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Novel Approaches to Improve Cellulase Biosynthesis for Biofuel Production – Adjusting Signal Transduction Pathways in the Biotechnological Workhorse *Trichoderma reesei*

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1. Introduction

Every living organism is destined to adjust its life style to the ecological niche of its habitat. Thereby the rotation of the earth, bringing about daily changes in light conditions, humidity and temperature, as well as nutrient requirements and the need for reproduction dominate the existence of creatures from bacteria to man. Adjustment of literally every biochemical pathway to the environmental conditions of the very spot an organism sees the light of day is of crucial importance for survival and successful competition against a plethora of rivals. However, not only adaptation as such but more importantly fast perception, correct interpretation, rating and an optimal response to environmental cues with efficient use of available resources for this task will ensure success in nature (Figure 1).

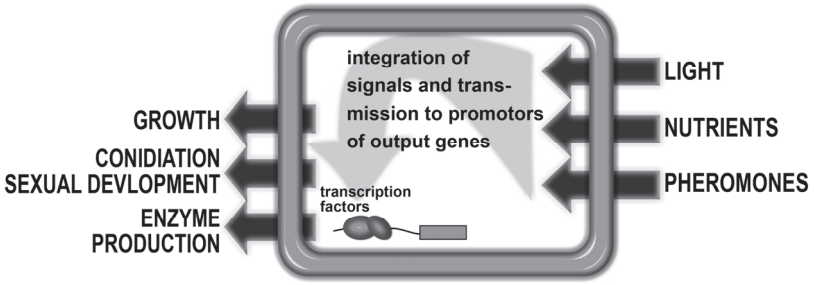

Fig. 1. Schematic representation of signal transduction and its consequences in fungi. Examples for environmental signals and possible adaptive reactions are shown.

In the kingdom of fungi, many species can be found that are masters of this game. One of them is *Trichoderma reesei* (anamorph of *Hypocrea jecorina*), a filamentous ascomycete possessing a very effective enzyme machinery for degradation of cellulose (Martinez et al., 2008; Schuster & Schmoll, 2010). If it would not have been for the so called cellulases of this

fungus, its name would probably only be known to a few specialists. But during World War II, *T. reesei* chose the cellulosic material of military tents and uniforms in the south pacific as its habitat. The efficient degradation of these materials – initially considered chemical warfare – was soon found to be accomplished by a fungus living in the warm and humid tropical rain forest of the Solomon Islands (Reese, 1976). After initial research efforts aimed at preventing degradation, the focus soon changed to promotion of decomposition for industrial use of cellulases (Reese, 1976). Notably, this fungus not only efficiently degrades cellulose in its natural habitat, the tropics, but also in the spartan environment of a shake flask containing minimal medium or a solid steel fermenter, which reflects a high potential for adaptation. Consequently, decades of research and industrial application made *T. reesei* one of the most prolific biotechnological workhorses in industry nowadays (Kubicek et al., 2009; Schmoll & Kubicek, 2003). However, *Trichoderma* spp. were also investigated for light dependent phenomena (Schmoll et al., 2010).

The cellulolytic and hemicellulolytic enzymes of *T. reesei* have been used for countless applications thereafter (Buchert et al., 1998; Galante et al., 1998a; Galante et al., 1998b), but only recently the need for alternative and sustainable fuel again boosted research with this fungus. Recent concerns about crop based-"first generation" biofuels enhanced the research on economically feasible "second generation" biofuels (Solomon, 2010; Somerville, 2007). The advantage of second generation biofuels is the usage of cheap, renewable and non-food biomass like cellulose from plants (Wilson, 2009). In the respective industrial process cellulases are applied for initial degradation of cellulosic plant material to fermentable sugars, which can then be further converted into bioethanol (Naik et al., 2010). The conversion process can be based on thermochemical processes or biochemical processes (Naik et al., 2010). The advantages of biochemical production of bioethanol are lower energy costs and that the production at smaller scales is possible, albeit the hydrolysis of cellulose to fermentable sugars is still the most expensive step in this process (Gomez et al., 2008).

In the following we explain how signal transduction pathways impact regulation of cellulase gene expression. Both known mechanisms and promising candidate factors/processes are discussed in order to provide a basis for evaluation of the relevance of a given signalling pathway or environmental cue for cellulase gene expression.

2. Cellulose, cellulases and *Trichoderma reesei*

Cellulose is a polysaccharide made up from D-glucose subunits in a polymerization degree of 10 000 or even higher. Therefore, this substrate cannot be transported into the cell for degradation. Additionally, the presence of the substrate outside the cell in its original form cannot be sensed. Hence *T. reesei* has developed an intriguing system for initial degradation of cellulose immediately upon landing of a spore on degradable material: Spores of *T. reesei* are covered with the cellulolytic enzymes, predominantly CBHII, which allows for an initial attack on cellulose (Kubicek et al., 1988; Messner et al., 1991) and also to detection of substrate by uptake of the characteristic degradation products. Once the cellulose signal is received, the powerful enzymatic machinery for plant cell wall degradation is launched and enables the spore to germinate and grow. Yet, the precise nature of this signal as well as the mechanism of its perception are still unknown, although the transglycosylation product of cellobiose, sophorose, is considered the natural inducer (Sternberg & Mandels, 1979). Additionally, the mode of perception of the signal remains obscure. While a G protein coupled receptor for sensing carbon has been identified in *N. crassa* (Li & Borkovich, 2006)

