F/G*. In addition, the transcript level of *cel7A*, *cel7B* and *cel7E* were also higher in the presence of cellulose than in another culture. Actually, HPLC analysis of the supernatant from cellulose culture showed several unidentified peaks in the chromatogram, along with the peaks of cellooligosaccharides. Identification and analysis of the effect of these compounds are necessary to understand the expression mode of these genes.

The quantitative comparison of induction and derepression of cellulolytic genes performed in this study proposed an importance of derepression, which is not considered as a key regulatory mechanism in *H. jecorina*, and thus indicates the difference of the regulatory mechanism of

cellulolytic gene expression between basidiomycetes and ascomycetes. Moreover, transcriptional induction caused specifically by cellotriose and cellotetraose was first demonstrated in basidiomycetes. The results obtained here illustrate the scheme of cellulose degradation by *P. chrysosporium* that Cel6A and Cel7D are mainly produced in early stage of cellulose degradation by carbon catabolite derepression (scheme 4.1). Cel7C and CDH are produced only small amount in this stage. After that, cellotriose and cellotetraose are formed by hydrolysis of cellulose and act as an inducer of the transcription of *cel7C*, *cel7D* and *cdh* at higher levels, although cellobiose could not act effectively for transcription, then the translated products of these genes work for a full-scale degradation of cellulose. Finally, cellulolytic gene expression was repressed by glucose, which is produced by BGLs. However, the effect of cellobiono-1,5-lactone, an oxidized product of cellobiose by the action of CDH is still remain unknown. Although cellobiono-1,5-lactone is an inducer of cellulases in *H. jecorina* (Iyayi, *et al*., 1989), it is not likely that it also work as an inducer in *P. chrysosporium*. As shown in Fig. 3.3, cellobiose, a precursor of cellobiono-1,5-lactone, was highly accumulated in 2-days of cultivation with cellulose, and thus, it is expected that cellobiono-1,5-lactone was mainly produced in 2 or 3-days of that cultivation. The transcripts of cellulolytic genes, however, were not appeared to be induced, rather repressed, in day 3 (Fig. 3.2). Accordingly, the inductive effect of cellobiono-1,5-lactone is estimated to be small or not at all in *P. chrysosporium*, though it may depends on the concentration of this lactone. Moreover, *cel7* genes seemed to have been already induced sufficiently when cdh was actively transcribed and produced the lactone. Since the efficiency of cellulose degradation by CBH is saturated unless the surface area of cellulose is extended even if excess amount of CBH is produced (Igarashi, *et al*., 2006), overproduction of CBH may bring no benefit in *P. chrysosporium*. Whatever the case, further investigation is required to shed light the effect of the lactone. The mechanism of Cel6A mediated initializing of cellulose degradation and triggering of another cellulolytic enzyme production is consisted with the previous finding in *H. jecorina* (El-gogary, *et al*., 1989, Seiboth, *et al*., 1992, Carle-Urioste, *et al*., 1997, Seiboth, *et al*., 1997). In fact, the Cel7A (CBHI in *H. jecorina*) deletion



Scheme 4.1 The regulation of cellulolytic gene expression under derepression, induction and repression.

mutant showed little transcriptional reduction of cellulase genes in *H. jecorina* (Seiboth, *et al*., 1997), indicating that CBHI is not necessary for the production of soluble inducer. In *P. chrysosporium*, however, CBHI gene (*cel7D*) was synergistically up-regulated with *cel6A* by carbon catabolite derepression, suggesting that Cel7D has a function of the inducer production as well as Cel6A. In contrast to these genes, the up-regulation of *cel7F*/*G* seems to be controlled dominantly by some other compounds in cellulose culture, and the transcription of *cel7A*, *cel7B* and *cel7E* were not appeared to be related to the induction or derepression. Thus, it is possibly suggested that there are the genes with a different roll among *cel7*s, which are concerned to cellulose degradation or not, even though they have a high level of sequence similarity. In addition to the cellulolytic genes investigated in this study, another cellulase genes, which encode endoglucanases, may have an important role in cellulolytic gene induction, because cellooligosaccharides probably produced by the activity of endoglucanase. Cel5A was also reported to be a critical enzyme to initialize the induction of cellulase genes in *H. jecorina* (Seiboth, *et al*., 1997). This result indicates the significance of endoglucanases in addition to *cel6A* in the triggering of cellulolytic gene expression, thus further investigation of the function of the genes encoding endoglucanases in *P. chrysosporium*

is important to understand the entire picture of cellulose degradation by several cellulolytic enzymes. Moreover, sugar transporting system needs extensive investigation, while little is known about transporter proteins or permiases in this fungi. Since the induction by means of cellotriose and cellotetraose is a unique feature among the cellulolytic fungi investigated to date, it is possible that the study of sugar assimilation system of *P. chrysosporium* leads us to the novel findings of the unique sugar transporters.

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2010年3月

List of publications

This thesis is based on the following articles, which are attached as references in Appendix section.

I **Suzuki, H., K. Igarashi, and M. Samejima.** 2008. Real-time quantitative analysis of carbon catabolite derepression of cellulolytic genes expressed in the basidiomycete *Phanerochaete chrysosporium*. Applied Microbiology and Biotechnology. **80**:99-106

II **Suzuki, H., K. Igarashi, and M. Samejima.** 2009. Quantitative transcriptional analysis of the genes encoding glycoside hydrolase family 7 cellulase isozymes in the basidiomycete *Phanerochaete chrysosporium*. FEMS Microbiology Letters. **299**:159-165

III **Suzuki, H., K. Igarashi, and M. Samejima.** 2010. Cellotriose and cellotetraose as inducers of the genes encoding cellobiohydrolases in the basidiomycete *Phanerochaete chrysosporium*. Applied and Environmental Microbiology. Submitted.

Appendix

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Real-time quantitative analysis of carbon catabolite derepression of cellulolytic genes expressed in the basidiomycete *Phanerochaete chrysosporium*

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Abstract Production of cellulolytic enzymes, such as cellobiohydrolases (CBH) and cellobiose dehydrogenase (CDH), by the basidiomycete *Phanerochaete chrysosporium* is significantly repressed in glucose-containing media; this is known as carbon catabolite repression. We have analyzed the glucose concentration dependence of transcript numbers of the cellulolytic genes (*cel6A*, *cel7D*, and *cdh*) and β-glucosidase gene (*bgl3A*) by means of real-time quantitative reverse transcriptase polymerase chain reaction to investigate the roll of carbon catabolite derepression in these gene expression. When the mycelium of *P. chrysosporium* grown in glucose culture was transferred to media containing various concentrations of glucose (0–5,000 μM), the expression levels of *cel6A*, *cel7D*, and *cdh* were drastically influenced by glucose, whereas no significant change was observed in *bgl3A*. The numbers of transcripts of *cel6A*, *cel7D*, and *cdh* increased exponentially during incubation for 6 h in the culture without glucose, and the rates of increase were 2.1 times per hour for *cel6A* transcripts and 2.7 times per hour for *cel7D* transcripts. Moreover, derepression of *cel6A* and *cel7D* was delayed (by 1.6 and 0.6 h, respectively) when the culture contained 50 μM glucose compared with that in the absence of glucose, suggesting that the promoter activities of *cel7D* and *cel6A* are distinct under conditions of carbon catabolite derepression.

Keywords Carbon catabolite derepression · *Phanerochaete chrysosporium* · Cellulose degradation · Real-time PCR

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Introduction

The white-rot basidiomycete, *Phanerochaete chrysosporium*, produces extracellular cellulases and oxidative enzymes related to cellulose degradation (Eriksson 1978). In the cellulolytic system of *P. chrysosporium*, cellobiohydrolases (CBH; EC 3.2.1.91) and endoglucanases (EG; EC 3.2.1.4) catalyze the hydrolysis of crystalline cellulose to cellobiose and cellooligosaccharides (Streamer et al. 1975; Uzcategui et al. 1991). In addition, cellobiose dehydrogenase (CDH; EC 1.1.99.18) oxidizes these products to the corresponding lactones (Ayers et al. 1978; Bao et al. 1993; Henriksson et al. 1998), which are further hydrolyzed to glucose and gluconolactone by extracellular β-glucosidase (BGL3A; EC 3.2.1.21; Deshpande et al. 1978; Smith and Gold 1979). In our previous report, however, we showed that BGL3A is functionally a glucan 1,3-β-glucosidase (Igarashi et al. 2003), playing an important role in fungal cell wall metabolism rather than cellulose degradation.

