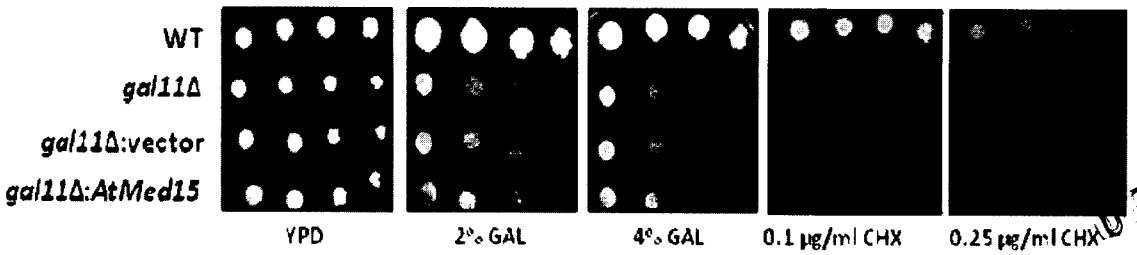
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ABSTRACT

"RECOMBINANT MICROORGANISMS AND USES THEREOF"

The present invention provides recombinant microorganism for bio-fuel production that express *Arabidopsis thaliana* MED15 protein. The recombinant microorganism also shows enhanced properties of flocculation, adhesiveness and growth. The present invention also provides a recombinant DNA molecule comprising the *AtMed15* gene operably linked to a promoter and a recombinant vector comprising the said recombinant DNA molecule. The present invention further provides a process for the production of biofuel from the said recombinant microorganisms.

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FIGURE 1

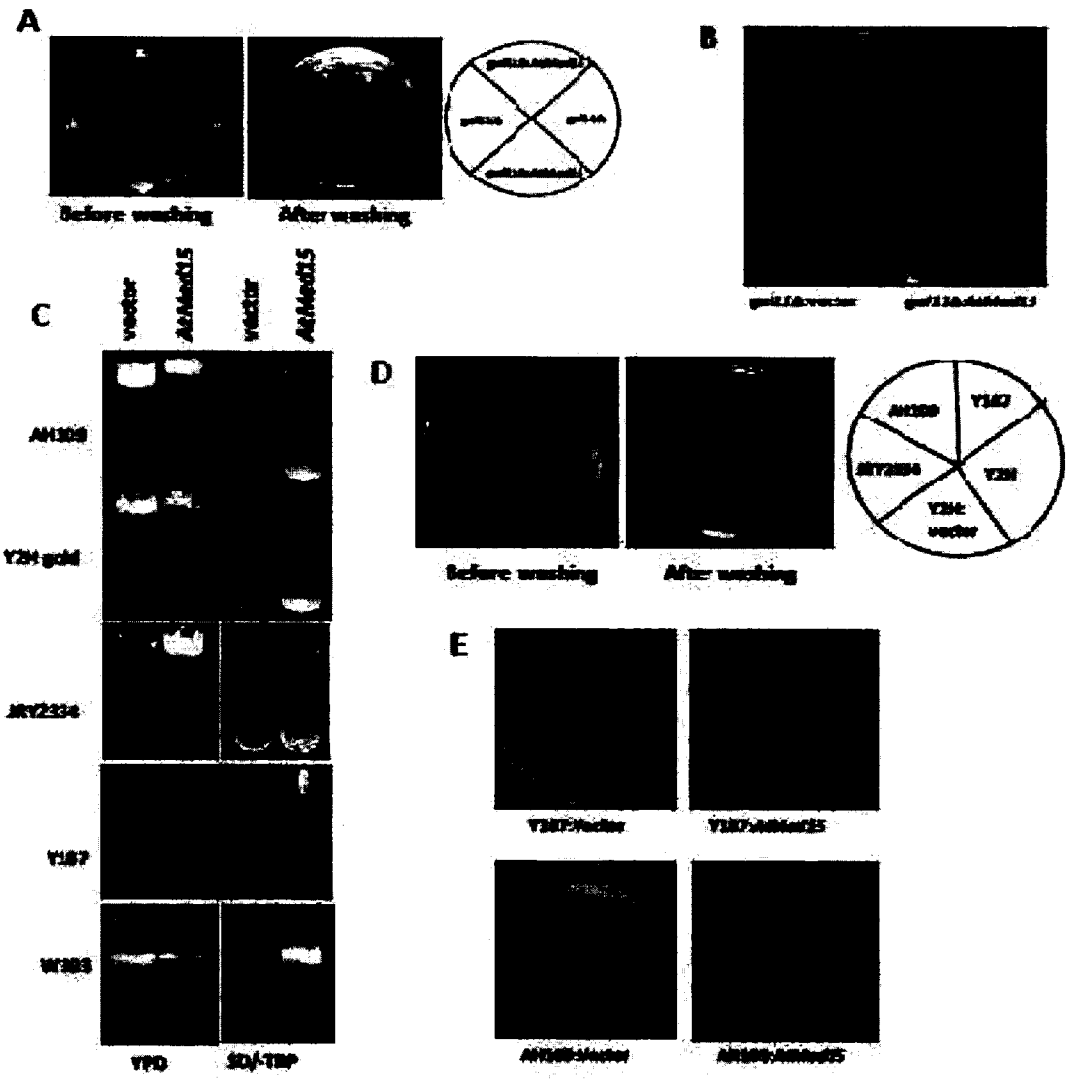
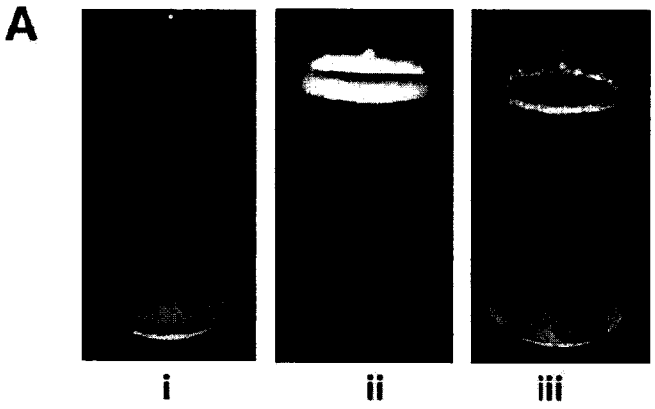
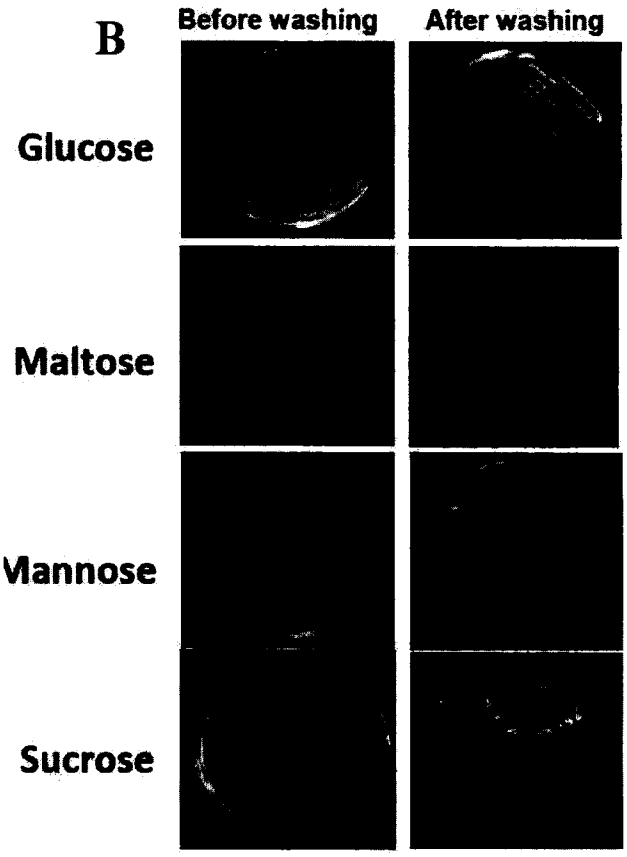


FIGURE 2

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I AH109:*AtMed15*
II *gal11Δ:AtMed15*
III Y187:*AtMed15*

FIGURE 3

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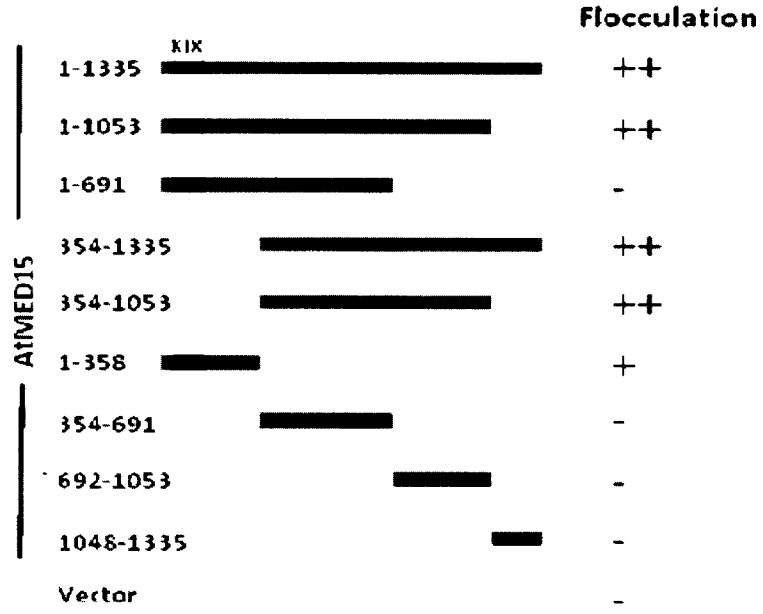


FIGURE 4

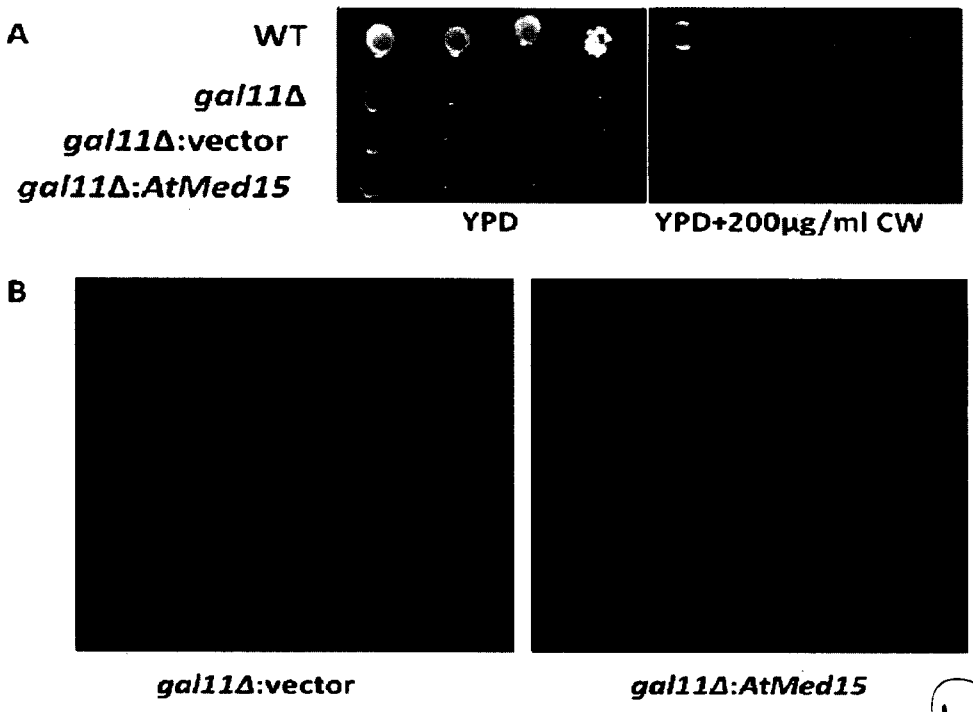


FIGURE 5

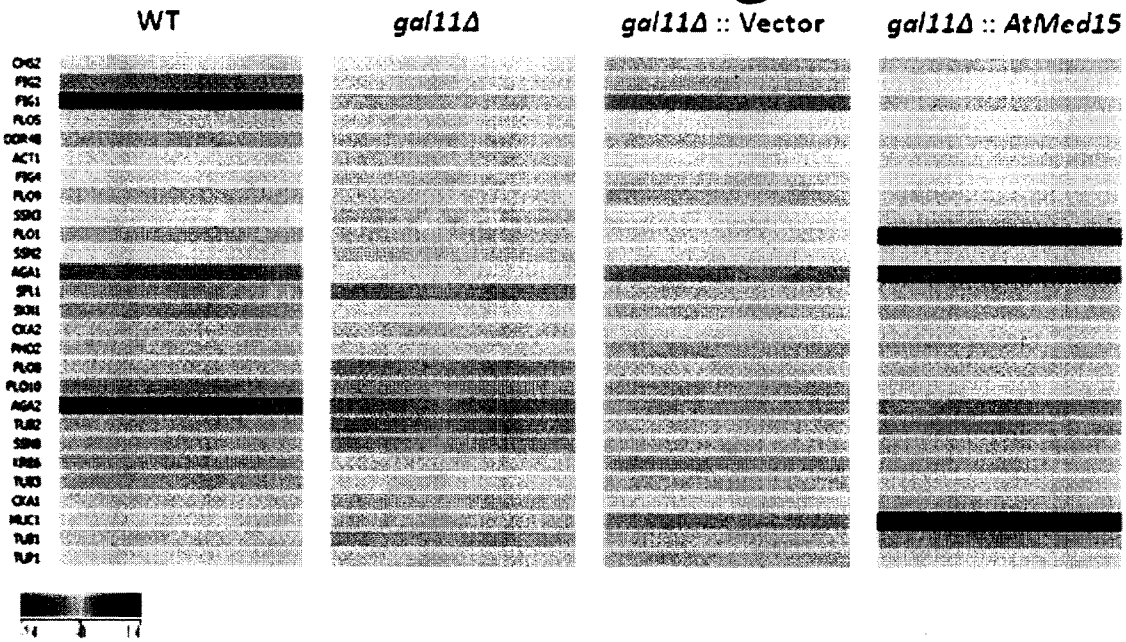
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A