and also homologues in many other filamentous fungi, analysis of the *T. reesei* genome revealed a deletion in the respective genomic region (Schmoll, 2008). So far no GPCR was found in *T. reesei*, which could have assumed the task of the obviously lost receptor. Consequently, it is reasonable to believe that detection of the presence of cellulose outside of the cell occurs upon import of degradation products and the respective signal is likely to be transmitted starting from a carbohydrate transporter or degradative enzyme inside the cell.
In contrast, the enzymatic machinery, which is launched upon detection of cellulose outside the cell, is well studied (Schmoll & Kubicek, 2003). Interestingly, compared to other ascomycetes, *T. reesei* has a very limited number of genes encoding cellulases and hemicellulases (Martinez et al., 2008), which is a real surprise considering its great potential of cellulose degradation.
However, not only the insoluble substrate cellulose induces cellulase gene expression. Already in the late fifties of the last decade also soluble carbon sources were found to induce cellulase gene expression (Mandels & Reese, 1957). In the following for cellobiose (Mandels & Reese, 1960), sophorose, which is a transglycosylation product of cellobiose (Mandels et al., 1962; Sternberg & Mandels, 1979; Vaheri et al., 1979), lactose (Mandels & Reese, 1957; Seiboth et al., 2007), L-sorbose (Kawamori et al., 1985), L-arabitol and several additional substrates (Margolles-Clark et al., 1997), a cellulase inducing potential in *T. reesei* was detected. Interestingly, the set of cellulolytic enzymes produced on these carbon sources is not necessarily the same as on cellulose as has been shown for sophorose and lactose (Messner et al., 1988; Schmoll & Kubicek, 2005; Sternberg & Mandels, 1980). These findings have several implications when considering signal transduction processes: Detection of these carbon sources in the environment causes *T. reesei* to produce cellulolytic enzymes and hence these different signals must be combined to cause a common output. Nevertheless, since the array of cellulases produced is incomplete on lactose and sophorose, certain fine tuning mechanisms, which adjust the cellulase mixture to slightly different substrates, are likely to be at work.
The industrial application of cellulases necessitated not only the optimization of the cultivation media but also the fungus itself was treated for higher performance. In research, not only QM6a, but also an early mutant strain enhanced for cellulase production, QM9414 as well as the hyperproducer strain RUTC30 (Seidl & Seiboth, 2010) are frequently used.

3. Regulation of cellulase gene expression

Besides evaluation of the influence of different carbon sources on expression of cellulase genes, the mechanism which triggers this process was also investigated at a molecular level.
Cloning of the major cellulase gene, *cbh1* (encoding cellobiohydrolase 1, today known as CEL7A) along with antibody-based analysis of this and further cellulolytic enzymes (Mischak et al., 1989; Shoemaker et al., 1983a; Shoemaker et al., 1983b; Teeri et al., 1987) established a crucial basis for elucidation of the mechanism of cellulase gene expression in *T. reesei*. The following studies concluded from the correlation of protein expression with mRNA abundance, that cellulase gene expression is predominantly regulated on the pre-translational level (El-Gogary et al., 1989; R. Messner & Kubicek, 1991; Morawetz et al., 1992). Nevertheless, recent results indicate that this assumption must be treated with caution and that posttranscriptional regulation in addition has to be considered (Gyalai-Korpos et al., 2010).

The main cellobiohydrolases *cbh1* and *cbh2*, as well as *egl1*, *egl2* and *egl5* are coordinately transcribed, with *cbh1* being the most highly expressed cellulase gene (Ilmén et al., 1997). Although the real inducer of cellulase production still remains to be determined, the transcription factors regulating the expression are well studied. Five transcription factors are implicated in this process: ACE1 and CRE1 are negative regulators and ACE2, XYR1 and the HAP2/3/5 complex are positive ones (Kubicek et al., 2009). These transcription factors not only bind to their target promotors under inducing conditions (for example cellulose or sophorose), but mostly also under repressing conditions (glucose) (Rauscher et al., 2006; Zeilinger et al., 1998).

Consequently, it is reasonable to assume that the activity of these transcription factors is (also) regulated by modification in response to changing conditions. These modifications, such as phosphorylation or ubiquitinylation can be the outcome of a signal transduction cascade. Phosphorylation in general is one of the central processes, by which environmental signals are transmitted or in other words by which switches on multiple proteins are moved into place to trigger a certain reaction to the environment. Kinases and phosphatases are thereby of crucial importance for signalling also in fungi (Dickman & Yarden, 1999; Kosti et al., 2010) and have even been termed the currency of signalling. In *T. reesei* DNA binding of ACE2 was suggested to require phosphorylation (Stricker et al., 2008b). However, neither the responsible kinase nor the signalling event causing its activation, are known. Another possible mechanism which predominantly acts via regulation of protein stability would be ubiquitinylation (Hochstrasser, 2009). Interestingly, first indications for an involvement of ubiquitinylation in regulation of cellulase gene expression have been found (Gremel et al., 2008). For both mechanisms, support comes from analysis of the amino acid sequences of the transcription factors known to be involved in regulation of cellulase gene expression: all of them comprise phosphorylation sites for cAMP dependent protein kinase, casein kinase II and protein kinase C (Table 1). XYR1 and HAP3 additionally contain probable PEST sequences (Table 1) that have been shown to act as ubiquitinylation and degradation signals (Rechsteiner & Rogers, 1996).

	cAMP and cGMP dependent phosphorylation sites	casein kinase II phosphorylation sites	protein kinase C phorphorylation sites	PEST sequence
XYR1	6	12	14	1
ACE1	1	7	2	none
ACE2	2	17	18	none
HAP2	2	2	7	none
HAP3	1	5	2	2
HAP5	1	2	1	none
CRE1	1	7	8	none

Table 1. Phosphorylation sites and PEST sequences of *T. reesei* transcription factors involved in regulation of cellulase gene expression. Phosphorylation sites were analyzed using GeneRunner v3.0 (Hastings). PEST-sequences were identified using the online tool epestfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind).

A further important regulatory process acting on the level of the promotor is chromatin remodelling. Also this mechanism was found to be involved in regulation of cellulase gene expression in *T. reesei* (Zeilinger et al., 2003).

In summary, there are numerous hints indicating direct action of signalling pathways on the transcription factors regulating cellulases, but it remains to be determined how they precisely work together to cause signal specific adjustment of cellulase gene expression.