Microbiological approaches have yielded considerable genetic and transcriptional information about cellulolytic genes in *P. chrysosporium*. Covert et al. reported that there are six genes encoding CBHI isozymes (*cel7A-F*) in the genome (Covert et al. 1992), and another *cel7* gene, *cel7G*, was found recently (Vanden Wymelenberg et al. 2006), whereas *cel6A*, *cdh*, and *bgl3A* (coding CBHII, CDH, and extracellular BGL, respectively) are each present as a single copy in the genome. The expression levels of *cel7* isozymes respond differently to culture conditions (Covert et al. 1992; Broda et al. 1995; Lamar et al. 1995; Vallim et al. 1998), and *cel7D* showed the highest expression level among *cel7*s in culture in the presence of cellulose (Vanden Wymelenberg et al. 1993). Transcriptional analysis in *P. chrysosporium* showed that, as in other filamentous fungi,

cellulolytic genes are highly expressed in submerged culture with cellulose as a sole carbon source (Sims et al. 1988; Covert et al. 1992; Sims et al. 1994; Tempelaars et al. 1994; Broda et al. 1995; Li et al. 1996). However, the mechanism of initial induction of cellulolytic gene expression in cellulose-degrading culture remains unclear because cellulose is an insoluble solid substrate and might not be recognizable directly by fungi. Cellobiose or cellooligosaccharides are candidate inducers of cellulolytic genes in *P. chrysosporium* (Eriksson 1978), but their effects are difficult to evaluate because these compounds are rapidly oxidized by CDH. In addition to the above inductive effects, glucose strongly represses transcription of cellulolytic genes (Sims et al. 1988; Sims et al. 1994; Tempelaars et al. 1994; Broda et al. 1995; Li et al. 1996; Yoshida et al. 2004). This is known as carbon catabolite repression. Thus, the production of cellulose-degrading enzymes of *P. chrysosporium* is considered to be regulated by induction and repression of transcription of the corresponding genes by the enzymes' products.

In the present study, we describe the significance of carbon catabolite derepression, which is the regulatory mechanism in addition to induction and repression for transcription of cellulolytic genes, by means of real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Moreover, expression of two CBH genes (*cel6A* and *cel7D*) was quantitatively analyzed in terms of the rate of increase of transcript numbers and the lag-time of derepression in the presence or absence of glucose.

Materials and methods

Fungal strain and culture conditions

Phanerochaete chrysosporium strain K-3 (Johnsrud and Eriksson 1985) was cultivated in 400 ml of modified Kremer and Wood medium (Kremer and Wood 1992), which did not contain urea or $MnSO_4$, for pre-culture. The medium contained (per liter) 2.2 g 2,2-dimethylsuccinic acid, 0.26 g $(NH_4)_2HPO_4$, 3.5 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 74 mg $CaCl_2{\cdot}2H_2O$, 10 mg $FeSO_4{\cdot}7H_2O$, 5 mg $MnSO_4{\cdot}7H_2O$, 5 mg $ZnSO_4{\cdot}7H_2O$, 1 mg $CoCl_2{\cdot}6H_2O$, and 0.1 mg thiamine HCl. Glucose (100 mM) was added to the medium as a sole carbon source. The inoculated medium was maintained at 37°C and shaken at 150 rpm for pre-grown. After 3 days of cultivation, the mycelia were harvested, washed with the medium containing no carbon source, and transferred to 400 ml of same medium without any carbon source (as a resting culture). After 6 h of cultivation, 5,000, 500, or 50 μM glucose was added to the medium and cultivated another 6 h (as a main culture).

Measurement of extracellular glucose concentration

A 5-ml aliquot of the main culture was harvested every hour, and the supernatant was boiled for 5 min to inactivate extracellular enzymes. Then, the glucose concentration was measured using Glucose CII-Test Wako (Wako, Japan) as follows: 50 μl of medium was mixed with 950 μl of coloring reagent, and the mixture was incubated for 10 min at 30°C. Glucose concentration was calculated from the absorbance using a value of ε_{505}=317 M^{-1} cm^{-1}.

Quantification of cellulolytic gene transcripts

The mycelia harvested from the main culture were immediately frozen in liquid nitrogen and stored at −80°C to extract mRNA. Total RNA was extracted from collected mycelia using an E.Z.N.A. Fungal RNA Kit (Omega Bio-tek, USA), and mRNA was purified with an Oligotex-dT30<super>mRNA Purification Kit (Takara Bio, Japan). First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Japan), 5′-RACE cDNA synthesis primer of a SMART™ RACE cDNA Amplification Kit (Clontech, USA) and 3′-RACE Adapter primer (Invitrogen, USA) and was used as the template. PCR was performed using TaKaRa Ex Taq (Takara Bio) with primer sets for *cel6A* (accession no. S76141), *cel7D* (accession no. L22656), *cdh* (accession no. X88897), *bgl3A* (accession no. AB081121), and actin gene (*act*; accession no. AB115328), as listed in Table 1. Real-time quantitative PCRs were performed in an ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems, USA), as described previously (Yoshida et al. 2004). The transcript number of actin gene was quantified as an internal standard. Primers and TaqMan probes for quantification of each transcript were designed using Primer Express™ version1.0 (PE Applied Biosystems) and are listed in Table 1.

Results

Expression levels of *cel6A*, *cel7D*, *cdh*, and *bgl3A* in cultures containing 5,000, 500, and 50 μM glucose (G5000, G500, and G50, respectively) or without glucose (G0) after main cultivation for 6 h were compared by RT-PCR, as shown in Fig. 1a. In the cases of *cel6A* and *cel7D*, G500, G50, and G0 resulted in stronger band intensities of transcripts than G5000. In the case of *cdh*, G0 resulted in moderate expression, and only weak signals were observed under other conditions. Weak expression of *bgl3A* was observed almost independently of glucose concentration.

Distinct differences of expression levels were observed among the genes, depending upon the glucose concentra-

Table 1 Primers and TaqMan probes for RT-PCR and real-time quantitative RT-PCR

Primers and TaqMan probes	
Primers for RT-PCR	
cel6A	
F	ATTGTTGCTCAGATTCAACAGTTC
R	CAGCGTCCGGCAGAG
cel7D	
F	CGAGGCTAATGTCGGCAA
R	TTAGTAGCACTGCGAGTAGTAAGGG
cdh	
F	TCGGTCAACGGAGCTCTCT
R	CGCCAGAAGTTGAGTTTGG
bgl3A	
F	CAACTCCGGAACAACCTACTTC
R	CGTTGCCTGCTTCCTGAC
act	
F	GCCGTGTTCCCATCCAT
R	CACTTGTAGATGGAGTTGTAGGTCGT
Primers for real-time RT-PCR	
cel6A	
F	GGAGAGTTCAGCATTGCCAAC
R	GCGGACATCAGGGAACTGTT
cel7D	
F	ACTGGTTACTGCGACTCGCA
R	AGTTGCCGACATTAGCCTCG
cdh	
F	GCAGGTGCTTCTCCCAAACTC
R	GGGCATAACGGGTAAAGCC
bgl3A	
F	GAGCAAGAGCATTTTCGGGA
R	TCCGTAGAGTTCATGCTCCGT
act	
F	ATGTGCAAGGCTGGCTTTG
R	GGCGACCAACGATGGATG
TaqMan probe[a]	
cel6A	CGGACAAGCCAACTACGAGAACTACATCGA
cel7D	TGCCCGAAGGACATCAAGTTCATTAACG
cdh	TGGCGCGCATACTCTGGTTCGG
bgl3A	TCCAGTTCGTCCAACGTCGATGACAG
act	ACGATGCTCCCCGTGCCGTGT