B

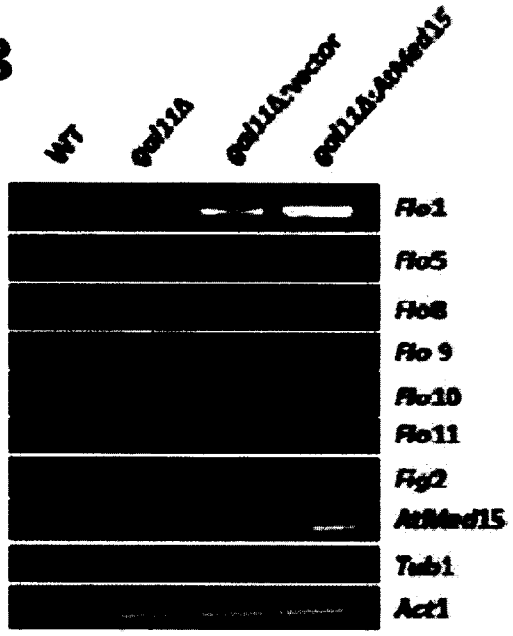


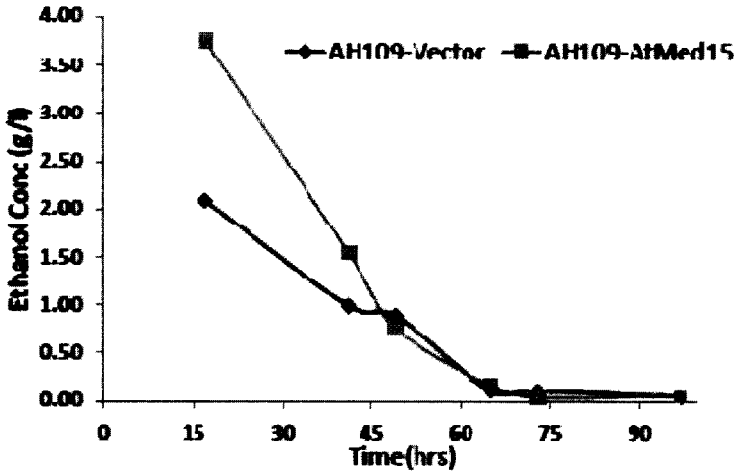
FIGURE 6

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A



B

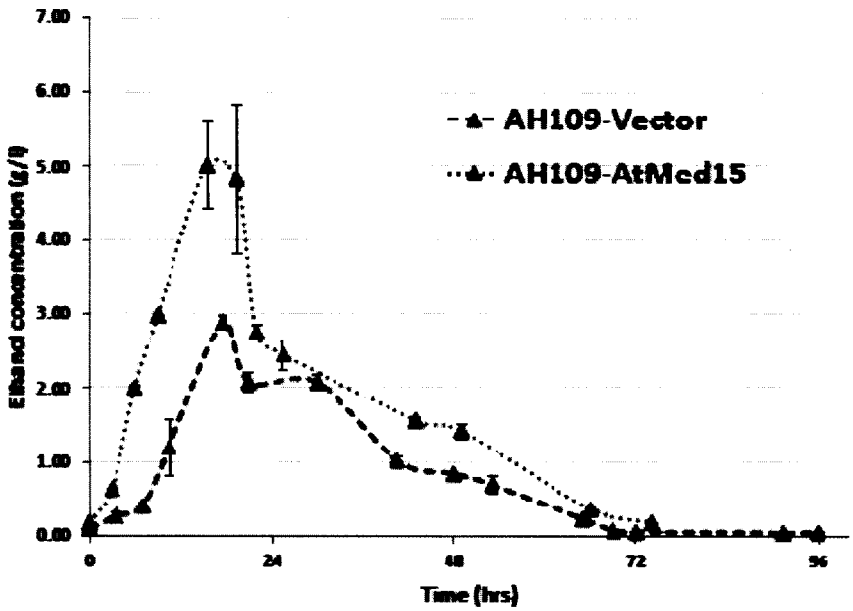


FIGURE 7

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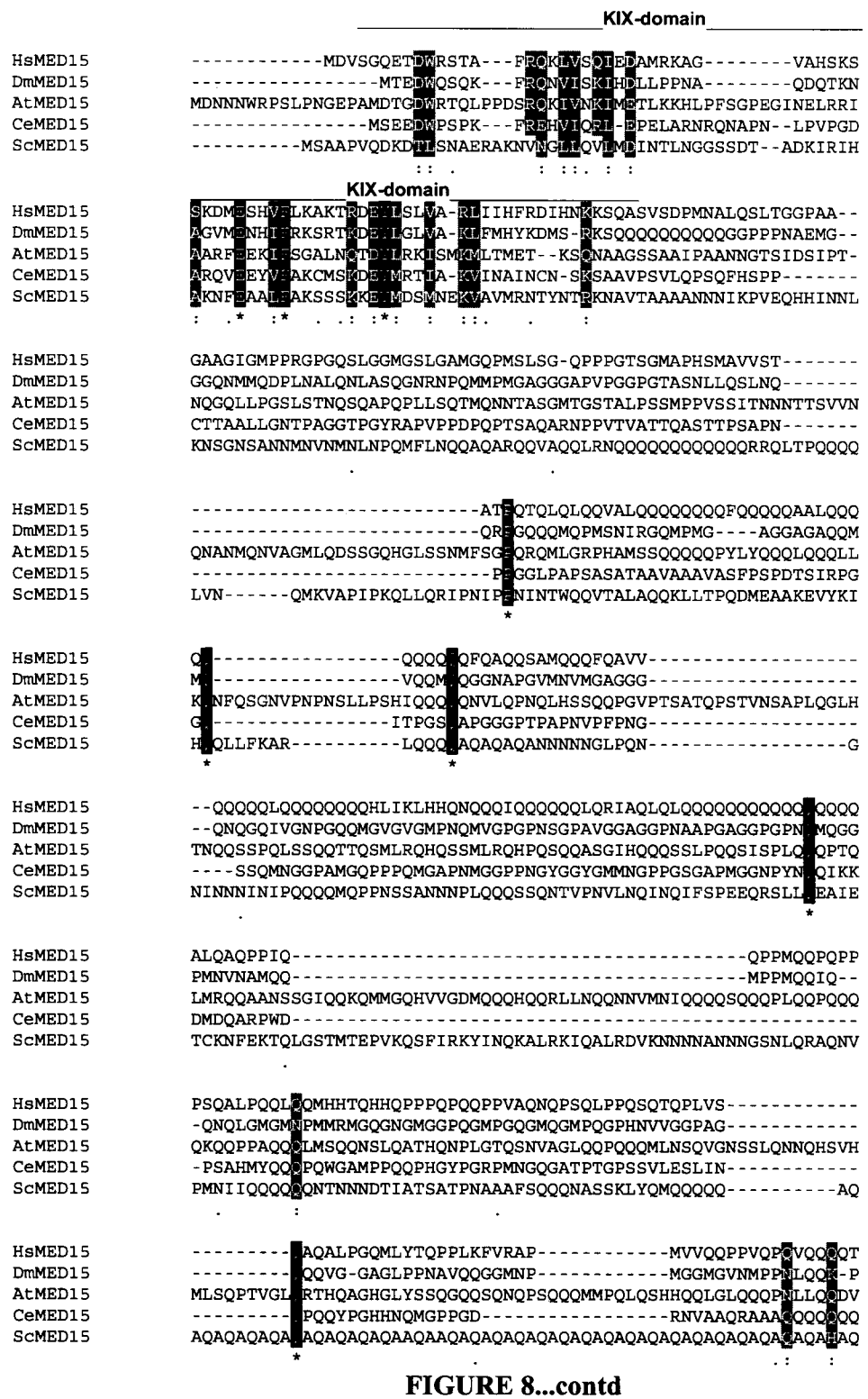


FIGURE 8...contd

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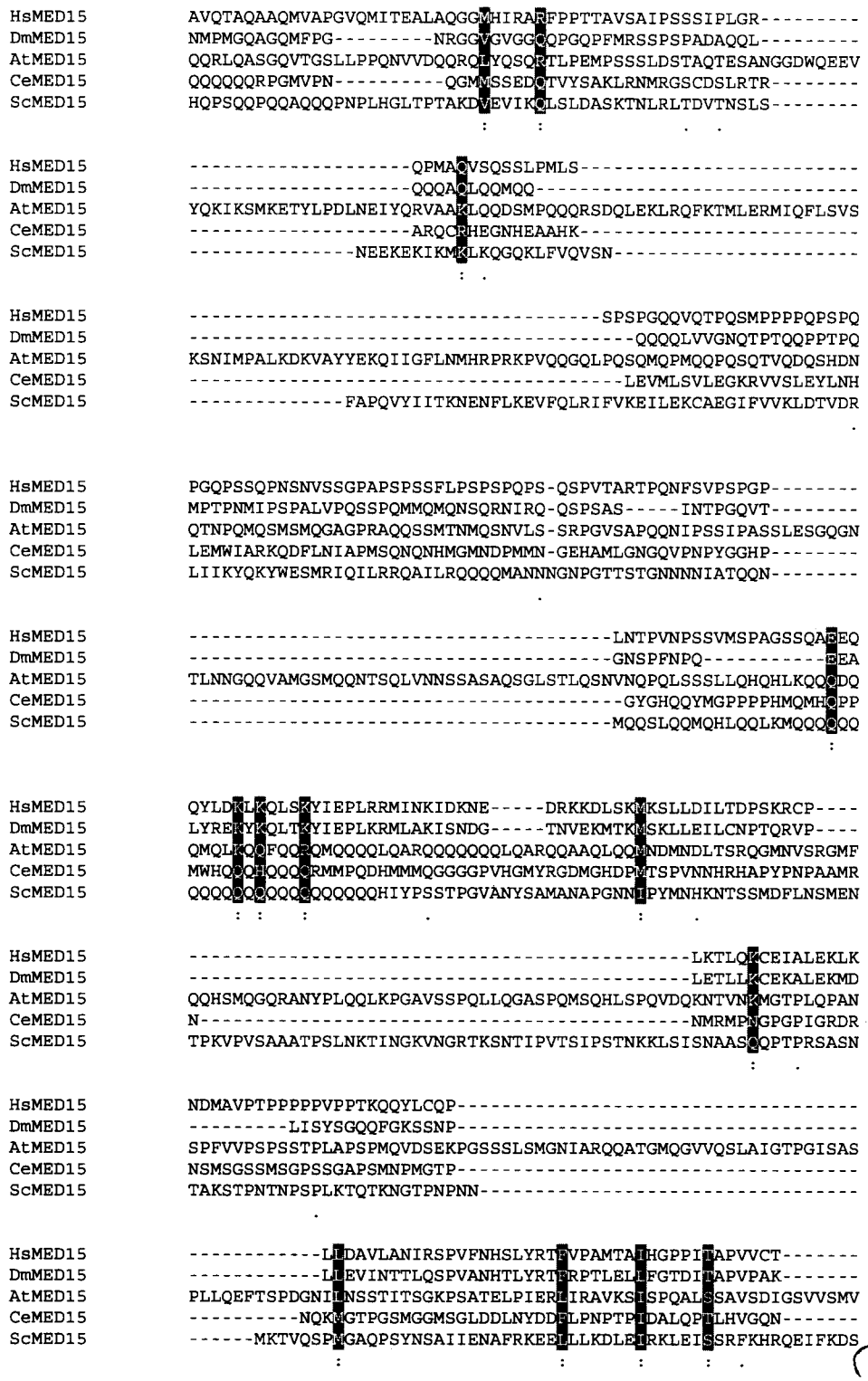