4. Evaluating the influence of signal transduction pathways on cellulase gene expression

Despite thorough investigation of cellulase induction, transcription, secretion, enzyme function and elucidation of important transcript factors, the role of classical signal transduction pathways in these processes only received little attention for quite some time. Although *T. reesei* protein kinase C (PKC) was among the first PKCs to be characterized in detail in filamentous fungi (Lendenfeld & Kubicek, 1998; Morawetz et al., 1996), this topic did not become a research focus in this fungus and information on characteristics and regulatory targets of protein kinases and phosphatases of *T. reesei* is still scarce. Nevertheless, the availability of the sequenced genome (Martinez et al., 2008) enabled an initial evaluation of the signalling inventory of this fungus (Schmoll, 2008), which for example revealed interesting differences to other ascomycetes in the number of available two component histidine kinases and casein kinases.
Additionally, early studies also revealed hints as to an involvement of Ca^{2+}-calmodulin signalling in regulation of plant cell wall degradation (Mach et al., 1998). Although in this study only transcription of a xylanase has been studied, coregulation of cellulases and xylanases under many conditions (Stricker et al., 2008a) suggests that also cellulases might be a target of Ca^{2+}-calmodulin signalling.
Detailed investigations aimed at an evaluation of the role of signal transduction pathways in cellulase regulation started less than 10 years ago. Basis for the first study were two *T. reesei* strains derived from QM6a. QM9414 represents an early improved cellulase producer strain, which produces higher amounts of cellulases than the wild-type, while QM9978 – resulting from the same mutation cycle - does not. Since the defect of QM9978 was neither found to be a mutation in the protein coding region of the major cellulase genes nor in their promotors (Torigoi et al., 1996; Zeilinger et al., 2000) the assumption was that a crucial signal transduction cascade must be perturbed in this strain. Consequently, a cDNA subtraction method (RaSH, rapid subtraction hybridization) was applied to compare transcription of QM9414 and QM9978 under conditions inducing cellulase expression (Schmoll et al., 2004). Although this study did not reveal the actual defect of QM9978, intriguing new insights into the signals influencing cellulase gene expression were gained, which are still subject to investigation.

4.1 Light positively influences cellulase transcription

The first and most surprising finding of the transcriptional comparison of QM9414 and QM9978 was the differential regulation of a putative light regulatory gene, encoding the orthologue of the *Neurospora crassa* photoreceptor VIVID (Schmoll et al., 2004). Detailed investigation of this gene, named *env1* (encoding ENVOY for "messenger") indeed confirmed a function in regulation of cellulase gene expression and moreover, also light itself was found to influence transcription of cellulase genes (Schmoll et al., 2005). However, this is not the only function of ENVOY. A follow up study showed that light regulated genes found in *T. reesei* can be ENVOY-mediated or independent and the effect can be both positive and negative. Intriguingly, also a function of ENVOY in darkness was detected (Schuster et al., 2007). From these findings, the question arises, which role light might play in the life of *T. reesei* and especially in cellulase expression.

In terms of signal transduction, this finding indicates that despite decades of laboratory cultivation and propagation, *T. reesei* still has not lost its evolutionary heritage. Although completely detached from its natural habitat, adjustment of organismal functions to light and darkness is still operational in *T. reesei* (Schmoll et al., 2010). The presence of this reaction to light in this industrial workhorse also suggests that a circadian rhythm is at work, which however remains to be proven. If so, the daily cycles of expression of certain genes, meant to anticipate dusk and dawn, would be an important feature to be considered and potentially exploited for strain improvement.

In general, light is used predominantly for two purposes. It serves as a source of energy by photosynthetic processes in plants and algae or as a source of information. Fungi are not able to exert photosynthesis and therefore they only use the information connected to the light signal. The presence of light indicates growth on an exposed surface in contrast to growing inside a rotting tree – one of the natural substrates of *T. reesei*. The fungus has to adapt to the different conditions on the surface, like altered humidity, temperature and different nutritional conditions. Furthermore, the chance to encounter a potential mating partner is higher outside a substrate and the entrainment of components of the circadian rhythm, which allows for adaptation to day and night is also induced by light (Brunner & Kaldi, 2008).

Fungi can sense different wavelengths of visible light – many of them developed perception machineries for blue, green and red light and therefore photoreceptor proteins are required. These are proteins that generate a signal in response to light and transfer this to appropriate targets to react to the changed light conditions (Herrera-Estrella & Horwitz, 2007). A photoreceptor consists of an apoprotein and a cofactor (chromophore). Dependent on the kind of chromophore, the photoreceptors have different absorption maxima for particular wavelength ranges (Herrera-Estrella & Horwitz, 2007). The importance of light for fungi becomes manifest in the widespread reaction of fungi to illumination. Thereby fungi not only react to the harmful effects of light, especially UV-light, by taking protective measures, they even adjust their metabolism – including virtually all metabolic pathways - in the presence of light (Tisch & Schmoll, 2010).

The immediate response to light at the molecular level involves early light responsive genes (ELRGs), transcription of which peaks around 45 minutes after illumination and late light response genes (LLRGs), which are transiently upregulated mainly around 90 minutes after the light pulse (Chen et al., 2009). Thereby, the early light response rather involves protective measures and thereafter also metabolic pathways seem to become adjusted to light conditions. In the light of these data, the fact that also cellulases are influenced by light perfectly fits and investigation of more factors of the light signalling machinery proved to be highly interesting.

In *N. crassa* three blue light photoreceptors were identified: WC-1 (white collar 1) and WC-2 (white collar-2), which are GATA family zinc finger transcription factors (Linden & Macino, 1997; Linden et al., 1997) and VIVID (Heintzen et al., 2001; Schwerdtfeger & Linden, 2003), the orthologue of *T. reesei* ENVOY. Upon blue light exposure, WC-1 and WC-2 form the white collar complex (WCC) and bind to light response elements in the promoter regions of target genes (He & Liu, 2005; Smith et al., 2010; Talora et al., 1999). VIVID interacts with the WCC and modulates its transcriptional activity (Chen et al., 2010a; Hunt et al., 2010).

The genome of *T. reesei* also comprises homologues of these two blue light receptors (encoded by *blr1* and *blr2*) (Castellanos et al., 2010; Schmoll, 2008). BLR1 possesses a PAS/LOV domain like ENVOY and WC-1 for blue light sensing and protein-protein interaction. Both BLR

proteins are necessary for full induction of *env1* transcription in light (Castellanos et al., 2010). The effect of BLR1 and BLR2 on transcription of *env1* strongly suggests also a function in cellulase gene regulation for these photoreceptor genes. Indeed, BLR1 and BLR2 positively impact the transcription of the major cellobiohydrolase of *T. reesei*, *cbh1* not only in light, but also in darkness. Interestingly, this effect on *cbh1* does not seem to be accomplished via regulation of *env1* (Castellanos et al., 2010). Homologues of the white collar proteins were identified in *Aspergillus*, *Fusarium* and other *Trichoderma* spp. (Casas-Flores et al., 2004; Castellanos et al., 2010; Purschwitz et al., 2008; Ruiz-Roldan et al., 2008; Schmoll, 2008). In *Trichoderma atroviride* the homologues of the WC proteins, the BLR proteins (blue light receptor 1 and 2), were shown to be necessary for blue light induced conidiation (Casas-Flores et al., 2004) and for carbon source dependent conidiation (Casas-Flores et al., 2006; Friedl et al., 2008a), which confirms a connection between light response and nutrient assimilation in fungi. Interestingly, also the photoreceptors of *T. atroviride* are involved in cellulase gene expression: Deletion of BLR-1 or BLR-2 causes strongly enhanced cellulase activities secreted into the culture medium (M. Friedl and M. Schmoll, unpublished).