[a] At its 5′ end, the probe contains 6FAM (6-carboxyfluorescein), the emission spectrum of which is quenched by TAMRA (6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine) at its 3′ end.

tion at the start of the main cultivation. The copy numbers of transcripts at the start (0 h) and after 6 h of the main cultivation were quantified to assess gene expression level, by means of real-time quantitative PCR. Figure 1b shows the copy numbers of *cel6A*, *cel7D*, *cdh*, and *bgl3A* mRNAs from 0 and 6 h. In the cases of *cel6A*, *cel7D*, and *cdh*, a higher concentration of glucose led to a smaller increase of the number of transcripts. For example, the transcript number of *cel6A* after 6 h was 210-fold higher than that at 0 h in G0, whereas it was 61-fold higher in G50 and 80-fold higher in G500 but only 3.3-fold higher in G5000. The transcript number of *cel7D* at 6 h was 310-fold higher than that at 0 h in G0 but only 0.97-fold in G5000. In the case of *cdh*, the corresponding values were 21-fold in G0 and 3.1-fold in G5000. On the other hand, there was no apparent relationship between transcript number and added glucose concentration in the case of *bgl3A*; the increases were 2.0-, 1.4-, 1.6-, and 1.9-fold in G0, G50, G500, and G5000, respectively.

The time course of extracellular glucose concentration in the main culture medium is shown in Fig. 2. In G5000, the amount of remaining glucose was 2,800 μM after cultivation for 6 h. In contrast, glucose in G500 and G50 was

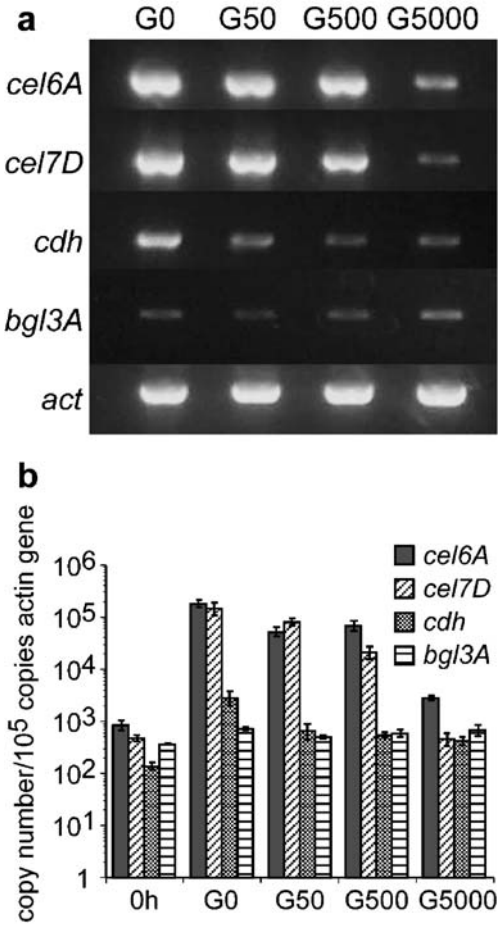

Fig. 1 Gene expression of *cel6A*, *cel7D*, *cdh* and *bgl3A* monitored by electrophoresis (**a**) and quantitative RT-PCR (**b**). **a** RT-PCR was performed using cDNAs from 6 h culture. Expression of the actin gene was analyzed as a control to compare expression levels between different culture conditions. **b** cDNAs immediately before the start of main cultivation (0 h) and after cultivation for 6 h under the indicated condition (G0, G50, G500, and G5000) were used. Copy number was normalized by the copy number of actin transcripts from the same sample. The *vertical axis* indicates the copy number of each gene per 10^5 copies of actin transcript. Each error bar shows the standard error in triplicate tests for each sample

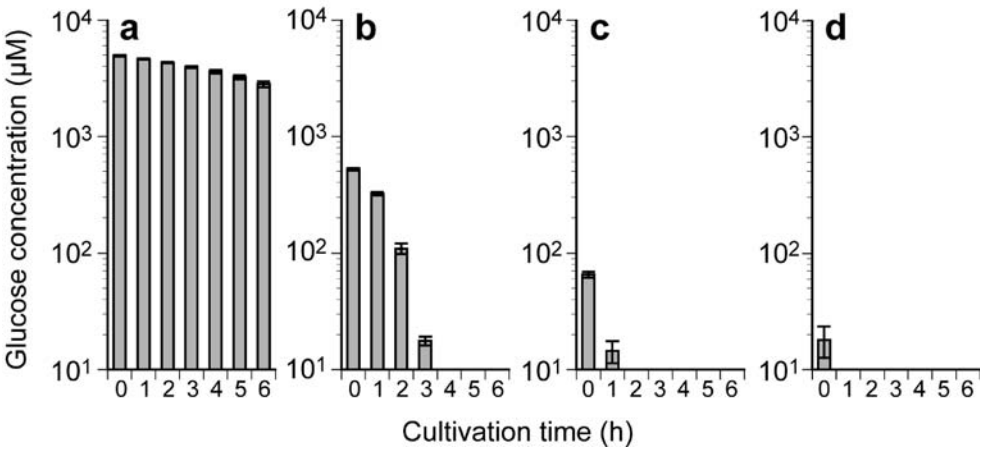

Fig. 2 Time course of extracellular glucose concentration in the main culture medium. Glucose 5,000 μM (**a**), 500 μM (**b**), 50 μM (**c**), or 0 μM (**d**) was added at the start of the cultivation. Glucose, which remained from the resting culture, was detected at the start point (0 h). Each error bar shows the standard error in triplicate tests for each sample

completely consumed after 4 and 2 h, respectively. In G0 culture, 18 μM glucose was detected at the start of the cultivation as a contamination from pre-grown culture. However, these amount of glucose was totally consumed within 1 h of the main cultivation. Therefore, G0 culture could be taken as a control experiment without any glucose in the present study. The glucose consumption was associated with high expression levels of *cel6A* and *cel7D* in the cells in G0, G50, and G500, while the expression of that genes were repressed in G5000 (Fig. 1).

Since the transcripts number of *cel6A*, *cel7D*, and *cdh* were greatly increased in G0 or G50, the time course of transcript levels was analyzed under these two conditions to examine the activation of gene expression. As shown in Fig. 3, the copy numbers of *cel6A*, *cel7D*, and *cdh* transcripts increased exponentially after 2 h. This time point is consistent with the time when glucose was totally consumed from the G50 medium. Similar exponential increases were observed in both G0 and G50, though the transcript numbers were larger in G0 than G50 at all time