FIGURE 8...contd

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```
HsMED15         ------------------------------------------------------------
DmMED15         ------------------------------------------------------------
AtMED15         DRIAGSAPGNGSRASVGEDLVAMTKCRLQARNFMTQEGMMATKKMKRHTTAMPLSVASLG
CeMED15         ------------------------------------------------------------
ScMED15         PMDLFMSTLG------------------------------------------DCLGIK

HsMED15         ------------------RKRRLEDDE--RQSIPSVLQGEVARLDPKFLVNLDPSHCSNN
DmMED15         ------------------RPRVEEKSTSFEQEVPHVLQGEIARLDTKFKVKLDTTSQINN
AtMED15         GSVGDNYKQFAGSETSDLESTATSDGKKARTETEHALLEEIKEINQFLIDTVVEISDDED
CeMED15         -------------------SMNAGPPVQRSNLNETARKELQILDARFEIDPNHQRHDAN
ScMED15         DEEMLTSCTIPKAVVDHINGSGKRKPTKAAQRARDQDSIDISIKDNKLVMKSKFNKSNRS
                                                 ::   : ::          .

HsMED15         GTVH---------------------------LICKLDDKDLPSVPPLELSSPADYFAQSP
DmMED15         KAIR---------------------------LICCLDDKRLPSVPPVSVSSPEEYFWQAP
AtMED15         AADPSEVAISSIGCEGTTVRFSFIAVSLSPALKAHLSSTQMSPIQPLRLLLPCSYFNGSP
CeMED15         HIIV---------------------------VCKIRNQQFP---PLRLVLPTTYFAGNV
ScMED15         YSIALSN------------------------VAAIFKGIGGNFKDLSTLLHSSSFSTSS
                                               . : .       :   *    *

HsMED15         LWIDRQWQYDANPFLQSVHRCMTSR-------LLQLPDKHSVTALLNTWAQSVHQACLSA
DmMED15         DCSLAEQEYSATPFLQTVQQALIAR-------ISKLPKNYSLSHLLDTWEMAVRQACSPQ
AtMED15         SLLDKLPVETSKENDLLSSKAMARFNIL----LRSLSQPMSLKDIAKTWDACARAVICEY
CeMED15         TVDRAVIDLDAYLYDLLQNSVYERLS---------RPGLSSITDYLNAWEEQVNQYQNQT
ScMED15         NMDVGNPRKRKASVLEISPQDSIASVLSPDSNIMSDSKKIKVDSPDDPEMTKSGATTSEK
                           :                        .   .:    ..:

HsMED15         A-----------------------------
DmMED15         SKPRAVCELSTLLGV---------------
AtMED15         AQQFGGGTFSSKYGTWEKYVAAS--------
CeMED15         SGGLDVAFNVGNDFFYDNLNL----------
ScMED15         QEVTNEAPFLTSGTSSEQFNVWDWNNWTSAT
```

FIGURE 8

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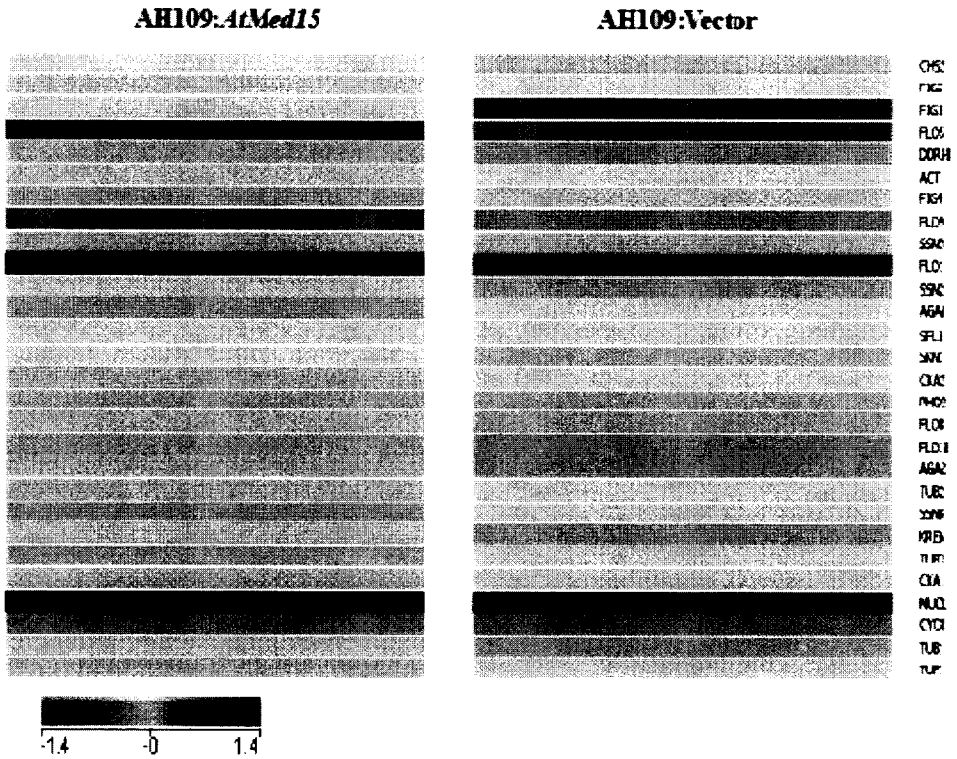


FIGURE 9

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FIELD OF INVENTION

The present invention relates to the field of industrial microbiology and the production of biofuels. The invention in particular, relates to genetically engineered microorganisms showing bioalcohol production and desired flocculation.

BACKGROUND OF THE INVENTION

The threat of reducing reserves of fossil fuels coupled with high consumption rates has led to alarming market fluctuations in crude oil prices leading to economic imbalances. Fossil fuel extraction and processing is inherently dangerous often leading to death or injury of workers in these industries. The toll of fossil fuels on human health and the environment is well documented, but our dependence on fossil fuels is extremely high. One recent study states the cost of pollution from coal exceeds the economic benefit of the electricity the coal produces (Rachel James, 09 August 2012; www.smartplanet.com). Another major threat is the threat of `peak oil`, the point at which the consumption rate of crude oil exceeds the supply rate, thus leading to significantly increased fuel cost results in an increased demand for alternative fuels. Today, many interest groups, including policy makers, industry planners, aware citizens, and the financial community, are interested in substituting petroleum-derived fuels with biomass-derived biofuels. Transitioning to cleaner energy technologies is safer for workers as well as better for public health, economic competitiveness, and the environment.

Plant biomass is an attractive feedstock for ethanol-fuel production by fermentation because it is renewable, and available at low costs and in large amounts. However, a few drawbacks of conventional biofuels are the competition between fuel and food for land and water resources. Massive diversion of food grains to feed cars has led to rise in prices of food and sparked into food riots in various countries. Another emerging concern about conventional biofuels is their potential for negative environmental impacts, since in some instances; they have actually been shown to be net contributors to carbon dioxide emissions because of the farming practices employed. Virgin

rainforests are being felled or burned to make way for oil plantations. Intensive cultivation using heavy doses of chemical fertilizers and pesticides can also degrade land and water resources.

The concept of using alcohol produced by microbial fermentation of sugars from agricultural biomass had its nascence at least two decades ago. The so-called 'second-generation' biofuels like cellulosic ethanol, which uses specially selected microbes and genetically engineered microbes to break down nonfood feedstocks like switchgrass, agricultural waste and wood chips, seem to have promising potential. While there are some promising developments, high costs have so far prohibited commercialisation (Jeremy Wakeford, 10th August 2012, www.engineeringnews.co.za).

There is a pressing need for major advances in second-generation technologies that overcome the food-fuel dilemma and environmental problems associated with traditional feedstock crops. The immediate challenge ahead is to identify target genes for genetic enhancement of bioalcohol production in the microbial system. Yeasts, particularly *Saccharomyces* yeasts, have traditionally been used for fermenting glucose-based feedstock to ethanol, and they are still considered the best microorganisms for that purpose.

US20110306105 describes designer cellulosomes for efficient hydrolysis of cellulosic material. The disclosure also provides methods for generating ethanol from a synthetic yeast consortium by direct fermentation of cellulose to ethanol with productivity, yield and final concentration close to that from glucose fermentation.

US20110165618 describes a genetically modified algae, fungus or bacteria, comprising a heterologous S-adenosylmethionine (SAM)-dependent methyl halide transferase gene, that is genetically modified to increase flux through a S-adenosyl-methionine (SAM) biosynthetic pathway; and/or genetically modified to increase the intracellular halide concentration. It also provides a process for producing methyl halide using the recombinant microorganisms.

US20100105573 describes a recombinant vector and microorganism including the isolated SNR84 gene, and a method of increasing volumetric productivity of biofuel from a galactose-containing carbon source using the isolated SNR84 gene, the recombinant vector or the recombinant microorganism. The patent also describes a method of screening yeast for genes associated with increased galactose catabolism when the genes are over-expressed.

Almost often in biotech industries, once the microbe has fulfilled its respective function, they are separated from the broth in the downstream processing. The flocculation phenomenon is exploited in such applications for an easy, convenient and extremely economical way to separate the flocs or aggregated microbes. Flocculation is a desirable and important characteristic that is unique to brewer's yeast. Yeast flocculation is a complicated process that is currently only partly understood. It requires the presence of at least two types of molecules on the yeast cell surface- the mannans that are present on the cell surface at all times and are encoded by the *MNN* genes and the flocculins (sugar binding proteins) which are the gene products of the *FLO* genes, and are activated only after depletion of nutrients. The flocculins bind to mannans on the surface of neighboring cells leading to the cross binding of cells and ultimately the formation of flocs, each consisting of several cells. The flocs sediment much faster relative to the free cells due to the reduced surface to volume ratio of the aggregated cells (Verstrepen, K.J. and Klis, F.M. (2006) Flocculation, adhesion and biofilm formation in yeasts. *Molecular Microbiology* 60(1), 5-15). Constant switching and adaptation of adhesion may be crucial for survival of the yeast in natural habitats but the instability of the flocculation profile is a true nightmare for industrial applications that rely on a constant and predictable yeast performance. A lot of efforts have been made and are being made to improvise the process of flocculation. Until now, other than natural selection of more flocculent strains, the improvement strategies involved over-expression of individual yeast flocculin genes like *Flo1 or Flo11* (Soares, E.V. (2010) Flocculation in *Saccharomyces cerevisiae*: a review. *Journal of Applied Microbiology.* 110, 1-18; Govender, P., Kroppenstedt, S. and Bauer, F.F. (2011) Novel wine-mediated *FL011*

focculation phenotype of commercial *Saccharomyces cerevisiae* wine yeast strains with modified *FLO* gene expression. *FEMS Microbiology Letters* 317, 117-126; Gomes, D.G., Guimarães, P.M.R., Pereira, F.B., Teixeira, J.A. and Domingues, L. (2012) Plasmid-mediate transfer of FL01 into industrial *Saccharomyces cerevisiae* PE-2 strain creates a strain useful for repeat-batch fermentations involving flocculation-sedimentation. *Bioresource Technology* 108,162-168).

There remains a need for more stable yeast which ferment carbon substrates to biofuel such as alcohol, and for enhanced biomass recovery of the transformed microorganisms which is environmentally responsible and a cost-effective process. The present invention addresses this need through novel recombinant microorganism in particular recombinant yeast expressing heterologous polynucleotide encoding a mediator complex subunit (MED15) protein, wherein the recombinant yeast exhibits enhanced flocculation properties and high bioalcohol production.

BRIEF DESCRIPTION OF ACCOMPANYING DRAWINGS

The following drawings form part of the present specification and are included to further illustrate aspects of the present invention. The invention may be better understood by reference to the drawings in combination with the detailed description of the specific embodiments presented herein.

Figure 1 shows that AtMED15 does not complement the function of GAL11 in yeast. Serially diluted equal amount of cells of WT (wild type), *gal11*Δ, *gal11*Δ yeast carrying vector (vector control) and *gal11*Δ yeast carrying vector harboring *AtMed15* cDNA, were spotted on YP agar media with galactose as carbon source or YPD agar media with cycloheximide (CHX) as xenobiotic. The plates were kept at 30°C for 3-5 days.

Figure 2 shows expression of *AtMed15* in yeast increases adhesiveness of cells and causes robust flocculation. **A.** Yeast cells were streaked on YPD agar plate and grown for 3 days. The plate was photographed before (left) and after (right) rinsing with water to wash off the loosely adhered cells. **B.** Flocculation is observed in transformed yeast cells grown in SD broth after 16 hours. C. Flocculation observed in Mat a (AH109,

Y2H Gold and JRY2334) and Mat α (Y187 and W303). Transformed yeast cells with *AtMed15* or vector control were grown in liquid YPD or SD selection media. D. Various transformed yeast strains were grown on SD agar media. The plate was photographed before (left) and after (middle) washing. Y2H Gold strain with the vector was used as control. E. Adhesiveness of cells was evaluated in overnight grown yeast cultures were transferred to petriplates, and rotated very slowly for few hours to see if the cells can form clumps.

Figure 3 shows effect of different factors on *AtMed15*-induced flocculation. **A.** (i) AH109:*AtMed15* yeast cells were grown in YPD broth, (ii) resuspended in buffer with EDTA, and then (iii) resuspended in buffer with $CaCl_2$. **B.** Yeast colonies were streaked and grown on solid media with different sugars as carbon source. The plates were photographed before (left panel) and after (right panel) washing.

Figure 4 shows mapping of AtMED15 region responsible for flocculation in yeast. AH109 yeast cells transformed with different fragments of *AtMed15* cDNA and were grown in YPD broth for flocculation analysis. AH109 cells transformed with vector alone were used as control. '++', '+' and '-' indicate strong, weak and no flocculation, respectively. Numerals in the left side indicate start and end amino acid position of the corresponding fragment.

Figure 5 shows that AtMED15 does not affect cell wall integrity in yeast. A. Serially diluted cells of WT (wild type), *gal11*Δ, *gal11*Δ vector control and *gal11*Δ transformed with *AtMed15* were spotted and grown on YPD or YPD with Calcofluor white. B. Yeast cells grown in YPD broth were treated with Calcofluor white and observed under confocal microscope.

Figure 6 shows expression of *AtMed15* increases transcript level of some flocculin genes in yeast. **A.** Heat map of normalized and baseline transformed average $\log_2$ signal values of genes encoding for flocculins/adhesins in different yeast cells. The color legend is shown at the bottom and gene names are on the left of the heat map. Expression levels of *Act1* and *Tub1* were analyzed as controls. Yeast strains are

mentioned on the top of the corresponding heat map. **B.** Semi-quantitative RT-PCR analysis of transcript level of few selected flocculin/adhesin genes in different yeast cells. *Tub1* and *Act1* were used as internal quantitative and qualitative controls, whereas *AtMed15* was amplified to show the specificity of the effect. The genes are mentioned on the right.

Figure 7 shows expression of *AtMed15* in yeast increases ethanol production. Equal volume and concentration of AH109 yeast vector control cells or harboring *AtMed15* were grown in YPD broth in **(A)** conical flask or **(B)** batch fermenter. Ethanol concentration was measured in the medium at regular time intervals.

Figure 8 shows the alignment of amino acid sequences of MED15 from different eukaryotes. Residues identical in all the proteins are highlighted with black colour and marked with '*', whereas similar residues are highlighted in grey colour and marked with ':'. KIX-domain is marked with a black line on the top of the sequences. Hs: *Homo sapiens*, Dm: *Drosophila melanogaster*, At: *Arabidopsis thaliana*, Ce: *Caenorhabditis elegans*, and Sc: *Saccharomyces cerevisiae*.

Figure 9 shows that the expression of *AtMed15* increases transcript level of flocculin genes in AH109. Heat map of normalized and baseline transformed average $\log_2$ signal values of genes is mentioned on the right, in the yeast strains AH109 vector control cells and AH109:*AtMed15* cells. The colour legend is shown at the bottom.

BRIEF DESCRIPTION OF SEQUENCE LISTING

SEQ ID NO: 1 shows nucleotide sequence of cDNA that encodes the *Arabidopsis thaliana* mediator complex MED15 protein (4008 bp).

SEQ ID NO: 2 shows amino acid sequence of *Arabidopsis thaliana* mediator complex MED15 protein (1335 aa).

SEQ ID NO: 3 shows the nucleotide sequence of the forward primer *AtMed15*F (29 nt)

SEQ ID NO: 4 shows the nucleotide sequence of the reverse primer *AtMed15R* (28 nt)

SEQ ID NO: 5 shows the nucleotide sequence of the reverse primer *AtMed15*_1_1080BamHI_R (25 nt)

SEQ ID NO: 6 shows the nucleotide sequence of the reverse primer *AtMed15*_1_2075BamHI_R (25 nt)

SEQ ID NO: 7 shows the nucleotide sequence of the reverse primer *AtMed15*_1_3160BamHI_R (26 nt)

SEQ ID NO: 8 shows the nucleotide sequence of the forward primer *AtMed15*_1_1060EcoRI_F (28 nt)

SEQ ID NO: 9 shows the nucleotide sequence of the forward primer *AtMed15*_1_2074EcoRI_F (25 nt)