4.2 How does *Trichoderma reesei* react to light?

The unexpected importance of light in metabolic processes of *T. reesei* necessitated a more detailed investigation of this effect in order to evaluate the possible relevance of this environmental cue for further studies (Schmoll et al., 2010). Analysis of light response of *T. reesei* upon growth on glycerol already revealed an adjustment of processes involved not only in protection from the harmful effects of light, but also of signalling pathways and metabolic processes (Schuster et al., 2007) In this study the central position of the light regulatory protein ENVOY became obvious.

New results under conditions more relevant for cellulase production, i. e. from cultivations on cellulose in light and darkness by microarray analysis further support significant adjustments of *T. reesei* to light (Tisch et al., 2011b, ms submitted). This genome wide analysis revealed 2.7 % of the genes to be differentially regulated in light and darkness in the *T. reesei* wild-type strain. Among the genes upregulated in light, a significant enrichment in the functions of DNA photolyase activity, carbohydrate metabolic activity (including cellulase activity and cellulose binding), regulation of oxidoreductase activity and sulphur metabolism was detected. Additionally, 6 genes involved in sexual development and 16 glycoside hydrolase genes are upregulated in light. Interestingly, glycoside hydrolase genes have also been detected among the genes downregulated in light and consequently, light cannot be considered a strictly positive or negative factor for expression of enzymes, but rather an environmental signal which triggers production of an altered enzyme mixture.

One of the most obvious effects of light on many fungi is the induction or enhancement of sporulation, or more generally speaking: Light is a crucial signal for the decision to undergo sexual development or reproduce asexually (Bayram et al., 2008; Blumenstein et al., 2005; Mooney & Yager, 1990; Seidl et al., 2009). Nevertheless, the genes regulated by light in *T. reesei* and other organisms (Chen et al., 2010a; Smith et al., 2010), which also involve adjustments of metabolic pathways, clearly indicate that sporulation is only one of many outputs of the signalling cascade initiating the adaptation to light.

4.3 Light-dependent modulation of cellulase gene expression is a conserved phenomenon in fungi

As described above, the light regulatory protein ENVOY is a homolog of VIVID, a photoreceptor that is well characterized in *N. crassa,* the model organism for studies on

photobiology and the circadian clock (Davis & Perkins, 2002). One of the crucial functions of VIVID is to act as a universal brake and repress both early and late light responses after prolonged illumination (Chen et al., 2009). Despite the similarity of ENVOY and VIVID, they are not fully functionally homologous, because *env1* could not complement a mutation in *vvd*. Additionally, the phenotypes of non functional mutants in these genes are strikingly different: While deletion of *env1* in *T. reesei* causes strongly decreased growth and obvious problems with adaptation to light, *N. crassa* strains lacking function *vvd* do not show such a growth defect in light, but enhanced carotenoid production (Schmoll et al., 2005; Shrode et al., 2001).
Nevertheless, since also *N. crassa* possesses an efficient machinery for degradation of cellulose (Eberhart et al., 1964; Eberhart et al., 1977), which was recently studied in detail (Tian et al., 2009), a comparison of the influence of light and the machinery transmitting the light signal on cellulase gene expression proved topical.
For *N. crassa*, as a model organism, there are a lot of genetic tools available, including a sequenced genome (Galagan et al., 2003) and an almost complete library of gene knock out strains (Colot et al., 2006). So far *N. crassa* was predominantly used for investigations of photobiology and the circadian clock (Chen et al., 2010b). Therefore, the combination of *N. crassa* as a model for light response and *T. reesei* as the model fungus for studies on plant cell wall degradation is an ideal fit for investigation of light modulated cellulase gene expression. Genome wide analysis of the effect of WC-1, WC-2 and VVD on cellulase gene expression indeed revealed that the light response machinery of *N. crassa* influences cellulase gene expression in the same way as in *T. reesei*. Surprisingly, while also in *N. crassa* WC-1, WC-2 and VVD exert a positive effect on transcription of the major cellulase gene, the cellulase mixture secreted into the culture medium by mutants in the respective genes was even more efficient than that in the wild-type (Schmoll et al., 2011, ms submitted). A similar effect was recently also observed in *T. reesei* (Gyalai-Korpos et al., 2010). In both cases, the light response machinery was suggested to accomplish this enhancement of cellulase activity despite decreased transcription of *cbh1* (or its orthologue) by alteration of the composition of the secreted enzyme mixture for higher efficiency dependent on light.
Consequently, the phenomenon of light modulated cellulase gene expression is likely to be conserved in fungi. However, the different phenotypes of mutants in *T. reesei env1* and *N. crassa vvd*, as well as in the photoreceptor genes (Castellanos et al., 2010) indicate that despite similar outcome, the signalling cascade achieving this aim is not identical in these two fungi.
Obviously, the environmental signal "light" starts a signal cascade which crosstalks with other factors like nutrition conditions leading to elevated levels of cellulase transcripts. Up to now, three of the crucial components of the light signalling pathway could be shown to be involved in transduction of the positive light signal to the cellulase promoter. The question arises, how the light signals are transferred to the cellulase promoters.

4.4 The role of heterotrimeric G protein signalling in the light transferring pathway

For a signal to take effect, it first has to be perceived and thereafter its transmission is necessary to reach its target. Considering a complex environment like a tropical rain forest clearly countless signals, like light intensity, humidity, temperature, carbon source, nitrogen source, pH or the potential presence of a mating partner are to be noticed by a filamentous fungus growing in this habitat. However, not every signal has the same significance under every condition. The complex network of signal transduction pathways in fungi not only

has to recognize these various signals. Also a rating and appropriate distribution of these signals is necessary in order to affect output pathways such as growth, development and enzyme production for optimal adaptation.
Results showing that nutritional and other environmental signals interfere with light signalling (Friedl et al., 2008a; Friedl et al., 2008b; Tisch & Schmoll, 2010), suggest a role for heterotrimeric G proteins in transduction of the light signal.
The G protein (guanine nucleotide binding protein) pathway is well studied and known as a connection pathway from the cell surface to intracellular targets (Figure 2) (Hamm, 1998; Li et al., 2007).