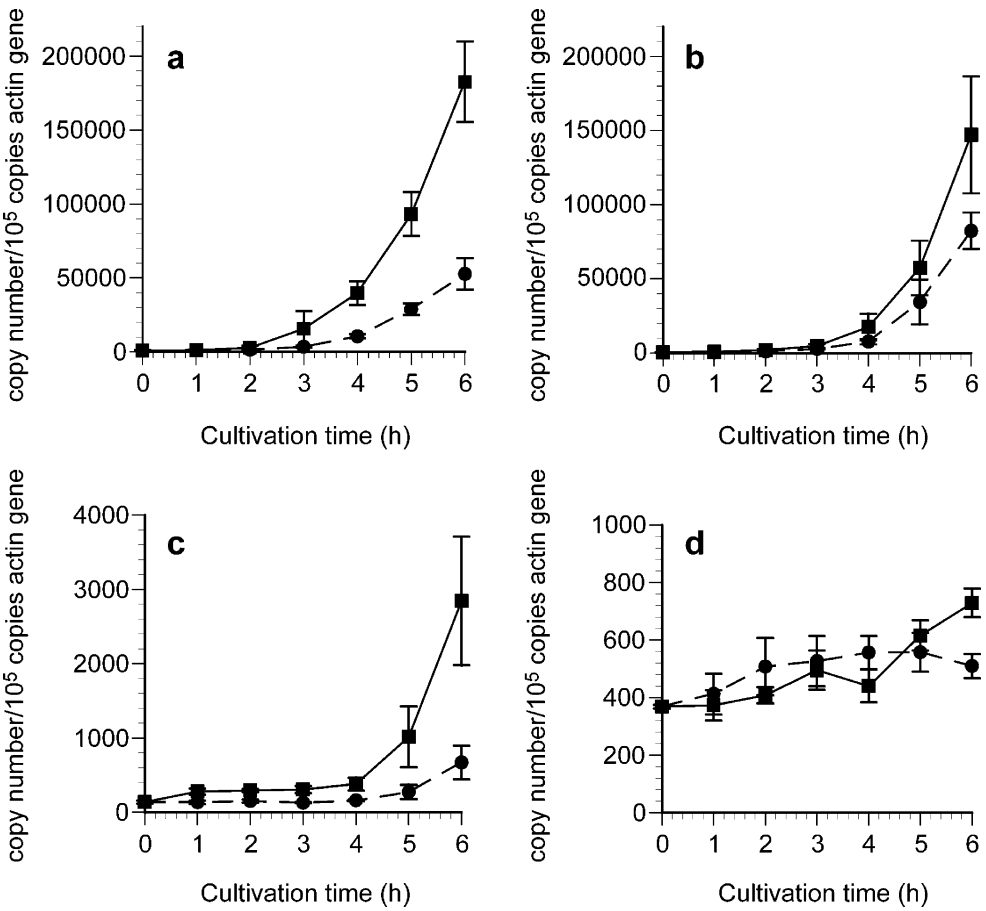

Fig. 3 Time course of *cel6A* (**a**), *cel7D* (**b**), *cdh* (**c**), and *bgl3A* (**d**) transcripts measured by quantitative RT-PCR. Transcript numbers of each gene in G0 (*square*) and G50 (*circle*) were normalized as described in Fig. 1b. Each error bar shows the standard error in triplicate tests for each sample

points. In contrast, the copy number of *bgl3A* transcripts showed no time dependence, and no obvious difference of transcript numbers was observed between G0 and G50.

The transcript numbers of *cel6A* and *cel7D* in G0 and G50 (Fig. 3) were re-plotted with an exponential *y*-axis (Fig. 4), and the parameters of the resulting plots ($y=a\times 10^{bx}$) are listed in Table 2. The rates of increase of transcripts of *cel6A* and *cel7D* were not affected by the glucose concentration of the culture and were 2.1 and 2.7 times per hour, respectively. Moreover, the distance between plots for G0 and G50, which indicates the time-lag of gene derepression caused by addition of 50 μM glucose, was 1.6 h for *cel6A* and 0.6 h for *cel7D*, indicating that the responses of *cel6A* and *cel7D* transcription to derepression are different.

Discussion

Cellulose biodegradation by the white-rot basidiomycete *P. chrysosproium* has been considered as a synergistic reaction of extracellular hydrolytic and oxidative enzymes, such as CBHs, EGs, CDH, and BGL (Eriksson 1978). However, the K_m of cellobiose for BGL3A is considerably higher than that for CDH, and BGL3A does not hydrolyze cellobionolactone, which is formed by the reaction of CDH, suggesting a kinetic disadvantage of BGL3A under cellulolytic conditions (Lymar et al. 1995; Igarashi et al. 2003). Moreover, transcription of *cdh* was promoted by cellobiose, whereas that of *bgl3A* was repressed, suggesting that BGL3A is not involved in the extracellular cellulose degradation system of this fungus (Yoshida et al. 2004). In the present work, we found that *bgl3A* expression is essentially independent of glucose concentration, indicating that *bgl3A* is regulated in a different manner from the other three cellulolytic genes and may not act in concert with the other cellulose-degrading enzymes.

Gene expression of cellulolytic enzymes is regulated by several transcriptional factors in *Hypocrea jecorina* and *Aspergillus* spp. (Schmoll and Kubicek 2003; Aro et al. 2005), and these are also thought to regulate in basidiomycete *Trametes versicolor* (Stapleton and Dobson 2003). As far as we know, however, there has been no report on the identification of cellulolytic gene regulators in basidiomycetes. In *H. jecorina* and *Aspergillus* spp., CRE is one of the cellulase repressors activated in the presence of glucose, and its binding motifs have been analyzed in some species, e.g., *Aspergillus nidulans* (CREA; SYGGRG; Dowzer and Kelly 1989; Dowzer and Kelly 1991; Cubero and Scazzocchio 1994) and *H. jecorina* (CREI; GTGGGG, CCCCAC; Strauss et al. 1995; Ilmen et al. 1996a,b; Takashima et al. 1996; Zeilinger et al. 2003). As glucose repression of cellulolytic genes in basidiomycetes seems to be similar to that of ascomycetes, it is expected that a repressor protein homologous to CRE regulates in basidiomycetes. In the present study, we searched CREA homologue by BLAST algorithm (tblastn) in the total genomic sequence of *P. chrysosporium*, which is publicly available on the JGI website at http://genome.jgi-psf.org/Phchr1/Phchr1.home.html, using *A. nidulans* CREA amino acid sequence (AAR02858) as a query. When the search was performed with expect value 1×10^{-10}, two CREA-like genes (scaffold 8:1050104-1052312 and scaffold 27:153078-154768) were found, and the identities of two homologues to *A. nidulans* CREA are 25% and 17%, respectively. Another C2H2 zinc-finger transcriptional factor with different function (74% identity to *A. nidulans* steA, a factor concerning cell wall metabolism, for example) was found when BLAST was performed with the expect value 1×10^{-9}, indicating that *P. chrysosporium* may have two CREA homologues, though it is still unknown whether these homologues truly act as the carbon catabolite repressors. Moreover, the promoter sequence of *cel6A*, *cel7D*, *cdh*, and *bgl3A* was analyzed to search

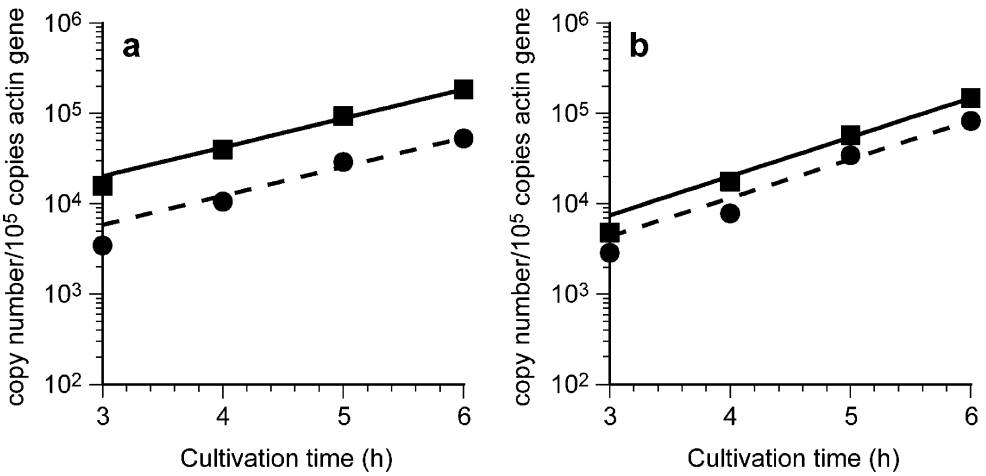

Fig. 4 Time course of *cel6A* (**a**) and *cel7D* (**b**) transcripts during exponential increase phase (3–6 h). Transcript numbers of each gene in G0 (*square*) and G50 (*circle*) (Fig. 3) were re-plotted with an exponential *y*-axis

Table 2 Parameters of regression curve ($y=a\times10^{bx}$)

		a	*b*	Slope (copy no./hour)[a]	Time for 10^4 copies (hour)
cel6A	G0	2,200±500	0.32±0.02	2.1	2.1
	G50	640±330	0.32±0.04	2.1	3.7
cel7D	G0	370±110	0.43±0.02	2.7	3.3
	G50	220±130	0.43±0.04	2.7	3.9

[a] The slope of each curve was calculated as 10^b.

putative CREA-like protein binding sites (defined as SYGGRG). The length of the promoters were determined as 2,000 bp for *cdh* and as the distance to an upstream gene 3′ end for *cel6A*, *cel7D*, and *bgl3A* because the promoter region of these genes, which functioned practically under the repression and derepression conditions, are not known in this moment. As a result, we found several putative binding sites in the promoter regions of *cel6A*, *cel7D*, and *cdh* in the genome database of *P. chrysosporium* (Fig. 5). In contrast, the promoter region of *bgl3A* has no such motif in the upstream region, consistent with the distinct behavior of *bgl3A* from the other genes under derepressing conditions.