SEQ ID NO: 10 shows the nucleotide sequence of the forward primer *AtMed15*_1_3142EcoRI_F (27 nt)

SEQ ID NO: 11 shows the nucleotide sequence of the forward primer *AtMed15*-RTF (21 nt)

SEQ ID NO: 12 shows the nucleotide sequence of the reverse primer *AtMed15*-RTR (21 nt)

SEQ ID NO: 13 shows the nucleotide sequence of the forward primer FLO1-RTF (21 nt)

SEQ ID NO: 14 shows the nucleotide sequence of the reverse primer FLO1-RTR (25 nt)

SEQ ID NO: 15 shows the nucleotide sequence of the forward primer FLO9-RTF (22 nt)

SEQ ID NO: 16 shows the nucleotide sequence of the reverse primer FLO9-RTR (24 nt)

SEQ ID NO: 17 shows the nucleotide sequence of the forward primer FLO5-RTF (23 nt)

SEQ ID NO: 18 shows the nucleotide sequence of the reverse primer FLO5-RTR (25 nt)

SEQ ID NO: 19 shows the nucleotide sequence of the forward primer FLO10-RTF (21 nt)

SEQ ID NO: 20 shows the nucleotide sequence of the reverse primer FLO10-RTR (20 nt)

SEQ ID NO: 21 shows the nucleotide sequence of the forward primer FLO8-RTF (24 nt)

SEQ ID NO: 22 shows the nucleotide sequence of the reverse primer FLO8-RTR (24 nt)

SEQ ID NO: 23 shows the nucleotide sequence of the forward primer FIG2-RTF (24 nt)

SEQ ID NO: 24 shows the nucleotide sequence of the reverse primer FIG2-RTR (24 nt)

SEQ ID NO: 25 shows the nucleotide sequence of the forward primer ACT1-F (20 nt)

SEQ ID NO: 26 shows the nucleotide sequence of the reverse primer ACT1-R (20 nt)

SEQ ID NO: 27 shows the nucleotide sequence of the forward primer TUB1-F (20 nt)

SEQ ID NO: 28 shows the nucleotide sequence of the reverse primer TUB1-R (20 nt)

DETAILED DESCRIPTION OF THE INVENTION

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Definitions

For convenience, before further description of the present invention, certain terms employed in the specification, examples and appended claims are collected here. These definitions should be read in light of the remainder of the disclosure and understood as by a person of skill in the art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art to which this invention belongs.

The articles "a", "an" and "the" are used to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

The terms "comprise" and "comprising" are used in the inclusive, open sense, meaning that additional elements may be included. It is not intended to be construed as "consists of only". Similarly, "comprise", "comprises", "comprising", "include", "includes", and "including" are interchangeable and not intended to be limiting.

The use of "or" means "and/or" unless stated otherwise.

The term "microorganism" includes prokaryotic and eukaryotic microbial species from the Domains Archaea, Bacteria and Eucarya, the latter including yeast and filamentous fungi, protozoa, algae, or higher Protista. The terms "microbial cells" and "microbes" are used interchangeably with the term microorganism.

The term "gene" is used broadly to refer to any segment of nucleic acid associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. For example, gene refers to a nucleic acid fragment that expresses mRNA or functional RNA, or encodes a specific protein, and which includes regulatory sequences. Genes also include non-expressed DNA segments that, for example, form recognition sequences for other proteins. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

A "heterologous gene" refers to a gene not normally found in the host organism but that is introduced by gene transfer.

"Coding sequence" refers to a DNA or RNA sequence that codes for a specific amino acid sequence and excludes the non-coding sequences. It may constitute an "uninterrupted coding sequence", i.e., lacking an intron, such as in a cDNA or it may include one or more introns bounded by appropriate splice junctions.

The term "transformation" means the transfer of nucleic acid (i.e., a nucleotide polymer) into a cell. As used herein, the term "genetic transformation" means the transfer and incorporation of DNA, especially recombinant DNA, into a cell.

The term "recombinant microorganism" and "recombinant host cell" are used interchangeably herein and refer to microorganisms that have been genetically modified to express or over-express endogenous polynucleotides, or to express heterologous polynucleotides, such as those included in a vector, or which have an alteration in expression of an endogenous gene.

The term "wild-type microorganism" describes a cell that occurs in nature, i.e. a cell that has not been genetically modified. A wild-type microorganism can be genetically modified to express or over-express a first target enzyme. This microorganism can act as a parental microorganism in the generation of a microorganism modified to express or over-express a second target enzyme. In turn, the microorganism modified to express or over-express a first and a second target enzyme can be modified to express or over-express a third target enzyme.

The term "control", "control microorganisms" and "vector control" as used herein are interchangeable and refer to microorganisms that carry only the vector but do not harbor the heterologous gene of interest. In the present invention, the gene of interest is *AtMed15*.

"Vector" is defined to include, inter alia, any plasmid, cosmid, phage, or binary vector in double or single stranded linear or circular form which may or may not be self

transmissible or mobilizable, and which can transform prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication). Also included are shuttle vectors by which is meant a DNA vehicle capable, naturally or by design, of replication in two different host organisms, which may be selected from actinomycetes and related species, bacteria and eukaryotic (e.g. higher plant, mammalian, yeast or fungal cells).

"Expression" refers to the transcription and/or translation of an endogenous gene, ORF or portion thereof in the host cell. In addition, expression refers to the transcription and stable accumulation of sense (mRNA) or functional RNA. Expression may also refer to the production of protein.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions, and methods are clearly within the scope of the invention, as described herein.

The inventors of the present invention initially carried out investigations on the function and role of the mediator complex subunit (MED15) protein of *Arabidopsis thaliana* and to understand its similarity to GAL11, which is involved in galactose metabolism in the yeast system. The Mediator complex or Mediator is a gigantic modular multiprotein complex, made up of 25 or more subunits (Bourbon, H-M. (2008) Comparative genomics supports a deep evolutionary origin for the large, four-module transcriptional mediator complex. *Nucleic Acids Research* 36, 3993-4008), required for regulation of transcription of RNA polymerase II dependent genes and functions as a co-factor for basal and induced expression of almost all RNA polymerase II-dependent genes (Thompson, C.M. and Young, R.A. (1995) General requirement for RNA polymerase II holoenzymes in vivo. *Proceedings of the National Academy of Sciences U.S.A.* 92, 4587-4590; Kornberg, R.D. (2007) The molecular basis of eukaryotic transcription. *Proceedings of the National Academy of Sciences U.S.A.* 104, 12955-12961; Malik, S. and Roeder, R.G. (2010) The metazoan Mediator co-activator

complex as an integrative hub for transcriptional regulation. *Nature Reviews Genetics* 11, 761-772; Conaway, R.C. and Conaway, J.W. (2011) Function and regulation of the Mediator complex. *Current Opinions in Genetics & Development.* 21, 225-230). The Mediator can function as a communication bridge that can relay regulatory signals from transcription factors bound to the regulatory elements to the RNA polymerase II machinery bound to the promoter of the gene. A striking evolutionary conservation in the structure of the complex from yeast to human is prevalent; however, very low similarity between the primary amino acid sequences of MED subunits across different kingdoms raises a question on the functional conservation of these individual subunits. The subunits of the complex form four distinct modules designated as head, middle, tail and kinase. The head, middle and tail modules form the relatively stable core structure, whereas the kinase module associates reversibly with the core structure. The kinase module sterically blocks the RNA Pol II interactions with the core Mediator and exerts a repressive function (Knuesel, M.T. Meyer, K.D. Bernecky, C. and Taatjes, D.J. (2009) The human CDK8 subcomplex is molecular switch that controls Mediator coactivator function. *Genes & Development* 23, 439-451; Elmlund, H., Baraznenok, V., Lindahl, M., Samuelsen, C.O., Koeck, P.J.B., Holmberg, S., Hebert, H., and Gustafsson, C.M. (2006) The cyclin-dependent kinase 8 module sterically blocks Mediator interactions with RNA polymerase II. *Proceedings of the National Academy of Sciences U.S.A.* 103, 15788-15793). The head and middle modules are involved in interactions with the core RNA polymerase II machinery (Davis, J.A., Takagi, Y., Kornberg, R.D. and Asturias, F.A. (2002) Structure of the yeast RNA polymerase II holoenzyme: Mediator conformation and polymerase interaction *Molecular Cell* 10, 409-415), whereas subunits of the tail modules interact with DNA-bound transcription activators and repressors (Myers, L.C., Gustafsson, C.M., Hayashibara, K.C., Brown, P.O. and Kornberg, R.D. (1999) Mediator protein mutations that selectively abolish activated transcription. *Proceedings of the National Academy of Sciences U.S.A.* 96, 67-72; Han, S.J., Lee, J.S., Kang, J.S. and Kim, Y.J. (2001) Med9/Ces2 and Gal11 modules of mediator are required for transcriptional repression of distint group of

genes. *The Journal of Biological Chemistry* 276, 37020-37026; Yang, F., Vought, B.W., Satterlee, J.S., Walker, A.K., Jim Sun, Z.Y., Watts, J.L., DeBeaumont, R., Saito, R.M., Hyberts, S.G., Yang, S., Macol, C., Iyer, L., Tjian, R., van den Heuvel, S., Hart, A.C., Wagner, G. and Naar, A.M. (2006) An ARC/Mediator subunit required for SREBP control of cholesterol and lipid homeostasis. *Nature* 442, 700-704; Thakur, J.K., Arthanari, H., Yang, F., Chau, K.H., Wagner, G., and Naar, A.M. (2009) Mediator subunit Gal11p/MED15 is required for fatty acid-dependent gene activation by yeast transcription factor Oaf1p. *The Journal of Biological Chemistry.* 284, 4422-4428; Thakur, J.K., Arthanari, H., Yang, F., Pan, S-J., Fan, X., Breger, J., Frueh, D.P., Gulshan, K., Li, D.K., Mylonakis, E., Struhl, K., Moye-Rowley, W.S., Cormack, B.P., Wagner, G. and Naar, A.M. (2008) A nuclear receptor-like pathway regulating multidrug resistance in fungi. *Nature* 452, 604-609). The tail module includes the MED2, MED3, MED14, MED15 and MED16 subunits.

The MED15 protein has evolved to process transcriptional signals for regulating distinct cellular processes. The *Med15* gene was initially identified in yeast as a positive regulator of the genes encoding proteins that are involved in galactose metabolism, and hence originally called *Gal11* (Nogi, Y. and Fukasawa, T. (1980) A novel mutation that affects utilization of galactose in *Saccharomyces cerevisiae. Current Genetics* 2, 115-120). GAL11 is a general cofactor involved in transcriptional activation of many functionally unrelated genes (Suzuki, Y. Nogi, Y. Abe, A. and Fukasawa, T. (1988) GAL11 protein, an auxillary transcription activator for genes encoding galactose-metabolizing enzymes in *Saccharomyces cereivisiae. Molecular and Cellular Biology* **8,** 4991-4999). Reports suggest that in eukaryotes ranging from simple unicellular yeasts to complex multicellular insects and mammals, MED15 is a node for diverse pathways relevant to processes like lipid metabolism, sugar metabolism and xenobiotic detoxification. MED15 has been shown to be targeted by the specific transcription factors to recruit transcriptional machinery at the cognate promoter to initiate the transcription process. Bioinformatic analysis of all the Mediator subunits and biochemical purification of Mediator complex from *Arabidopsis* suggest

that plant genomes also code for MED15, however, until now there is no report on function of MED15 in plants.

In the present invention, unexpected and surprising results were obtained when the inventors attempted to express the *Arabidopsis thaliana* MED15 protein in the yeast system of *Saccharomyces cerevisiae* and evaluate the functional role of *Med15* with that of *Gal11* of the yeast system. Expression of *Arabidopsis Med15* cDNA (*AtMed15)* having nucleotide sequence as set forth in SEQ ID NO: 2 in *Gal11*Δ strain of *Saccharomyces cerevisiae* drastically modified the morphology of the yeast cells. *AtMed15* could not complement the function of *ScGal11* for its involvement in galactose utilization as carbon source and resistance against cycloheximide. However, surprisingly, the expression of *AtMed15* in yeast brought about morphological changes in the yeast cell. The transformed yeast cells adhered strongly on the surface of the agar media, and showed robust flocculation in the liquid media. The transformed yeast cells showed enhanced invasive growth and adhesiveness on solid medium. There was also significant increase in ethanol production by the transformed yeast cells compared to the non-transformed cells. Mating type of the strains influenced the morphological expression of the *AtMed15*-transformed *Saccharomyces cerevisiae Gal11*Δ strain cells. Robust changes in adhesion and flocculation morphology were observed in Mat a strains when compared to Mat α strains. Flocculation was observed even when yeast cells were grown in different carbon sources, or at different pH or temperatures. Expression of flocculation related *Flo* genes like *Flo1* and *Flo11*, but not *Flo5, Flo8* and *Flo10,* was up-regulated in the *AtMed15*-transformed cells, suggesting a transcriptional activator or co-activator like function of AtMED15 for selected genes in yeast cells. There was increased ethanol production by the yeast expressing *AtMed15.* The maximum ethanol produced by the recombinant microorganism carrying *AtMed15* was 4.43 – 5.84 gram/litre compared to 2.79 – 2.99 gram/litre produced by the vector control microorganisms. Ethanol production level for wild type *Saccharomyces cerevisiae* is similar to vector control. The increase in yield of ethanol production was 60% to 80%, preferably in the range of 65% to 75% compared to the ethanol production