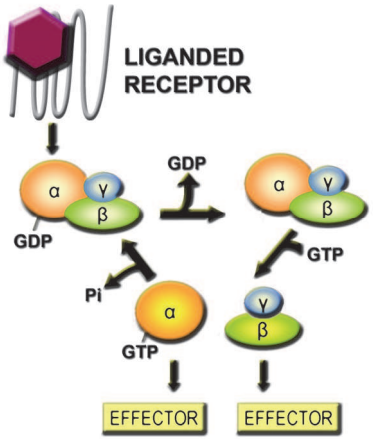

Fig. 2. Binding of a ligand to a G protein coupled receptor (GPCR) leads to a conformational change of the G protein alpha subunit and thereby exchanging GDP (guanosine diphosphate) to GTP (guanosine triphosphate). The GTP bound G alpha subunit is activated and releases the G protein beta and gamma subunits. G beta and gamma work as a dimer to affect further targets. The intrinsic GTPase activity of G alpha hydrolyzes GTP to GDP to terminate the signalling cycle and enables the G beta gamma dimer to reassociate with the G alpha subunit. As a heterotrimeric G protein it binds to the GPCR the circle of G protein signalling is closed (Hamm, 1998; Sprang, 1997).

Fungi have a versatile array of G protein coupled receptors for perception of environmental signals. They belong to six classes: Ste2-like pheromone receptors, Ste3-like pheromone receptors, carbon/amino acid receptors, putative nutrient receptors, cAMP receptor-like and microbial opsins (Xue et al., 2008), not including the PTH11-like receptors first identified in *Magnaporthe grisea* and involved in pathogenicity (Kulkarni et al., 2005). So far, only nutrients, pheromones and light could be confirmed as activating ligands of GPCRs (Li et al., 2007; Xue et al., 2008). The transmitting factors, the G protein alpha subunits, are classified in three groups (I, II, III) with group I and III being similar to mammalian G alpha-i and G alpha-s subunits. In contrast to the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans*, the genome of which comprises three G alpha subunits each, *S. cerevisiae* only contains two different G alpha subunits (Bölker, 1998; Li et al., 2007).
The genome of *T. reesei* comprises three G alpha subunits (GNA1, GNA2 and GNA3), one G beta subunit (GNB1) and one G gamma subunit (GNG1) (Schmoll, 2008). In 1995 it was

already shown that a G alpha subunit CPG-1 is required for cellulase mediated induction of *cbh1* transcription in *Cryphonectria parasitica* (Wang & Nuss, 1995). Additionally, an effect of cAMP on cellulase gene expression had been discovered (Sestak & Farkas, 1993). Since intracellular cAMP levels are modulated by class III G alpha subunits, also a participation of this subgroup in regulation of cellulase gene expression was likely.

Consequently, an involvement of the heterotrimeric G proteins in regulation of cellulase transcription was investigated in *T. reesei*. Indeed, GNA1 was found to exert an influence on cellulase gene expression (Seibel et al., 2009). However, the most interesting outcome of this study was that this influence is different in light and darkness. Deletion of *gna1* caused strongly enhanced *cbh1* transcript levels in darkness, but abolished *cbh1* transcription in light. Interestingly, also carbon source dependent feedback regulation of *gna1* upon activation was observed. These results reflect the need for integration of different environmental signals to adjust the output – for example cellulase gene expression. Obviously, the light signal is important for cellulase gene expression and for the signal transmitted by GNA1. Since despite constitutive activation of GNA1 (i. e. constant transmission of the signal), still an inducer is needed, GNA1 cannot be the transmitter of the cellulose signal and the actual nature of the signal transmitted by GNA1 remains to be determined (Seibel et al., 2009). Also, whatever this signal is, the carbon source on which the fungus grows is important for its relevance: Feedback regulation of the transcript of *gna1* is one step in setting the sensitivity of the GNA1-related signal(s) by increasing transcription upon activation and hence leads to stronger amplification of the signal.

The second G protein alpha subunit (belonging to subgroup III), GNA3, was also found to be involved in regulation of cellulase gene expression (Schmoll et al., 2009): Constitutive activation of GNA3 revealed a strong positive effect of this G protein and hence its cognate signal on *cbh1* transcript levels. Intriguingly, this positive effect was only observed in light. In darkness, neither constitutive activation nor knock-down nor overexpression had any effect.

This light-dependent effect of both GNA1 and GNA3 indicated that a light regulatory factor must be involved in their regulatory output – most likely acting as a checkpoint for signal transmission. First indications as to such a function came from the study on GNA3, which showed an influence of ENVOY (Schmoll et al., 2009). In the following, ENVOY indeed turned out to interfere with transcriptional regulation of both *gna1* and *gna3*, albeit in a different way. It was required for efficient feedback regulation of *gna1*, but not *gna3*, which nevertheless showed enhanced light response in the absence of *env1* (Tisch et al., 2011a). This central position of ENVOY in this regulatory mechanism is reflected by the phenotype of double mutant strains expressing constitutively active G alpha subunits but lacking ENVOY: In darkness, they exhibit the phenotype of constitutive activation of GNA1 or GNA3. In light ENVOY clearly is of major importance, since these strains show the typical poor growth and sporulation as strains lacking *env1*. Consequently, ENVOY represents a major checkpoint of signal transduction via the heterotrimeric G protein pathway in *T. reesei*. The mechanism by which this crucial impact is likely to be mediated involves the regulation of cAMP levels that are adjusted also by ENVOY which seems to contribute to this mechanism by exerting a negative effect on the function of phosphodiesterases.

Knowing that nutrient signalling and light response are connected, a further factor, known to be involved in both, transmission of light signals and the heterotrimeric G protein pathway, came into focus: phosducin like proteins. Phosducins were initially isolated from photoreceptor cells from the retina of mammals (Lee et al., 1984; Lee et al., 1987) and assume the function of a co-chaperone to regulate the correct folding of G beta and gamma (Lukov

et al., 2006; Lukov et al., 2005), which makes them the ideal candidate for transduction of the light signal to the G proteins. Interestingly, these proteins had previously not been investigated for a function in light response in fungi.
In *T. reesei*, the class I phosducin like protein PhLP1 and the G protein beta and gamma subunits GNB1 and GNG1 act in the same pathway and additionally perform their function in a light dependent way (Tisch et al., 2011b, ms submitted). Indeed, cellulase gene transcription was found to be positively influenced by PhLP1 and hence G beta and gamma folding. Interestingly, this analysis showed that regulation of glycoside hydrolase transcript levels is among the major targets of PhLP1-GNB1-GNG1. Consequently, not only the major cellulases *cbh1* and *cbh2* (Schmoll et al., 2009; Seibel et al., 2009) are subject to regulation in response to nutrient signals transmitted by heterotrimeric G protein signalling, this important pathway targets carbon source utilization at a broad scale.
Additionally, the operation and output of this pathway is light dependent with the major function in light, but also some impact in darkness. Hence, these results emphasize that for research on carbon source degradation aiming at an improvement of cellulase production, consideration of light dependent processes is of crucial importance. Otherwise, beneficial mechanisms might remain undiscovered or misinterpreted due to an interference of random light pulses with transcript profiles.