In filamentous fungi, glucose generally serves as a repressor of cellulolytic genes, and derepression of gene expression is provoked when the glucose concentration falls (Broda et al. 1995; Ilmen et al. 1997). In some cases, however, lower expression level of *cbh* (*cel7A* and *cel7B*) has been detected constitutively (Covert et al. 1992), although the actual number of transcripts may be small compared to those of *cel7C* or *cel7D*. The presence of glucose in the culture or environment may be recognized as concentration or as intake flux by fungi, and it was reported that concentration is more influential than flux for yeast (Meijer et al. 1998). However, the relationship between the glucose concentration and the levels of cellulase gene expression were not investigated quantitatively. Accordingly, we monitored the change of glucose concentration with cultivation time and analyzed the correlation with cellulolytic gene expression to examine the effect of glucose derepression. Moreover, transcript numbers of *cel6A* and *cel7D* were analyzed and compared with the derepression rate of the two genes and the lag time at two glucose concentrations (G0 and G50). The time course analysis of cellulolytic gene expression was performed during the main culture, which was started after 6 h of the resting cultivation (without any carbon source). The reason for the necessity of this resting cultivation is that glucose, which is contained in pre-grown culture, was seemed to be remained in or out of the fungal cells even after washing the mycelia. Indeed, the increases of cellulolytic gene transcripts did not occur during the resting cultivation and were observed after 2 h in the main cultivation. This indicates that glucose was not completely washed out, and glucose repression may still be effective throughout resting cultivation. The remaining glucose appears to be consumed totally by the fungus during main cultivation, and then carbon catabolite derepression occurs. In the previous study, we have reported that the copy number of *cdh* transcripts showed no significant difference between the culture with 2% glucose and no carbon (Yoshida et al. 2004). In the present paper, however, we observed that the number of *cdh* transcripts was clearly increased during the cultivation with no carbon source. The contradiction between these two investigations could be because of the resting cultivation. In the previous report, glucose from the pre-grown culture or produced by degradation of hyphae was carried over into the transferred medium, so that carbon catabolite repression may continue. In contrast, as the resting cultivation avoid the undesirable influence of glucose contamination, expression of cellulolytic genes were not repressed and increased by a function of carbon catabolite derepression. This fact suggest the importance of the resting culture to eliminate the effects of pre-culture conditions.

The derepression rates of *cel6A* and *cel7D* were specific to each gene and were not influenced by the glucose concentration in the culture medium. The lag times

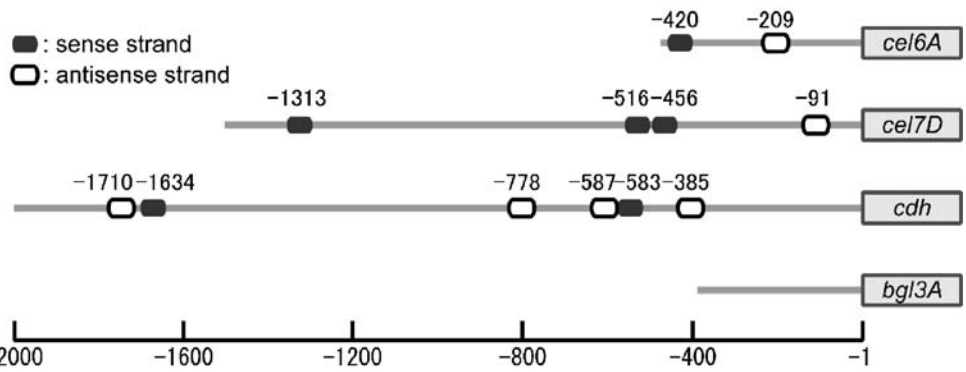

Fig. 5 Occurrence and distribution of SYGGRG sequence (putative CREA binding region) in cellulolytic gene promoters. *Filled marker* indicates a binding site on the sense strand and *open marker* means antisense strand. Numbers of the nucleotides begin with the first nucleotide of the start codon as +1. Each number above the marker indicates the position of 3′ end of each binding sites in the promoter

indicated that the response of *cel6A* was slower than that of *cel7D*. Since gene transcription is controlled by the promoter regions, the quantitative analysis of derepression rate and lag time of gene expression could be able to illustrate the character of the promoter activity. It is well known that cellulase production and cellulolytic gene expression in filamentous fungi are controlled by the induction and repression of the relevant genes. Several oligosaccharides have been proposed as candidate inducers in fungi. For example, sophorose induces cellulase expression in *H. jecorina* (Mandels et al. 1962), gentiobiose induces cellulase of *Penicillium purpurogenum* (Kurasawa et al. 1992), cellobiose probably act as an inducer in several fungi (Eberhart et al. 1977; Eriksson 1978; Canevascini et al. 1979; Rho et al. 1982; Seiboth et al. 1997), and cellopentaose is recently reported as a probable inducer for EG in *Polyporus arcularius* (Ohnishi et al. 2007). In the present study, however, we observed a drastic increase of cellulolytic gene expression in the absence of inducers. Two possibilities are suggested for the mechanism of derepression, release of repressor, or activation without releasing the repressor, though it is impossible to conclude the true mechanism from the results obtained in the present study. At this moment, because putative CREA-binding sites exist at the upstream of the cellulolytic gene, the increase of these cellulolytic gene expressions should be considered as the result of down-regulation of carbon catabolite repressors under derepression condition similarly to the other cellulolytic ascomycetes. Although the molecular mechanism of the release of the repression is still unknown, the presented results clearly indicate that carbon catabolite derepression plays a key role in cellulolytic gene regulation as well as induction or repression. Moreover, quantitative analysis of gene expression in the presence of various concentrations of carbon sources appears to be an effective approach for characterizing gene expression characteristics and promoter activities.

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RESEARCH LETTER

Quantitative transcriptional analysis of the genes encoding glycoside hydrolase family 7 cellulase isozymes in the basidiomycete *Phanerochaete chrysosporium*

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Abstract

Cellulolytic fungi generally secrete a cellulase mixture consisting mainly of glycoside hydrolase family 7 cellulases (Cel7s) during degradation of crystalline cellulose. Although several Cel7s have been investigated so far, the marked similarity in their amino acid and nucleotide sequences makes independent quantitative analysis difficult. Here, we present a real-time PCR method for the detection and quantification of Cel7 genes (*cel7A-F*/*G*) in the basidiomycete *Phanerochaete chrysosporium* using PCR primer sets designed based on the 3′ untranslated region sequences. It was confirmed by agarose gel electrophoresis, sequencing, and dissociation curve analysis of the PCR products that each *cel7* transcript was specifically amplified by the corresponding primers. We applied this real-time reverse-transcription PCR method using the presented primer sets to evaluate quantitatively the expression changes of *cel7* genes in *P. chrysosporium* under conditions of carbon catabolite derepression.