by the vector control microorganisms. Manipulating classical genes like alcohol dehydrogenase may increase the ethanol production levels but would not modulate the flocculation levels. In addition to increasing the ethanol production, *AtMed15* causes flocculation in yeast which can make downstream processes easier and economical.

An embodiment of the present invention relates to a recombinant microorganism for bio-ethanol production wherein the microorganism comprises a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2.

Another embodiment of the present invention relates to a recombinant DNA molecule comprising polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the polynucleotide is operably linked to a promoter.

A further embodiment of the present invention relates to a process for production of bio-ethanol, wherein said process comprises (a) culturing a recombinant microorganism comprising a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, in a culture medium under suitable conditions; and (b) recovering bio-ethanol from the culture medium.

A recombinant strain of *Saccharomyces cerevisiae* designated as *Saccharomyces cerevisiae* AHAtM15 has been deposited under Budapest treaty under accession number MTCC 5718 on 28/09/2012 at Microbial Type Culture Collection (MTCC), Chandigarh, India.

Suitable embodiments of the present invention are now described. While specific configurations and arrangements are discussed, it should be understood that this is done for illustrative purposes only. A person skilled in the relevant art will recognize that other configurations and arrangements can be used without departing from the spirit and scope of the invention. As such, the spirit and scope of the appended claims should not be limited to the description of the preferred embodiment contained therein.

An embodiment of the present invention provides a recombinant microorganism for bio-ethanol production, wherein said microorganism comprises a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2.

In another embodiment of the present invention there is provided a recombinant microorganism for bio-ethanol production, wherein said microorganism comprises a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the nucleotide sequence of the heterologous polynucleotide is as set forth in SEQ ID NO: 1.

In yet another embodiment of the present invention there is provided a recombinant microorganism for bio-ethanol production, wherein said microorganism comprises a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the heterologous polynucleotide is obtained from *Arabidopsis thaliana* and *Oryza sativa.*

In still another embodiment of the present invention there is provided a recombinant microorganism for bio-ethanol production, wherein said microorganism comprises a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the microorganism is selected from the group consisting of *Saccharomyces*, *Kluyveromyces, Torulopsis, Yamadazyma, Hansenula, Candida, Pichia, Yarrowia, Trichoderma, Erwinia, Klebsiella, Xanthomonas, E.coli Clostridium, Enterobacter, Synechococcus, Synechocystis, Cyanothece. Microcoleus, Scenedesmus, Arthrospira, Spirulina* and *Anabaena.*

In another embodiment of the present invention there is provided a recombinant microorganism for bio-ethanol production, wherein said microorganism comprises a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the microorganism is *Saccharomyces.*

In yet another embodiment of the present invention there is provided a recombinant microorganism for bio-ethanol production, wherein said microorganism comprises a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the microorganism is *Saccharomyces cerevisiae*.

In yet another embodiment of the present invention there is provided a recombinant microorganism for bio-ethanol production comprising a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the microorganism *Saccharomyces cerevisiae* exhibits flocculation.

Another embodiment of the present invention provides a recombinant DNA molecule comprising polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the polynucleotide is operably linked to a promoter.

In another embodiment of the present invention there is provided a recombinant DNA molecule comprising polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2 operably linked to a promoter, wherein the nucleotide sequence of said polynucleotide is as set forth in SEQ ID NO: 1.

In another embodiment of the present invention there is provided a recombinant vector comprising the recombinant DNA molecule comprising polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the polynucleotide is operably linked to a promoter.

In another embodiment of the present invention there is provided a recombinant vector comprising the recombinant DNA molecule comprising the polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2 operably linked to a promoter, wherein the nucleotide sequence of said polynucleotide is as set forth in SEQ ID NO: 1.

Another embodiment of the present invention provides a process for production of bio-ethanol, wherein said process comprises (a) culturing a recombinant microorganism comprising a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, in a culture medium under suitable conditions; and (b) recovering bio-ethanol from the culture medium.

In another embodiment of the present invention there is provided a process for production of bio-ethanol, wherein said process comprises (a) culturing a recombinant microorganism comprising a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, in a culture medium under suitable conditions; and (b) recovering bio-ethanol from the culture medium, wherein the microorganism is selected from the group consisting of *Saccharomyces, Kluyveromyces, Torulopsis, Yamadazyma, Hansenula, Candida, Pichia, Yarrowia, Trichoderma, Erwinia, Klebsiella, Xanthomonas, E.coli Clostridium, Enterobacter, Synechococcus, Synechocystis, Cyanothece. Microcoleus, Scenedesmus, Arthrospira, Spirulina* and *Anabaena.*

In yet another embodiment of the present invention there is provided a process for production of bio-ethanol, wherein said process comprises (a) culturing a recombinant microorganism comprising a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, in a culture medium under suitable conditions; and (b) recovering bio-ethanol from the culture medium, wherein the microorganism is *Saccharomyces cerevisiae.*

Further embodiment of the present invention provides a process for production of biofuel from biomass using a recombinant yeast comprising a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the process comprises inoculating the recombinant yeast carrying the *AtMed15* gene with the biomass samples in a culture medium under suitable conditions; and recovering the biofuel.

Further embodiment of the present invention provides a process for production of bioethanol from biomass using a recombinant yeast comprising a heterologous polynucleotide encoding a mediator complex subunit (MED15) protein having amino acid sequence as set forth in SEQ ID NO: 2, wherein the process comprises inoculating the recombinant yeast carrying the *AtMed15* gene with the biomass samples in a culture medium under suitable conditions; and recovering the bioethanol.

AtMED15 cannot complement some functions of ScGAL11 in yeast

Functional complementation experiments showed that AtMED15 was not functionally similar to ScGAL11. The *AtMed15* having SEQ ID NO: 2 was cloned in yeast vector pGHM for introduction into *ScGal11*Δ strain, and the transformed cells were grown on media containing galactose as a carbon source. The transformed yeast cells behaved similar to the *ScGal11*Δ strains and the vector control and were unable to grow well in the galactose medium, as shown in Figure 1. In yeast, master regulators of multidrug resistance, PDR1 and PDR3, require GAL11 for their transcriptional activity. So, *ScGal11*Δ yeast cells are very sensitive to different drugs including cycloheximide. As evident from the last two panels in Figure 1, transformation of *ScGal11*Δ cells with *AtMed15* did not restore resistance to cycloheximide in the *ScGal11*Δ yeast strain. The *AtMed15* cDNA could not complement the functions of *ScGal11* (yeast *Med15*) in galactose metabolism and multidrug resistance as seen in Figure 1.

Expression of *AtMed15* induces flocculation and enhances adhesiveness in yeast

The *Scgal11*Δ yeast cells transformed with *AtMed15* displayed significant flocculation while growing in the liquid media (Figure 2B). Similar changes were observed in the wild type Mat a strains AH109, Y2HGold, and JRY2334, suggesting the general ability of AtMED15 to affect the yeast cells irrespective of presence and absence of indigenous *Gal11* gene, as shown in Figures 2C and 2D. However, expression of *AtMed15* in Mat α type strains like Y187 and W303 did not show such robust changes (Figures 2C and 2D), suggesting that the effect was more prominent in the strains of mating type Mat a.

Expression of *AtMed15* in *Scgal11*Δ yeast enhanced cell-cell adhesion and adherence on agar media, and induced strong flocculation and clumping in liquid media. The cells became stickier and adhered to the surface of the media more strongly as assessed by the washing experiments, suggesting enhanced invasive growth in the agar media as shown in Figure 2D. Mat a strains of yeast cells expressing *AtMed15* aggregated and formed clumps showing increased cell-cell adhesion, however, this effect was very nominal in Mat α strain Y187 expressing *AtMed15*, as shown in Figure 2E.

Factors affecting flocculation in transformed yeast

Cations play very important role in yeast flocculation by promoting flocculation. The free and labile Ca^{2+} ions in the media enable cell wall bound lectins / mannoproteins adopt their active conformation, which in turn interacts with carbohydrate residues of neighboring cells to cause flocculation. Addition of EDTA in the culture media caused dispersion of flocs in the flocculant yeast culture of transformed *Scgal11*Δ yeast cells, as shown in Figure 3A. The flocculation was reversible, as addition of excess $CaCl_2$ in the medium could induce re-formation of flocs.

pH of the growth medium does not affect yeast flocculation. The *AtMed15*-transformed yeast cells were grown in culture media with the adjusted pH from 1.5 to 9.0 and flocculation was observed to be similar over the entire pH range, as seen in Table 4. At lower pH, since number of cells was less, lesser flocs were observed but the degree of flocculation remained the same.

Temperature affects flocculation by breaking cell-cell interactions. However, *AtMed15*-transformed yeast cells in liquid media incubated at different temperatures for 8 hours, did not show considerable difference in the extent of flocculation, as seen in Table 5. A pH of 1.5 and temperature of 50^0C was lethal for both the transformed and control yeast cells. In these conditions, less number of yeast cells was observed in the culture media, but the surviving cells were able to form flocs, as shown in Tables 4 and 5. Overall, there was no effect of pH and temperature on flocculation in *AtMed15*-transformed yeast cells.

Sugars affect flocculation by modulating cell-cell interactions. The transformed yeast cells were grown in liquid and agar media with different carbon sources like glucose, maltose, sucrose or mannose, and flocculation in liquid broth and adhesiveness on solid agar surface were observed. In all these culture media, expression of AtMED15 could induce flocculation and enhance invasive growth in Mat a type yeast strains, as seen in Figure 3B and Table 6.

Mapping of AtMED15 region responsible for flocculation

In order to find out the active fragment of AtMED15 responsible for flocculation in yeast culture, different fragments of this protein were expressed in the yeast cells. The carboxyl-end deletion of residues from position 1054 to 1335 did not affect flocculation much, but removal of residues from 692 to 1335 position completely abolished flocculation. The middle region from position 354 to 1053 could induce flocculation, indicating its importance in flocculation and also suggesting that residues from 691 to 1053 position might be necessary for this process. But surprisingly, expression of a fragment containing residues just from 692 to 1053 did not induce flocculation, revealing the fact that these residues alone were not sufficient to induce flocculation. Similarly, the fragment containing residues 354 to 691 was also not able to induce flocculation. The amino terminal region from residue 1 to residue 358 could induce very little flocculation, indicating that the region 359-691 might have an inhibitory effect on flocculation. It seems that peptide containing the 354-1053 residues, this inhibitory effect of fragment containing 359-691 residues is nullified by the presence of 692-1053 residues. Thus, in AtMED15, the middle region of the protein spanning residues from 354 to 1053 is required to induce flocculation in yeast cells, as seen in Figure 4. Further *in-silico* analysis did not reveal the presence of any characteristic domain or feature in this region.