4.5 The light signalling pathway and the cAMP pathway are interconnected

While initial results clearly confirmed the early steps of signal transmission (the heterotrimeric G protein pathway and the photoreceptors) to be important for adjustment of cellulase levels to be produced, the question remained how the respective signals might reach their target. Hence the gap between the signal being relayed in the upper part of the cascade and regulation of transcription of cellulase genes by transcription factors deserves closer attention.
One of the downstream targets of G protein signalling is the 3'-5'-cyclic adenosine monophosphate (cAMP) pathway (Bölker, 1998; Li et al., 2007). cAMP represents an important secondary messenger used for signalling processes from bacteria to man. In fungi, cAMP plays diverse roles in the cell – it is involved in development, virulence, pathogenicity, sporulation, and light responses (D'Souza & Heitman, 2001). With respect to plant cell wall degradation it is interesting that it was shown already decades ago, that cAMP affects xylanase production in *Cryptococcus albidus* - addition of exogenous cAMP results in a 1.5 to 2 fold increase (Morosoli et al., 1989).
Also for *T. reesei* cAMP is crucial in regulation of plant cell wall degrading enzymes: an early report showed an involvement of cAMP in regulation of cellulase gene expression (Sestak & Farkas, 1993) and is in accordance with the function of GNA3 in regulating cAMP levels and its function in this process (Schmoll et al., 2009). For *T. atroviride* it was shown that the light response pathway crosstalks with the cAMP pathway (Friedl et al., 2008a; Casas-Flores et al., 2006) and in *T. reesei*, GNA3 as well as ENVOY have an effect on cAMP levels (Schmoll et al., 2009; Tisch et al., 2011a). Therefore the cAMP pathway represents an important downstream regulatory pathway to be investigated for improvement of cellulase production.
The most important task of cAMP is to activate cAMP dependent protein kinase A (PKA), which in turn initiates a phosphorylation cascade and activates/inactivates further target genes. cAMP is produced by adenylate cyclase from ATP and degraded by phosphodiesterase (Houslay & Adams, 2003; Pall, 1981). Intriguingly, the steady state levels of intracellular cAMP are subject to a sophisticated fine tuning mechanism: PDE and PKA establish a negative feedback loop (Hicks et al., 2005; Wang et al., 2005), which seems to be targeted by ENV1 via

negative impact on PDE in light (Tisch et al., 2011a). Adenylate cyclase is either stimulated by G alpha, beta and gamma subunits or inhibited by G alpha subunits (Ivey et al., 1999; Neves et al., 2002; Schmoll et al., 2009).

In *T. reesei*, the function of the two major components of the cAMP pathway has recently been studied with an emphasis on the involvement in regulation of cellulase gene expression (Schuster et al., 2011, ms submitted). Both adenylate cyclase (ACY1) and protein kinase A (catalytic subunit, PKAC1) were found to be important factors in the cellulase regulon. Intriguingly, both factors enhance the light responsiveness of cellulase gene transcription, i. e. the difference between cellulase transcription in light and darkness. Moreover, while ACY1 exerts a consistently positive effect, PKAC1 acts positively in light and negatively in darkness. Their effect on cellulase transcription is likely mediated by regulation of the transcription factor XYR1 via phosphorylation an upstream factor modulating the abundance of the *xyr1* transcript. Consequently, PKAC1 is a crucial light dependent checkpoint downstream of the heterotrimeric G protein pathway.

This differential effect of components of the cAMP pathway in dependence of light highlights its importance and a connection of this pathway to light dependent processes has been shown previously: Interestingly, cAMP levels and regulatory targets of cAMP react to different light conditions. The operation of adenylate cyclase of *T. reesei*, which is a membrane bound enzyme, is stimulated by light (Kolarova et al., 1992). PKA activity of *T. atroviride* is increased in light and overexpression of the regulatory subunit of PKA results in a blocking of light response genes (Casas-Flores et al., 2006). The protein kinase A of *N. crassa* is a part of the circadian clock by stabilizing the white collar complex (WCC) and the negative regulating element FREQUENCY (Huang et al., 2007; Mehra et al., 2009).

Due to the fact that PKA is involved in the circadian clock in *N. crassa* (Huang et al., 2007) and also for *Trichoderma* a connection between PKA activity and light response was shown (Casas-Flores et al., 2006), a feedback loop is likely (Figure 3). First, light is sensed and transduced by the BLR proteins and ENVOY. In parallel, the G proteins, which regulate

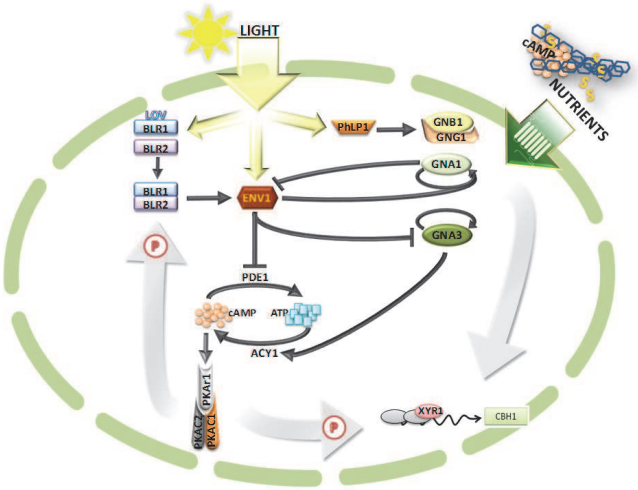

Fig. 3. Model for integration of nutrient signals and light signals regulating cellulase gene expression.