Introduction

Cellulolytic fungi secrete multiple cellulose-hydrolyzing enzymes, generally called cellulases, during cellulose degradation (Eriksson *et al.*, 1990; Coughlan, 1991; Tomme *et al.*, 1995), and these enzymes are mainly categorized as glycoside hydrolases (GHs), which cleave the glycosidic bonds of various polysaccharides. Recently, they have been grouped into families based on the amino acid sequences and possible secondary structure predicted by hydrophobic cluster analysis (Henrissat, 1991; Henrissat & Bairoch, 1996; Henrissat & Davies, 1997). A fungal cellulase mixture consists of a high proportion of enzymes belonging to GH family 7, which typically account for $>50\%$ of the total secreted protein, emphasizing the importance of the investigation of this family to achieve a better understanding of fungal cellulose utilization.

The ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is one of the best-studied cellulolytic microorganisms and is known to produce two enzymes belonging to GH family 7, i.e. cellobiohydrolase I (EC 3.2.1.91; *Tr* Cel7A) and endoglucanase I (EC 3.2.1.4; *Tr* Cel7B) (Shoemaker *et al.*, 1983; Teeri *et al.*, 1983; Penttila *et al.*, 1986). There have been several reports on quantification of CBHI and EGI in culture fluid using polyclonal and monoclonal antibodies (Messner *et al.*, 1988; Mischak *et al.*, 1989; Kolbe & Kubicek, 1990; Luderer *et al.*, 1991). Moreover, Northern hybridization was performed for detection of their mRNA (Ilmen *et al.*, 1997) and is now a widely used method for the detection and semi-quantification of *cel7* transcripts.

In the case of the wood-rotting basidiomycete *Phanerochaete chrysosporium*, on the other hand, the situation regarding quantification of Cel7s is more complicated. *Phanerochaete chrysosporium* produces multicomponent extracellular cellulases (Eriksson & Pettersson, 1975a, b; Eriksson, 1978). In the genetic analysis of *P. chrysosporium*, Covert *et al.* (1992a) identified six genes encoding Cel7 isozymes (*cel7A-F*) in the genome, and this was confirmed by the total genome sequencing of this fungus (Martinez *et al.*, 2004). Recently, an additional gene was found and named *cel7G*; this is a duplication of *cel7F*, because the nucleic acid sequence is exactly the same, suggesting that this fungus carries a total of seven genes possibly encoding Cel7s (Vanden Wymelenberg *et al.*, 2006). A detailed investigation

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of each individual enzyme's function and the corresponding gene expression will be needed to understand this multicomponent cellulolytic system. However, the protein characteristics of Cel7 isozymes have not been investigated, except for two major isozymes (Cel7C and D) (Eriksson & Pettersson, 1975a; Streamer *et al.*, 1975; Uzcategui *et al.*, 1991; Igarashi *et al.*, 1998), mainly because the minor Cel7 isozymes are extremely difficult to separate from each other. Transcription of these Cel7s has been examined by means of Northern analysis (Sims *et al.*, 1988), reverse transcription (RT)-PCR (Tempelaars *et al.*, 1994; Broda *et al.*, 1995), and competitive RT-PCR (Covert *et al.*, 1992b; Vanden Wymelenberg *et al.*, 1993; Lamar *et al.*, 1995; Vallim *et al.*, 1998). Recently, expressed sequence tag analysis (Sato *et al.*, 2009) and microarray analysis (Vanden Wymelenberg *et al.*, 2009) were performed for the quantification of *cel7* gene transcripts. However, quantitative analysis by real-time PCR has not been performed for these genes, because the *cel7* genes show high levels of sequence identity (at least around 70%), making it difficult to design specific primer sets for real-time PCR amplification (Table 1).

In the present study, we developed a method to quantify *cel7* gene transcripts individually in *P. chrysosporium* using real-time RT-PCR with specific primer sets designed based on the sequences of the 3′ untranslated regions (3′-UTRs) of these genes, which show little similarity. The developed method was applied to analyze the time courses of the expression of *cel7*s under conditions of carbon catabolite derepression. The results demonstrated that the method could accurately quantify *cel7* gene transcripts.

Materials and methods

Fungal strain and culture conditions

Phanerochaete chrysosporium strain K-3 (Johnsrud & Eriksson, 1985) was cultivated in 400 mL of modified Kremer and Wood medium (Kremer & Wood, 1992) for preculture and resting culture, as described previously (Suzuki *et al.*, 2008). After 6 h of resting cultivation, mycelia were cultivated for another 6 h without any carbon source (main culture). A 5-mL aliquot of the main culture was harvested every hour for cDNA preparation from collected mycelia. cDNA was constructed as described previously (Suzuki *et al.*, 2008) and used as the template for real-time quantitative RT-PCR.

Table 1. Nucleic acid sequence identity between *cel7* genes (%)

	cel7A	*cel7B*	*cel7C*	*cel7D*	*cel7E*	*cel7F/G*
cel7B	67		66	66	63	64
cel7C	73	73		93	82	81
cel7D	71	71	83		81	81
cel7E	70	69	81	81		91
cel7F/G	72	71	84	82	83	

The numbers with the white background indicate catalytic domain sequence identities, and those with the gray background indicate CBM sequence identities.

Identification of 3′-UTR sequences

The cDNA prepared from the cellulose-grown mycelia was used as the PCR template for 3′ rapid amplification of cDNA ends (RACE). The 3′-UTRs of *cel7A*, *cel7B*, *cel7C*, *cel7D*, *cel7E*, and *cel7F/G* genes (GenBank accession nos X54411 for both *cel7A* and *cel7B*, Z22528, L22656, Z11727, and Z11729) were amplified using an abridged universal amplification primer (Invitrogen) and gene-specific primers designed based on the total genomic sequences, as listed in Table 2. The reaction was performed for 35 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 68 °C for 30 s using KOD-plus Ver. 2 (Toyobo). Approximately 900 bp of the downstream region of *cel7A*, *cel7C*, *cel7D*, and *cel7F/G* genes was amplified from total genomic DNA of *P. chrysosporium* using the gene-specific primer sets (Table 2). The reaction was performed for 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 68 °C for 60 s. All PCR products were cloned in pCR4Blunt-TOPO using a Zero Blunt TOPO PCR cloning kit (Invitrogen) and *Escherichia coli* JM109, and were sequenced using DTCS Quick Start Master Mix with DNA sequencer CEQ 8800 (Beckman Coulter).

Verification of the specificity of the primer sets for real-time quantitative RT-PCR

PCR was performed using the *cel7A-F/G* primer sets for real-time RT-PCR to verify their specificity. The template DNA of this PCR was pCR4Blunt-TOPO containing 3′-RACE products of *cel7A*, *cel7C*, *cel7D*, *cel7E*, and *cel7F/G* and the same vector containing *cel7B* gene ORF. The reaction was performed for 15 cycles of denaturation at 95 °C for 30 s,

Table 2. Gene-specific primers for 3′-RACE and genomic PCR

Primers for 3′-RACE	
cel7A	GTTCAGGCCTGCGTGAATATTG
cel7B	ACTATTCTCAGTGTATCTAATGCTG
cel7C	AGTGCTACTAAGTTACGGAATGGAT
cel7D	TGCTACTAAGAGGCGCTTGGACAT
cel7E	TCGCAGTGCTACTAGATGGTGTAC
cel7F/G	TACTCCCAGTGCTACTAAGTCGC
Primers for genomic PCR*	
cel7A	ATGTATCCACCGCCAGTTCACG
cel7C	GATGGATACTCTGTTGGAAGGAC
cel7D	ACCGACGATGGCTACAACATCG
cel7F/G	CGCTTTGCACAAACACATCAAGC

*The listed primers for genomic PCR match the reverse sequence. The primers for 3′-RACE were used as the forward primers for each genomic PCR.

annealing at 60 °C for 30 s, and extension at 72 °C for 20 s using Takara Ex Taq (Takara Bio). RT-PCR was also performed using cDNA from 2-day-old cellulose-grown mycelia to confirm specific amplification with each primer set. The reaction was performed for 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. The RT-PCR products were sequenced as described above. Dissociation curve analysis was performed in a Mx3000P Real-time QPCR System (Stratagene) as follows: 5 μL of template solution was mixed with 20 μL of solution containing 12.5 μL of Brilliant II SYBR Green QPCR Master Mix (Stratagene), 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, 0.375 μL of ROX reference dye solution, and 6.125 μL of sterile-distilled water. The mixtures were initially incubated at 95 °C for 15 min, followed by amplification for up to 40 cycles of 95 °C for 20 s and 60 °C for 30 s. After thermal cycling, reaction mixtures were heated from 60 to 94 °C for 20 min at a constant rate and the fluorescence of SYBR Green I was measured continuously during heating. Fluorescence data were analyzed using MxPro ver.4.0 software (Stratagene) to plot the dissociation curves. The template cDNA was prepared from 2–5-day-old cellulose-grown culture for triplicate tests.