Yeast cell wall integrity is not affected by AtMED15

Flocculation is a property related to the characteristics of cell wall and the transformed cells were tested for their sensitivity towards Calcofluor White. Expression of *AtMed15*

in yeast cells did not affect the growth in the presence of Calcofluor White on the solid agar medium as seen in Figure 5A. Observation of the Calcofluor White-stained transformed cells under confocal microscope revealed no difference in the staining pattern of chitin in the cell wall of the transformed cells compared with that of vector control, as shown in Figure 5B. Expression of *AtMed15* does not affect the cell wall integrity of the transformed yeast cells.

Expression of *AtMed15* in yeast affected expression of *Flo* genes

Atleast 28 genes are associated with flocculation or cell aggregation processes in yeast. The most important group of genes responsible for flocculation code for flocculin or adhesion proteins *viz* FLO1, FLO5, FLO9 and FLO10. Based on sequences, these proteins are very similar to each other. The second group of genes important for flocculation code for proteins likes FLO11, FIG2 and AGA1. These proteins are different from each other. The FLO8 protein is a transcription factor that regulates expression of many *Flo* genes.

Gene expression analysis by microarray shows that deletion of *Gal11* caused reduction in the transcript level of some flocculin, adhesion and mannoprotein genes, suggesting that GAL11 is required for the expression of such genes and so might be an important factor to regulate flocculation and cell-cell adhesion in yeast. This is not surprising since GAL11 as a Mediator subunit does function as a co-regulator for many genes in yeast. Expression of flocculin and adhesion related genes in *AtMed15*-transformed *gal11*Δ yeast cells and vector control was evaluated by microarray analysis. There was increased transcription of *Flo1*, *Flo11/Muc1* and *Aga1* genes in the transformed yeast cells compared to vector control and untransformed *gal11*Δ cells, as shown in Figure 6A.

The gene expression data was confirmed by semi-quantitative RT-PCR analysis of few selected genes, as seen in Figure 6B. Amplification of *Act1* and *Tub1* transcripts served as control. Almost similar results were obtained after microarray analysis of *AtMed15* induced expression of genes in AH109 cells, as seen in Figure 9. There was no

noticeable effect on the transcript level of many other flocculation/adhesion related genes. Expression of *AtMed15* might be inducing flocculation in yeast culture partly by up-regulating the transcription of some selected flocculin/adhesin and mannoprotein coding genes.

In yeast, GAL11 is required for transcriptional regulation of flocculin/adhesion genes and this function is countered by subunits of CDK module of the Mediator complex. Based on the known mechanisms of gene regulation by GAL11, it is plausible to think that transcription factors like FLO8 that regulates flocculation, might be targeting GAL11 for their transcriptional activity. Expression of AtMED15 in yeast caused enhanced stickiness of yeast colonies on agar surface, increased cell-cell adhesion resulting in clump formation, and strong flocculation of cells in liquid culture by increasing the transcript levels of *Flo* genes, suggesting that AtMED15 could complement function of GAL11 in regulation of flocculin/adhesion genes in yeast. Thus, there is a possibility that, like GAL11 in yeast, AtMED15 plays important role in cell-cell adhesion in *Arabidopsis*.

This is the first report where a gene from another kingdom, *AtMed15* from Arabidopsis, is used to induce flocculation in yeast. Importantly, expression of *AtMed15* caused increase in transcript level of more than one *Flo* gene in yeast, so the effect is more robust. The AtMED15-induced flocculation in yeast is constitutive as flocculation was observed throughout the cell growth, is not affected by wide fluctuation of pH and temperature, and is immune to presence of different carbon sources. These characteristics can make AtMED15-driven flocculation suitable for exploitation in bioreactors as the cells are self-immobilized in flocs/pellets.

Subunit MED15 which is present in the tail module of Mediator complex has been found to be very important for transcriptional regulation of many processes in yeast and metazoans. However, there is not much of primary amino acid sequence similarity between AtMED15 and other characterized MED15 proteins from yeast and metazoans,

but there is homology only in the amino terminal region which code for a KIX domain, as shown in Figure 8.

AtMED15 increased ethanol production in yeast culture

The fermentation efficiency of the transformed yeast cells was significantly higher in both small scale flask culture and large scale batch culture in a fermentor, compared to the vector control, as shown in Figure 7. The ethanol production increases by more than 50% during the course of cell growth and fermentation, more preferably by 70% to 75% as shown in Figure 7. The maximum ethanol obtained in the case of recombinant microorganism carrying *AtMed15* was in the range of 4.43 - 5.84 gram/litre 5.02 gram/litre, range of 2.79 - 2.99 gram/litre by vector control cells and the range of 2.29 - 2.33 gram/litre in wild-type yeast strains.

Currently known industrial production levels of bio-ethanol or biofuels use completely different media like corn starch, rice starch, sugarcane extract etc. Many researchers also use very rich media with more than 25% sugar for increasing the fermentation. The effect of AtMed15 on bio-ethanol production in such rich media is yet to be seen. However, the microorganisms of the present invention have shown increased yield of bio-ethanol in a non-enriched culture medium. Therefore, their potential in enriched culture medium can be optimally exploited for bio-ethanol production.

Although the subject matter has been described in considerable detail with reference to certain preferred embodiments thereof, other embodiments are possible. As such, the spirit and scope of the appended claims should not be limited to the description of the preferred embodiment contained therein.

EXAMPLES

The disclosure will now be illustrated with working examples, which is intended to illustrate the working of disclosure and not intended to take restrictively to imply any limitations on the scope of the present disclosure. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly

understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods, devices and materials are described herein.

Cloning of the *AtMed15* gene from *Arabidopsis thaliana*

Plant materials, microbial materials, plasmids, culture media and growth conditions

Seedlings of *Arabidopsis thaliana* ecotype Columbia (col 0) were grown under culture conditions of 22±2°C with a 16 h light and 8 h dark photoperiod.

The *Escherichia coli* strain DH5α was used as a host for all plasmid construction and maintenance studies. The plasmids and oligonucleotides used in this study are listed in Table 1 and Table 2. The plasmids used in this study, pGBKT7 and pGADT7 were obtained from Clontech. The vector pGHM was made from pGADT7 by replacing the *Hind*III fragment (~800bp) with new MCS containing *Eco*RI, *Nde*I and *Xho*I sites.

The yeast strains were grown at 30^0C on YPD media (1% yeast extract, 2% bactopeptone, and 2% glucose) or SD-selection media (0.67% yeast nitrogen base without amino acids, supplemented with 2% glucose and the required amino acids).

Construction of recombinant vector

Total RNA was extracted from young leaves of two weeks old *Arabidopsis thaliana* plants. The full-length cDNA of the *AtMed15* gene was amplified by Reverse Transcriptase PCR using the forward primer *AtMed15*F having sequence as set forth in SEQ ID NO: 3 and the reverse primer *AtMed15*R, having sequence as set forth in SEQ ID NO: 4. The primers span complete coding sequence i.e. 1 to 4008 bases in the CDS of AtMed15; from 68 base to 4076 base in the cDNA of AtMed1. The run protocol for PCR is as provided in Table 7.

The size of the PCR product (amplicon) was 4027 bp. This includes the restriction sites included in the primers. The amplicon was resolved in 1% agarose gel, and then the DNA molecule was purified from the gel piece using QIAquick spin column (Qiagen).

The purified PCR product was further cloned into the region between the *Eco*RI and *Bam*HI sites in the multiple cloning site of pGBKT7 and pGADT7 plasmid vectors to make the plasmid constructs, pGBKT7:*AtMed15* and pGADT7:*AtMed15*, respectively. For ligation, the vector plasmids, pGBKT7 or pGADT7 and the full length *AtMed15* CDS, with the sticky ends generated by digestion with *Eco*RI and *Bam*HI, were mixed together in a molar ration of 1:3 along with 5 units of ligase (Fermentas), and then incubated at 16°C for 16 hours.

The pGADT7:*AtMed15* plasmid construct was digested with *Eco*RI and *Sal*I and resolved on 1% agarose gel. The 4010 bases long AtMed15 CDS with sticky ends of *Eco*RI and *Sal*I was purified using QIAquick spin column (Qiagen) and mixed with pGHM vector with the sticky ends generated by digestion with *Eco*RI and *Xho*I, along with 5 units of ligase. The ligation mix was incubated at 16°C for 16 hours. This ligation mix was used to transform competent DH5α E. coli cells for propagation of pGHM:*AtMed15* plasmid.

For the expression of the truncated *AtMed15*, the fragment was cloned in between *Eco*RI and *Bam*HI sites of pGBKT7 vector. Different fragments of *AtMed15* CDS were amplified by using combinations of primers, SEQ ID NO: 5, 6, 7, 8, 9 and 10, digested with *Eco*RI and *Bam*HI, purified from 1% agarose gel, and then ligated in *Eco*RI and *Bam*HI sites of pGBKT7 as explained above. These recombinant plasmids, pGBKT7:*AtMed15* fragments were introduced into AH109 yeast cells using EZ-transformation Kit (MP Biomedicals, USA) and the positive yeast cells were selected on SD–Trp media. All the recombinant plasmids were confirmed by sequencing.

Saccharomyces cerevisiae transformation

The *Saccharomyces cerevisiae* strains used in this study are listed in Table1. Yeast transformations were performed using the EZ-Transformation Kit (MP Biomedicals, LLC, USA) as per the manufacturer's instructions. 1 mL of overnight grown yeast cells were pelleted by centrifugation at 2500 rpm for 2 min in a microfuge tube of 1.5 mL volume. The yeast pellet was resuspended in 125 µL of EZ-transformation mix and then

mixed thoroughly with 1 μg of plasmid DNA and 5 μL of carrier DNA. The tube was incubated at 42^{0}C for 30 minutes. Following this, the yeast cells were spread on selection media.

Flocculation and phenotypic growth assays

Adhesiveness of cells (cell-cell adhesion) and clump formation was evaluated in transformed and control yeast cells. 10 mL of the overnight grown yeast cultures were transferred to petriplates, and rotated at 20 rpm for 4 hrs at 30^{0}C (Figure 2).

Flocculation in cells was evaluated after 16 hours. Cells were grown in culture medium containing 2% dextrose, pH 5.8 and at 30^{0}C and rotated at 200 rpm. It was observed that flocculation was shown by the transformed cells at all times. Non transformed, non-vector harboring, wild type yeast cells do not show flocculation, similar to the vector control cells (Figure 3).

Effect of pH on flocculation

Yeast cells were grown in 10 mL YPD liquid media under different pH of 1.5, 4.8, 6.9 and 9.0 at 30^{0}C for 2 days to evaluate the effect of pH on yeast morphological characteristics. pH 1.5 proved lethal to the yeast cells but the surviving cells showed flocculation. Flocculation was observed over the entire pH range, as seen in Table 4. Overall, there was no effect of pH on flocculation in *AtMed15*-transformed yeast cells compared to the vector control.

Effect of temperature on flocculation

Yeast cells were grown overnight in 5ml YPD liquid medium at 30^{0}C, and then transferred to incubator shaker set at different temperatures (4^{0}C, 30^{0}C and 60^{0}C) and incubated for another 8 hours at constant shaking. Extent of flocculation was visually observed at different intervals of time. *AtMed15*-transformed yeast cells in liquid media incubated at different temperatures for 8 hours did not show considerable difference in the extent of flocculation, as seen in Table 5. A temperature of 50^{0}C was lethal for transformed and control yeast cells. In these conditions, less number of yeast cells was

observed in the culture media, but the surviving cells were able to form flocs, as shown in Table 5.

For adhesive growth assays, yeast strains were grown on YP agar medium (1% yeast extract, 2% bactopeptone) containing 2% of different carbon sources (glucose, sucrose, maltose and mannose) and incubated at 30^0C for 5 days. The plates were then rinsed with a thin layer of water. The plates were photographed before and after washing.

Effect of cations on flocculation

AH109:vector and AH109:*AtMed15* yeast cells were grown overnight in YPD medium. 1 mL of overnight grown yeast culture was aliquoted in a 1.5 mL microfuge tube and pelleted at 2500 rpm for 3 minutes. The yeast pellet was resuspended in 1 mL PBS (phosphate buffer saline). To this, 30 µL of 500 mM EDTA was added and thoroughly mixed. Flocculation was visually observed after 1 hour (Figure 3A).

The yeast cells were again pelleted at 2500 rpm for 3 minutes and then resuspended in 1 mL of fresh PBS buffer. To this, 200 µL of 100 mM $CaCl_2$ was added, and flocculation was visually observed after 1 hour (Figure 3A).