5. Nutrient availability and the output of signalling cascades

Early studies on *T. reesei*, which are the basis for the high levels of cellulase production possible today, provide detailed investigations on the nutrients and trace elements beneficial for cellulase induction and secretion. However, the molecular basis of the need and function of these nutrients is largely unknown, as is the interdependence with different environmental conditions.

With respect to the carbon source, it becomes obvious now, that the effect of one environmental cue cannot be considered isolated from other important conditions: Regulation of cellulase gene transcription is stimulated by light on cellulose (Schmoll et al., 2005), but decreased in light on lactose (Schuster et al., 2011, ms submitted).

The cost efficiency for cellulase production prompted investigations about cellulase production on different carbon sources. A very cheap renewable carbon source for cellulase fermentations is lactose, because it is a by-product from cheese production (Kubicek et al., 2009; Seiboth et al., 2007). Most interestingly, although the products of cellulose and lactose conversion are the same (glucose), the influence of light on cellulase transcription is in *T. reesei* is not (Schmoll et al., 2005; Schuster et al., 2011, ms submitted). On cellulose, which resembles the natural substrate, light positively influences cellulase transcription, whereas on lactose, which cannot be considered a natural carbon source, the cellulase transcription is enhanced in darkness.

Although this phenomenon is in agreement with studies already reported decades ago (Carlile, 1965), showing that the influence of light on growth is dependent on the carbon source, the mechanisms triggering this differential response will be intriguing targets of further research.

Only recently a few further studies provide a first glimpse on the relevance of chemicals other than the major carbon source for cellulase gene expression at the molecular level.

The screening of new regulators of cellulase gene transcription identified a protein involved in regulation of sulphur metabolism as a potential signalling factor (Gremel et al., 2008). The respective protein is called LIMPET (encoded by *lim1*) and encodes a WD-repeat/F-box protein, a putative E3 ubiquitin ligase. LIMPET shares similarity with *N. crassa* SCON-2, *A. nidulans* SconB and *S. cerevisiae* Met30p, which are members of a sequential control mechanism for regulation of catabolite repression of sulphur metabolism (Marzluf, 2001). Consequently, analysis of the role of the sulphur source for cellulase gene expression proved reasonable.

Interestingly, the uptake of sulphate was found to be dependent on the light status and even essential for growth of *T. reesei* in light. Moreover, the concentration of methionine, which can be used as alternative sulphur source, is important for regulation of cellulase gene transcription. But most strikingly, addition of 5 mM methionine to the medium abolished transcription of *cbh1* in light and even has a slightly positive effect on *cbh1* in darkness. Consequently, methionine or possibly the sulphur source in general is not only perceived as a nutritional signal. The presence of methionine is of different relevance for cellulase gene expression in light and darkness (Gremel et al., 2008). This surprising result further indicates that similar differences between the effect of a nutrient or even trace element under illumination and in constant darkness may occur. Consequently, routine evaluation of a given medium in open shakers can show effects, which are deleterious for the yield or maybe worse in a dark large scale fermentor or even remain undetected despite highly beneficial.

This study also provides further hints as to the regulatory mechanism of cellulase gene expression. LIMPET represents a component of the ubiquitinylation pathway, which can

either activate a protein or destine it for degradation. The reaction of the *lim1* transcript to both cellulase inducing conditions and sulphur limitation indicates an interconnection between the respective regulatory pathways via a mechanism that acts on the cellulase transcription factors in response to changing availability of sulphur sources in the environment. However, the physiological significance of the differential and strong response of cellulase transcription to high levels of methionine in light and darkness remain to be determined.

6. Nutrition, reproduction and the rotation of earth – the footprints of evolution in *T. reesei*

It would be reasonable to assume that only nutritional signals would be relevant for the levels of plant cell wall degrading enzymes, needed to sustain growth and development of a fungus. However, an early screening for signal transduction genes involved in regulation of cellulase gene expression, identified a pheromone precursor gene in *T. reesei* (Schmoll et al., 2004). This signal – indicating the presence of a potential mating partner - is transmitted by the heterotrimeric G protein pathway (Bölker & Kahmann, 1993; Li et al., 2007; Xue et al., 2008), which was already shown to be important for cellulase regulation (Schmoll et al., 2009; Seibel et al., 2009; Tisch et al., 2011a).

This is in agreement with the assumption that a shared signalling pathway may also lead to shared output effects. In the following, this pheromone precursor gene (*hpp1*) turned out to represent a so far undescribed class of pheromones (h-type pheromones, in addition to a-type and alpha-type), which – at least in *T. reesei* - assume a-type function (Schmoll et al., 2010). With respect to relevance for cellulase gene expression, analyses are still in progress, and first results indicate that indeed, the availability of the pheromone precursor genes has an influence on regulation of cellulase gene expression (A. Schuster and M. Schmoll, unpublished results). Although the reaction to an extracellular substrate and the detection of a mating partner may seem to be unrelated events, one has to keep in mind that in many cases, deterioration of environmental conditions such as lack of nutrients initiates sexual development in an attempt to improve the genetic equipment for coping with the harsh conditions (Aanen & Hoekstra, 2007). Additionally, the encounter of a mating partner can lead to pheromone induced growth arrest (Skulachev, 2002), which cannot be considered isolated from metabolic regulation. In the special case of *T. reesei* the decision between asexual development (sporulation) or sexual development also has direct implications for cellulase gene expression, since the spores of this fungus become covered with cellulases (Kubicek et al., 1988; Messner & Kubicek, 1991). Therefore the sexual cycle and its relevance for production of plant cell wall degrading enzymes in *T. reesei* warrant further investigations.

The presence of a peptide pheromone precursor gene in *T. reesei*, indicated that sexual development is likely to be possible, although all previous attempts had failed and *T. reesei* had been considered asexual (Kuhls et al., 1996). Indeed, crossing of the original isolate from the Solomon Islands, QM6a, which is also the parental strain of all strains used in research and industry, with a wild-type isolate was achieved. Interestingly, light is required for mating of *T. reesei* (Seidl et al., 2009). Accordingly, also the transcription of the pheromone precursor gene *hpp1* is light responsive. Consequently, it is obvious that *T. reesei* - despite decades of application in research and industry and growth under most unnatural conditions – is still bound to its evolutionary obligations to adjust its life to the availability

of nutrient, the need for reproduction and the necessary resources to ensure its success as well as to the rotation of earth by responding to light and darkness.