Quantification of *cel7* gene transcripts

Real-time quantitative PCRs were performed in an Mx3000P Real-time QPCR System as described for dissociation curve analysis. For each standard curve, the dilution series of pCR4Blunt-TOPO vectors described above was used as templates. The transcript number of the actin gene was quantified as an internal standard using the following primers: actin-F 5′-GCATGTGCAAGGCTGGCTTTG-3′ and actin-R 5′-AGGGCGACCAACGATGGATG-3′. Fluorescence intensity analysis was performed with MxPro ver. 4.0 software.

Results and discussion

Identification of 3′-UTR of *cel7* genes

Design of specific primer sets for PCR amplification of *cel7* genes encountered two difficulties, i.e. the high similarity of *cel7* gene sequences and genetic polymorphism because *P. chrysosporium* K-3 is a heterokaryotic strain. As shown in Table 1, the nucleic acid sequence identities between *cel7A*-*cel7F/G* catalytic domains and carbohydrate-binding modules (CBMs) are about 70–80%. In fact, we failed to find appropriate sequences in the coding regions of *cel7* genes for the specific primer sets for SYBR Green I-mediated real-time PCR amplification. Therefore, the 3′-UTR sequences of *cel7* genes were required to evaluate the possibility of using them for specific primer design. The 3′-RACE method was applied for the identification of 3′-UTRs using gene-specific primers (as shown in Table 2) and the universal amplification primer. As a result, 3′-UTRs of *cel7A*, *cel7C*, *cel7D*, *cel7E*, and *cel7F/G* were successfully amplified and sequenced. However, the 3′-UTR of *cel7B* was not amplified in 3′-RACE, because of its low expression level. To detect the genetic polymorphism of each gene, approximately 900 bp of the genomic sequences downstream of the respective stop codons was amplified using specifically designed primers (Table 2) and then sequenced. The genomic PCR was performed for *cel7A*, *cel7C*, *cel7D*, and *cel7F/G*, because the genetic polymorphism in *cel7E* 3′-UTR had already been detected by 3′-RACE analysis. As a result, polymorphisms were detected in *cel7A*, *cel7D*, and *cel7F/G*, but no nucleic acid alteration was found in *cel7C*. We then compared the sequence identities of 3′-UTRs between *cel7* genes (except *cel7B*), as well as catalytic domains and CBMs, and found that they showed $< 10\%$ identity. This result suggested that it is possible to design specific primer sets based on the 3′-UTR region of *cel7A*, *C*, *D*, *E*, and *F*/*G*. For *cel7B*, the sequence of its CBM showed lower similarity than that of other *cel7* genes (Table 1), and therefore we considered that a specific primer set could be designed based on its CBM and flanking linker region.

Primer design for specific amplification of *cel7* genes

The primer sets were designed to amplify *cel7* genes specifically in real-time PCR using their 3′-UTR sequences, except in the case of *cel7B*, for which the linker region and CBM were used. Figure 1a shows the structures of the 3′-ends of *cel7* genes and the positions of the primer sets. The designed primer sequences are listed in Table 3. To verify the specificity of each primer set, PCR was performed using these primer sets and the template vectors harboring each target sequence reciprocally (Fig. 1b). When the primer set for *cel7A* was used, only the vector harboring the *cel7A* 3′-RACE product was amplified. This indicated that the *cel7A* primer set did not cause nonspecific amplification of other *cel7* targets. The other five primer sets also did not amplify other *cel7* targets and only amplified their own target sequences. Furthermore, RT-PCR was performed using cDNA prepared from a 2-day-old cellulose-grown culture as a template. Figure 2a shows the RT-PCR products generated with the primer sets for *cel7A* – *cel7F/G*. A single band was observed in each lane at an appropriate location, indicating that the primer sets for *cel7* genes successfully amplified their target genes from the cDNA sample. These RT-PCR products were sequenced and confirmed to be the specific products. Moreover, dissociation curve analysis of *cel7* RT-PCR products was performed to ascertain whether minor nonspecific products were accumulated (Fig. 2b). All

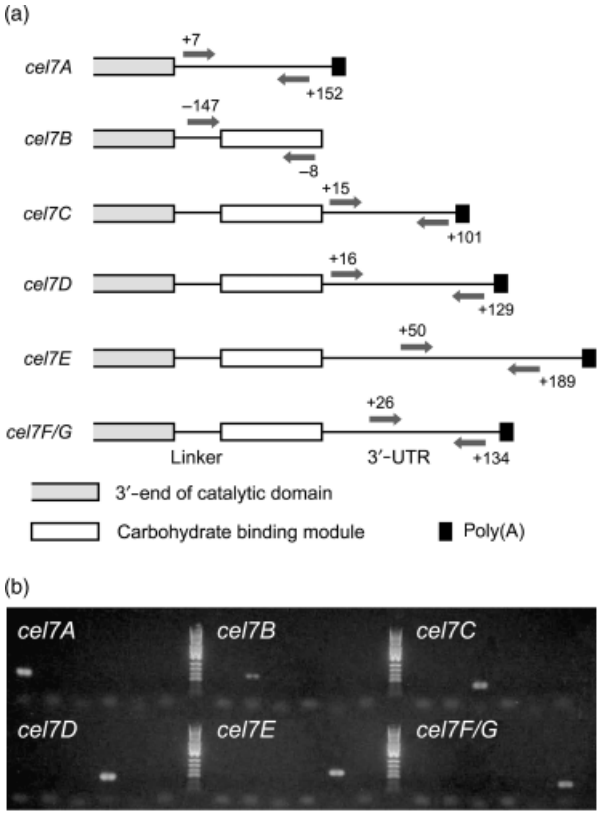

Fig. 1. (a) Structures of the 3′-ends of *cel7* genes. Arrows represent primer locations. The number presented beside each arrow indicates the position of the 5′-end of each consensus sequence for the primer. Numbers of nucleotides begin with the last nucleotide of the stop codon as − 1. The specificity of designed primer sets was validated by PCR (b). The agarose gel shown here is divided into six blocks with *cel7* gene names. Each gene name presented on the gel indicates the primer set of the corresponding gene used in that block. There are six lanes in one block, and each lane shows the PCR product using the template vector harboring the target sequence of *cel7A*, *cel7B*, *cel7C*, *cel7D*, *cel7E*, or *cel7F/G*.

products showed a single peak in the dissociation curve, indicating that no nonspecific product was amplified. Consequently, it was established that these primer sets specifically amplify *cel7* genes without generating any significant amount of nonspecific products.

Quantitative analysis of *cel7* gene expression under carbon catabolite derepression

A previous study of *cel7* gene transcription in *P. chrysosporium* showed that, as in other cellulolytic fungi, the genes are highly expressed in a submerged cultures with cellulose as a carbon source and are strongly repressed by glucose (Sims *et al.*, 1988, 1994; Covert *et al.*, 1992b; Vanden Wymelenberg *et al.*, 1993; Broda *et al.*, 1995). Furthermore, it is reported that they were differently expressed during cultivation with cellulose or aspen wood chips (Covert *et al.*, 1992b; Vanden

Table 3. Gene-specific primers for real-time quantitative RT-PCR

cel7A	
F	GTGTGTAACTTAGATTCGTGGTGT
R	TTCGATACCATGGGCTGACGAC
cel7B	
F	GGATCCGCACCTGCATCTTCA
R	CACTGAGAATAGTAGATATTGAGAG
cel7C	
F	GCGGGGGTTGGATTTGTTGCAC
R	AGGAACAAAGAAATATGATTTGAGGT
cel7D	
F	TGCAGCCTCGAAAATAGCAGACT
R	GTATGTGTGCATTTCACGGATCG
cel7E	
F	TTGGCGCAGACGGGATTTTCC
R	CACGCAAACTACCCCTTTCCA
cel7F/G	
F	AGGGTGTTTCTTCTCTTTTTGGCT
R	GTATATCTTTCTGCTGATTCTACAG

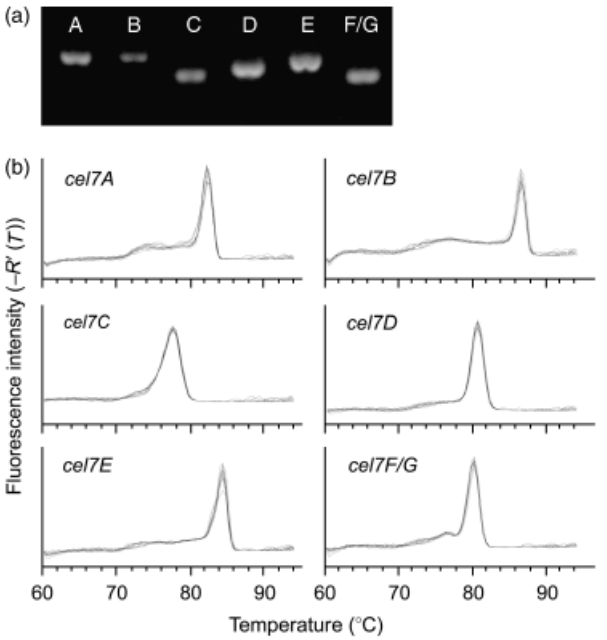

Fig. 2. Verification of the specificity of our primer sets. (a) RT-PCR amplification of *cel7* genes from cDNA template using the primer sets designed in this study. Each lane shows the RT-PCR product using the primer set for *cel7A* (A), *cel7B* (B), *cel7C* (C), *cel7D* (D), *cel7E* (E), or *cel7F/G* (F). (b) Dissociation curve analysis of RT-PCR products using SYBR Green I. Decreasing fluorescence intensity of SYBR Green I was measured for each product from 60 to 94 °C. Derivative plots of dissociation curves are indicated.

Wymelenberg *et al.*, 1993; Vallim *et al.*, 1998). These results indicate the complexity of *cel7* gene regulation, and a detailed investigation with more sensitive detection is essential to understand the Cel7 enzyme system in *P. chrysosporium*. Analysis of the expression of each gene by real-time

quantitative RT-PCR under various culture conditions would be one of the most informative approaches. Recently, we reported the quantitative analysis of derepression of cellulolytic genes in *P. chrysosporium* (Suzuki *et al.*, 2008), and the drastic increase of *cel7D* transcripts under derepression led to our interest in the derepressed expression behavior of other *cel7* genes. Therefore, we performed real-time RT-PCR amplification of *cel7* genes using our novel primer sets to analyze the gene expression patterns quantitatively during cultivation of *P. chrysosporium* under carbon catabolite derepression. However, *cel7G* cannot be distinguished from *cel7F* because they have identical sequences, including the 5′- and 3′-UTR. Thus, the quantity of the *cel7F/G* transcript should be regarded as the sum total of *cel7F* and *cel7G*. The time course of transcript levels was analyzed in a medium lacking a carbon source to assess the effect of derepression. As shown in Fig. 3, the time course of expression appeared to be different for each *cel7* gene. *cel7D* transcripts showed repressed expression in the early stage of cultivation, followed by an exponential increase after 2 h of cultivation (Fig. 3d), in agreement with the previous result using the TaqMan probe method (Suzuki *et al.*, 2008). In the case of *cel7C*, gene expression remained repressed up to 5 h of cultivation (Fig. 3c). The number of transcripts of *cel7C* was 35-fold higher than that of *cel7D* in the repressed stage, in spite of the fact that *cel7D* is known to be the most highly expressed *cbh* gene in a cellulose-grown culture (Vanden Wymelenberg *et al.*, 1993). In contrast, *cel7C* transcripts were 3.5-fold lower than those of *cel7D* at 6 h of cultivation under derepressed conditions. These results indicate that *cel7C* is less susceptible than *cel7D* to glucose-mediated repression and derepression. On the other hand, *cel7A*, *cel7B*, *cel7E*, and *cel7F* showed no clear increase of transcripts during 6 h of cultivation (Fig. 3a, b, e, and f). The numbers of transcripts of *cel7A* and *cel7E* were close to the repressed level of *cel7D* transcripts, and the level of *cel7F* transcripts was 10-fold higher than that of *cel7E*. The number of *cel7B* transcripts showed the lowest level during the entire cultivation. These results indicate that *cel7A*, *cel7B*, *cel7E*, and *cel7F* are not regulated by the derepression machinery, at least under these culture conditions, so that the regulatory differences among these genes need further investigation.

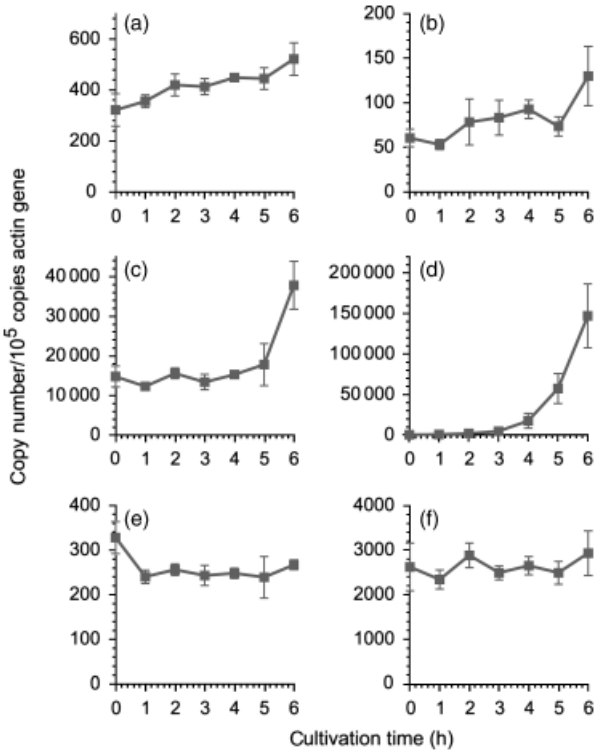

Fig. 3. Time courses of *cel7A* (a), *cel7B* (b), *cel7C* (c), *cel7D* (d), *cel7E* (e), and *cel7F/G* (f) transcripts measured by quantitative real-time RT-PCR. cDNAs from culture without any carbon source were used. Transcript numbers of each gene were normalized by the copy number of actin transcripts from the same sample. The vertical axis indicates the copy number of each gene per 10^5 copies of actin transcript. Each error bar shows the SD in triplicate tests for each cultivation.

Conclusion

A specific quantification method for the expression of *cel7* genes in *P. chrysosporium* was developed in this study. Using this method, even a low-expressed gene transcript, such as that of *cel7B*, could be detected quantitatively and independently. Therefore, this real-time PCR assay is applicable for quantitative evaluation of *cel7* genes under a variety of cultivation conditions and is expected to be a helpful tool for elucidating the cellulose degradation mechanism of *P. chrysosporium*. Moreover, the 3′-UTR-based primer design strategy should be applicable to other cellulolytic fungi, which have multiple cellulases with similar sequences.

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