7. Perspectives and challenges

The relevance of signal transduction pathways for regulation of cellulase gene expression as reviewed here only uncovered a small part of the complex network, which determines the adjustment of *T. reesei* to its environment – be it a tropical rainforest, a shake flask or a steel fermentor (Table 2). Nevertheless, these studies also provide guidelines and directions for exploiting the natural signal transduction pathways to cheat *T. reesei* by mimicking conditions requiring huge amounts of cellulases to be produced.

gene	function	delivered signal	light dependent	effect on cellulase gene transcription		publication(s)
				light	darkness	
gna3	G protein alpha subunit 3	nutrients	yes	positive	no effect	Schmoll et al., 2009
gna1	G protein alpha subunit 1	nutrients	yes	positive	positive	Seibel et al., 2009
env1	ENVOY, light regulatory protein	light	yes	positive	positive	Schmoll et al., 2005
blr1	Blue light receptor 1	light	yes	positive	positive	Castellanos et al., 2010
blr2	Blue light receptor 2	light	yes	positive	positive	Castellanos et al., 2010
phlp1	Phosducin like protein	nutrients	yes	positive	positive	Tisch et al., 2011b
gnb1	G protein beta subunit	nutrients / pheromones	yes	positive	positive	Tisch et al., 2011b
gng1	G protein gamma subunit	nutrients / pheromones	yes	positive	positive	Tisch et al., 2011b
acy1	adenylate cyclase 1	nutrients	yes	positive	positive	Schuster et al., 2011
pkac1	protein kinase A, catalytic subunit	nutrients	yes	positive	negative	Schuster et al., 2011
lim1	LIMPET, putative E3 ubiquitin ligase	sulphur	yes	unknown	unknown	Gremel et al., 2008

Table 2. Genes involved in cellulase signalling. Data obtained from indicated publications.

On the one hand there are still numerous signal transduction pathways (Bahn et al., 2007; Schmoll, 2008) and related regulatory mechanisms, which remain to be studied with respect to their impact on plant cell wall degradation. The reports summarized here can only be considered a beginning in unravelling the interdependence of signal transduction pathways, enzyme production and growth. Several more signalling pathways, such as MAP-kinase cascades, two component phosphorelays or calcium signalling still await evaluation with respect to regulation of plant cell wall degrading enzymes. Additionally, mechanisms of posttranslational modification of proteins involved in signal transduction and transcriptional regulation deserves increased attention. It would only be logical if different signal transduction pathways would influence each other by activation, deactivation or degradation of intermittent steps in their cascade.

Reports on the function of two component phosphorelay systems and MAPkinase pathways and their involvement with the circadian clock (Catlett et al., 2003; de Paula et al., 2008; Jones et al., 2007) indicate functions in regulation of metabolic pathways of these signalling cascades. Also recent findings on the functions of the COP9 signalosome in *A. nidulans*, which is a crucial regulator of ubiquitin ligases (Braus et al., 2010; Nahlik et al., 2010) open up excitingly new topics for investigation of cell signalling with the involvement of protein modification. Additionally, the recently described mechanisms of regulation of mRNA

stability by decapping (Morozov et al., 2010) may also play a role in posttranscriptional regulation of cellulase gene expression in *T. reesei* (Gyalai-Korpos et al., 2010).

On the other hand, studies on signal transduction pathways and especially light response in recent years, clearly showed that light is an important environmental cue, which cannot be neglected when analyzing *T. reesei*. Even though light response is not a primary field of research when intending to improve industrial production of cellulases, *T. reesei* still obeys to its evolutionary heritage and reacts to light with unexpectedly broad adjustments of metabolic processes, which also involve alterations in transcript levels of plant cell wall degrading enzymes. Consequently, when investigating regulatory processes in cellulase production, establishment of controlled light conditions is equally important as careful preparation of growth media with respect to precise amounts of carbon source, nitrogen source or trace elements. Otherwise, random light effects – comparable to significant impurities in nutrient sources - may cause unreliable interpretations or unpredictable and costly problems when upscaling laboratory cultivations (in transparent shake flasks or fermentors) to the production scale in large, constantly dark steel fermentors.

Despite the intriguing insights into signal transduction pathways of *T. reesei* and their influence on cellulase gene expression, the sometimes significantly increased *cbh1* transcript levels in several mutant strains do not result in equally enhanced efficiency in plant cell wall degradation. So far only few hints as to the reason of this phenomenon are available. Interestingly, several reports indicate that pretranslational regulation of cellulase gene expression may not be the general standard. Therefore, it will be of crucial importance to elucidate the molecular mechanism behind the discrepancy between cellulase regulation on the transcriptional level and efficiency of the secreted plant cell wall degrading enzymes. At the same time, knowledge on this mechanism will uncover important bottlenecks in cellulase production and provide the means to harvest the real potential of *T. reesei* and likely also of other industrially important fungi.

The application of light-dependent results in an industrial setting can be a problem. Large scale steel fermenters can hardly be equipped with sufficiently effective light sources to reproduce laboratory conditions. Therefore one future challenge will be to uncover the interconnections between light response and enzyme expression in more detail to allow for modification of the respective pathways in a way that a light-dependent increase in cellulase production can be realized also in a dark steel fermentor.

It will be interesting to see, whether genes known to impact cellulase gene expression by acting as signal transmitters can still enhance the already efficient industrial production strains derived from the wild-type QM6a. Considering the multiple mutation cycles used for strain improvement, it is very likely that also signalling pathways contributed to the high production of cellulase mixtures, which can be achieved nowadays. Beneficial mutations detected in the course of studies on signal transduction may already be present in producer strains. Nevertheless, more detailed understanding of the mechanism of the complex signalling network in *T. reesei*, can still be used to optimize pathways, which may be perturbed by mutation, identify bottlenecks, improve the use of alternative substrates by targeted adjustment of signalling, and achieve optimal use of resources for most cost efficient cellulase biosynthesis in *T. reesei*.

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This book aspires to be a comprehensive summary of current biofuels issues and thereby contribute to the understanding of this important topic. Readers will find themes including biofuels development efforts, their implications for the food industry, current and future biofuels crops, the successful Brazilian ethanol program, insights of the first, second, third and fourth biofuel generations, advanced biofuel production techniques, related waste treatment, emissions and environmental impacts, water consumption, produced allergens and toxins. Additionally, the biofuel policy discussion is expected to be continuing in the foreseeable future and the reading of the biofuels features dealt with in this book, are recommended for anyone interested in understanding this diverse and developing theme.

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