Effect of sugar source on flocculation

Growth in media with different sugars: single colony of freshly grown yeast cells (AH109:AtMed15, gal11Δ:AtMed15 or Y187:AtMed15) were grown in 10 ml of liquid media containing 2% glucose, maltose, mannose or sucrose as carbon source for 16 hours, and then flocculation was observed.

For analysis of adhesion on agar surface, single colony of yeast (AH109:*AtMed15*, *gal11Δ:AtMed15* or Y187:*AtMed15*) was streaked on the agar surface of media containing 2% of glucose, maltose, mannose or sucrose as carbon source, and then grown at 30 0C for 3 days. The adhesiveness of these yeast cells on the solid agar surface was analysed by rinsing the plates with thin layer water.

Calcoflour White experiment

Chitin and β-glucan are major components of inner layer of yeast cell wall, and staining with Calcofluor White allows a qualitative estimation of chitin accumulation in the cell wall. Yeast cells, wild-type, *gal11*Δ, *gal11*Δ:vector or *gal11*Δ:*AtMed15*, were grown overnight in YPD medium. Cells were diluted to 0.2 OD at 600. The cells were serially diluted 10 times and 2 µL of each dilution was spotted on the surface of YPD agar or YPD agar with 200 µg/mL Calcafluor White (Sigma). The plates were incubated at 30^0C for 3 days. For observations under microsope, Calcafluor White solution (upto final concentration of 200 µg/mL) was added in 50 mL of overnight grown yeast culture. One drop of yeast culture was put on slide and observed under confocal microscope (Figure 5).

Gene expression analysis of flocculin/adhesion genes

Microarray Analysis of gene expression

Total RNA was isolated from 50ml of overnight grown yeast cultures, using RNeasy mini kit (Qiagen) as per instructions given in the manual. Quality of RNA was assessed on RNA Nanochip (Agilent) using 2100 Bioanalyzer (Agilent). High quality RNA was amplified and labeled with Cy3 using One Color Spike Mix (Agilent) and then used to hybridize G4813A Yeast Microarray slide (Agilent) as per manufacturer's instruction. After washing, the arrays were scanned in Agilent Microarray Scanner G25665CA, and the strength of signals was analyzed by Feature Extraction Software (Agilent). Following this, the raw data were imported to GeneSpring GX 11.5.1 software (Agilent) for detailed analysis and heat map generation. Expression of *AtMed15* caused increase in transcript level of more than one *Flo* gene in yeast, so the effect is more robust. In microarray experiment, 22 genes were analysed (Figure 6).

Semi-quantitative RT-PCR analysis

5 µL of total RNA (1µg/mL) was taken in a microfuge tube and heated at 65 0C for 10 minutes and then immediately chilled in ice. To this, 11 µL of first-strand reaction mix (GE Healthcare), 1 µL of 200 mM DTT, and 1 µL of 5 µg/µL oligo d(T)18 primer were mixed in a total volume of 33 µL. After this the tube was kept at 37^0C for 1 hour for the

synthesis of cDNA. This cDNA solution was used as a template for PCR amplification of specific genes using specific primers mentioned in the table 3 (SEQ ID 11 to SEQ ID 28).

The cDNA thus prepared was used as template for PCR analysis.

Only a few genes were evaluated in semi-quantitative RT-PCR experiment. It was observed that *Flo5, Flo8* and *Flo10* did not show did not show any up-regulation due to expression of AtMed15 but the *Flo1* and *Flo11* genes were up-regulated in the *AtMed15*-transformed cells, suggesting a transcriptional activator or co-activator like function of AtMED15 for selected genes in yeast cells (Figure 6).

Ethanol production by the recombinant yeast carrying *AtMed15*

The transformed yeast cells AH109:AtMed15 and vector control AH109:vector cells were grown separately at 30^0C in a fermentor (3L jacketed glass vessel) for batch fermentation experiments. YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose) was used after being sterilized in the fermentor by autoclaving. The pH probe was calibrated before autoclaving the fermentor. The pH of medium was maintained at 6.5± 0.05 by automatic addition of 2N NaOH and 2N H_3PO_4. 2-3 drops of sterilized silicone antifoam was added to prevent excessive foaming.

An Applikon AD101, a 3L jacketed glass vessel with control unit was used for batch cultivation of cells for ethanol production.

Aerobic condition was maintained by keeping dissolved oxygen (DO) level more than 20 percent and impeller speed of 800 rpm. The DO probe was calibrated by one point calibration. Single colony from YPD agar plate was taken and pre-culture was done in 50 ml medium in conical flask at 220 rpm on rotary shaker at 30^oC±0.1^{o}C for 24 hours. Equal volumes of cells of equal optical density were inoculated aseptically into reactor to maintain consistency in control and test run. Samples were drawn from the sampling port aseptically and filtered using syringe filter to get rid of all the cells at regular

interval of 24 hrs as shown in Figure 7B. Ethanol was estimated in the filtrate by gas chromatography (GC) using Stabilwax Restek capillary column.

The maximum ethanol obtained in the case of recombinant microorganism carrying *AtMed15* was 5.02 gram/litre in comparison to 2.89 gram/litre produced by that carrying just the vector. There was increase of about 73.7% in the production of ethanol by the transformed yeast cells compared to the vector control cells by batch fermentation. The yield of ethanol by wild type, non-transformed, yeast cells was in the range of 2.29 – 2.33 gram/litre.

In conical flask cultures, the amount of ethanol produced by AH109:*AtMed15* was in the range of 3.85 - 4.20 grams/litre and by AH109: vector cells the amount of ethanol produced was in the range of 2.89 - 3.03 grams/l.

Bio-ethanol production form biomass using the recombinant yeast carrying *AtMed15*

A variety of biomass of different crops/plants and/or algal sources can be used for producing ethanol. Among these are crops such as sugarcane, corn and other starch crops, cellulosic biomass feedstock such as herbaceous and woody plants, agricultural and forestry residues and municipal solid waste and industrial waste streams. The fermentive capacity of recombinant yeast carrying *AtMed15* was evaluated by inoculating the recombinant yeast carrying *AtMed15* with the biomass samples. Aliquots were collected at regular intervals and monitored for ethanol production.

Table 1: Yeast strains used for the present invention

Strain	Genotype	Source
BY4741	MATa *his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura*3Δ0	Giaever *et al.* 2002
BY4741 *gal11Δ*	BY4741 *gal11Δ*	Thakur *et al* 2008
AH109	MATa *trp*1-901*leu*2-3 112 *ura*3-52 *his*3-200 *gal*4Δ *gal*80Δ *LYS*2:: $GAL1_{UAS}$-$GAL1_{TATA}$-*HIS*3 *MEL*1$GAL2_{UAS}$-$GAL2_{TATA}$-*ADE*2 *URA*3:: $MEL1_{UAS}$-$MEL1_{TATA}$-*lacZ*	Clonetech
JRY2334	MATa *ade*2-1 *his*3-11 15 *leu*2-3 112 *trp*1-1 *ura*3-1 *can*1-100	Thomas and Rothstein, 1989
Y2HGOLD	MAT*a trp*1-901 *leu2*-3 112 *ura*3-52 *his*3-	Clonetech

	200 *gal*4 *Δ gal*80*Δ LYS2: : GAL*1$_{UAS}$–*Gal*1$_{TATA}$–*His*3, *GAL*2$_{UAS}$–*Gal*2$_{TATA}$–*Ade*2 *URA*3:: *MEL*1$_{UAS}$–*Mel*1$_{TATA}$ *AUR*1-*C MEL*1	
YI87	MATα ura3-52 *his*3-200 *ade* 2-101 *trp* 1-901 *leu2-3* 112 *gal*4Δ met$^-$*gal*80Δ *URA*3:: *GAL1*$_{UAS}$-*GAL*1$_{TATA}$-*lacZ MEL*1	Clonetech
W303	MAT α *ade*2-1 *ura*3-1 *his*3-11 15 *trp*1-1 *leu*2-3 112 *can*1-100	Thomas and Rothstein, 1989

Table 2: List of plasmids used in the present invention

Plasmid	Description	Reference
pGBKT7	*GAL*4 $_{(1-147)}$ DNA-BD, *TRP1*, kan^r, c-Myc epitope tag	Clonetech
pGADT7	*GAL4* $_{(768-881)}$ AD, *LEU2*, amp^r, HA epitope tag	Clonetech
pGBKT7-*AtMed15*	*AtMed15* $_{(1-1335)}$ (full-length CDS) in pGBKT7	present invention
pGHM	*LEU2*, amp^r, ADH1 promoter	present invention
pGHM -*AtMed15*	*AtMed15* in pGHM	present invention
pGHM -*egfp*	*egfp* in pGHM	present invention

Table 3: List of primers

Cloning (5'-3')	
SEQ ID NO: 3 *AtMed15*F	GCGAATTCATGGATAATAACAATTGGAGG
SEQ ID NO: 4 *AtMed15*R	GCGGATCCATTCAGGAAGCTGCTACATC
Deletion Analysis primers (5'-3')	
SEQ ID NO: 5 *AtMed15*_1_1080BamHI_R	ATGGATCCGCATTAATTGTGTAGGC
SEQ ID NO: 6 *AtMed15*_1_2075BamHI_R	ATGGATCCTTGCTGTACTGGCTTCC
SEQ ID NO: 7 *AtMed15*_1_3160BamHI_R	TAGGATCCGGCTCTAATAAGGCGTTC
SEQ ID NO: 8 *AtMed15*_1_1060EcoRI_F	TAGAATTCCAGCCTACACAATTAATGCG
SEQ ID NO: 9 *AtMed15*_1_2074EcoRI_F	TAGAATTCGGGCAGCTTCCGCAATC
SEQ ID NO: 10 *AtMed15*_1_3142EcoRI_F	TAGAATTCGAACGCCTTATTAGAGCCG
Semi-quantitative RT-PCR (5'-3')	
SEQ ID NO: 11 *AtMed15*-RTF SEQ ID NO: 12 *AtMed15*-RTR	GTCTCCTATTCAACCATTGCG GTGCGTACTCACAGATCACTG

SEQ ID NO: 13 FLO1-RTF	AGGTCTAATCAGCACTACAAC
SEQ ID NO: 14 FLO1-RTR	CGAAGTGAAAGTGCCAGTCCATGGT
SEQ ID NO: 15 FLO9-RTF	AGTCACTTCTTCTCTAGTCACT
SEQ ID NO: 16 FLO9-RTR	AGTAATTTCCTGACTTGTTGTTGC
SEQ ID NO: 17 FLO5-RTF	ACCACTGCATATTTTTGGTAATC
SEQ ID NO: 18 FLO5-RTR	ATCTACTGTTGCAAAAGAAAACGTG
SEQ ID NO: 19 FLO10-RTF	AATAGCGACAATGTTTCTGCT
SEQ ID NO: 20 FLO10-RTR	CACTATTGGTTAGTAGATGA
SEQ ID NO: 21 FLO8-RTF	TTCAGCCCTCATCCAATGTGGGTA
SEQ ID NO: 22 FLO8-RTR	ATCCGGTCCTTGGTCTTCAACCAT
SEQ ID NO: 23 FIG2-RTF	TAGCGTTCCACAAGCCTCCTCATT
SEQ ID NO: 24 FIG2-RTR	AGTGGTTTGTGACAGCGGAAGAGA
SEQ ID NO: 25 ACT1-F	TGTCACCAACTGGGACGATA
SEQ ID NO: 26 ACT1-R	CGTGAGTAACACCATCACCG
SEQ ID NO: 27 TUB1-F	TTGGTGTCCAACTGGTTTCA
SEQ ID NO: 28 TUB1-R	GGCACCCACTTCGATGTAAT

Table 4: Table showing the flocculation of *AtMed15*-transformed yeast cells under various pH where '++' and '+' indicate strong and less flocculation respectively.

Sl. No.	pH	Flocculation
1	1.5	+
2	4.8	++
3	6.9	++
4	9.0	++

Table 5: Table showing the flocculation of *AtMed15*-transformed yeast cells under various temperatures where '++' and '+' indicate strong and less flocculation respectively.

Sl. No.	Temp (°C)	Flocculation
1	4	++
2	30	++
3	50	+

Table 6: Table showing the flocculation of yeast cells in the presence of various sugars where'+' and '-' indicate presence and absence of flocculation, respectively.

S. No.	Yeast strain	Glucose	Sucrose	Maltose	Mannose
1	AH109	-	-	-	-
2	AH109:vector	-	-	-	-
3	AH109:AtMed15	+	+	+	+
4	gal11Δ	-	-	-	-
5	gal11Δ:vector	-	-	-	-
6	gal11Δ:AtMed15	+	+	+	+
7	Y187	-	-	-	-
8	Y187:vector	-	-	-	-
9	Y187:AtMed15	-	-	-	-

Table 7: Table showing the run protocol for polymerase chain reaction

Steps	Temperature and time	No. of cycles
Initial denaturation	94^0C, 3 min	1 cycle
Annealing & Extension	94^0C, 20 seconds 57^0C, 45 seconds 68^0C, 4 minutes	30 cycles
Hold	68^0C, 10 minutes	1 cycle

SEQ ID NO: 1 shows nucleotide sequence of cDNA that encodes the *Arabidopsis thaliana* mediator complex MED15 protein (4008 bp).

ATGGATAATA ACAATTGGAG GCCTTCTCTT CCAAACGGAG AGCCTGCCAT GGATACAGGT
GACTGGAGAA CGCAATTGCC ACCTGATTCT CGTCAGAAGA TCGTCAACAA GATAATGGAA
ACACTAAAGA AGCACCTTCC ATTTTCCGGA CCAGAGGGAA TTAACGAGCT CAGGAGAATT
GCAGCTAGAT TCGAGGAGAA AATTTTCAGC GGTGCTCTTA ACCAGACTGA TTACCTTCGG
AAAATATCAA TGAAGATGCT GACAATGGAG ACTAAATCAC AAAATGCAGC TGGTTCTTCC
GCAGCTATCC CTGCCGCTAA TAATGGCACA TCCATAGATT CGATACCCAC CAATCAAGGC
CAACTTCTTC CAGGATCGTT ATCAACCAAT CAATCTCAAG CACCTCAGCC GTTGCTGTCC
CAAACCATGC AGAATAATAC TGCCTCTGGA ATGACGGGTT CTACTGCTTT ACCATCTTCC
ATGCCACCTG TTTCTTCCAT AACCAATAAT AACACCACAA GCGTTGTGAA CCAGAATGCC
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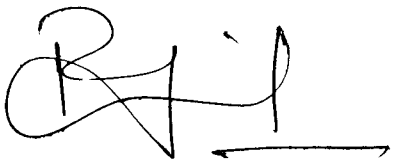
AACATGTTTT CAGGACCCCA AAGGCAGATG CTGGGTAGGC CACATGCTAT GTCTTCACAG
CAACAACAGC AGCCATATCT TTACCAGCAG CAGCTACAGC AGCAACTTCT CAAGCAGAAC
TTCCAGTCAG GGAATGTTCC CAATCCCAAT TCGCTTTTGC CATCACACAT TCAACAACAG
CAGCAAAATG TGCTGCAGCC TAATCAACTG CATTCATCTC AACAACCTGG TGTTCCAACA
TCTGCGACTC AGCCCTCCAC TGTGAACTCA GCTCCTCTCC AAGGTCTCCA CACCAATCAG
CAATCAAGTC CGCAATTGTC TTCTCAGCAG ACGACACAAT CTATGCTTCG TCAGCATCAA
TCGTCGATGC TAAGGCAACA TCCGCAATCA CAACAAGCCT CTGGTATCCA TCAGCAGCAA
TCTTCATTGC CGCAACAATC AATTTCTCCTC TACAGCAGC AGCCTACACA ATTAATGCGG
CAACAAGCTG CAAATAGCTC AGGCATCCAA CAGAAGCAGA TGATGGGGCA GCATGTTGTT
GGGGATATGC AGCAGCAACA TCAGCAAAGG TTACTGAACC AACAAAATAA TGTTATGAAC
ATACAACAGC AGCAGTCGCA GCAGCAACCA CTGCAGCAGC CACAGCAACA GCAGAAACAG
CAGCCACCGG CCCAGCAGCA GTTGATGTCT CAACAAAACA GCCTCCAGGC AACGCATCAG
AACCCACTGG GCACTCAAAG CAATGTTGCA GGATTGCAGC AACCACAACA ACAGATGCTC
AATTCCCAGG TTGGCAATTC GAGTTTGCAG AATAACCAGC ACTCGGTGCA CATGTTATCA
CAACCAACGG TTGGGCTGCA ACGAACACAT CAGGCTGGCC ATGGCTTGTA TTCTTCTCAG
GGACAACAGT CACAAAATCA GCCATCACAA CAGCAGATGA TGCCACAGCT TCAATCGCAT
CATCAGCAGC TGGGTTTGCA ACAACAACCT AATCTGCTAC AACAGGATGT GCAACAAAGG
CTACAAGCTT CAGGCCAAGT CACTGGTTCC CTGCTTCCAC CTCAAAATGT TGTGGACCAA
CAGAGACAAC TATATCAATC CCAAAGAACC CTTCCGGAGA TGCCATCATC ATCGCTGGAT
TCGACAGCAC AGACGGAAAG TGCAAATGGA GGTGATTGGC AAGAGGAGGT TTACCAAAAG
ATCAAATCTA TGAAAGAGAC TTACTTACCA GATCTGAATG AAATCTACCA GAGAGTTGCA
GCAAAGTTGC AGCAAGATTC TATGCCACAG CAACAAAGAT CAGATCAGCT TGAGAAACTG
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ATCATGCCCG CATTAAAAGA TAAGGTGGCT TATTATGAGA AGCAGATTAT AGGTTTCTTA
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CCTATGCAGC AACCACAATC TCAGACAGTT CAAGATCAAT CCCATGATAA CCAAACAAAT
CCGCAGATGC AATCAATGAG CATGCAGGGT GCTGGGCCAA GGGCACAACA GAGTAGTATG
ACAAATATGC AGAGCAATGT TCTATCATCT CGTCCTGGAG TTTCAGCTCC ACAGCAGAAC
ATTCCCAGTT CCATACCGGC TTCTAGTTTA GAATCAGGCC AAGGAAATAC CTTGAACAAT
GGACAGCAGG TTGCCATGGG ATCTATGCAA CAAAATACTT CTCAACTAGT AAATAACAGT
TCTGCCTCTG CTCAAAGTGG GTTGAGCACA CTGCAGTCGA ATGTGAATCA ACCCCAGTTA
AGTTCCAGTT TGCTTCAGCA TCAGCACCTC AAGCAACAGC AAGATCAACA AATGCAGCTC
AAACAGCAAT TTCAACAGCG CCAGATGCAA CAGCAACAGT TGCAAGCAAG ACAGCAACAG
CAACAGCAAC AGTTGCAAGC AAGACAGCAA GCGGCACAAT TACAGCAGAT GAATGATATG
AATGATTTAA CATCGAGGCA GGGGATGAAT GTCAGTCGTG GGATGTTTCA GCAACATTCT

ATGCAGGGTC AGCGTGCGAA TTATCCTCTT CAACAGTTAA AACCAGGAGC TGTTTCGTCG
CCTCAACTTC TGCAAGGTGC ATCTCCTCAG ATGTCACAAC ATTTGTCTCC TCAGGTTGAC
CAGAAAAATA CTGTCAACAA GATGGGAACT CCATTGCAAC CTGCAAATTC CCCTTTTGTT
GTCCCATCTC CTTCTTCAAC CCCCTTGGCT CCGTCCCCTA TGCAAGTTGA CTCTGAGAAA
CCTGGTTCTT CTTCGTTGTC AATGGGAAAT ATTGCACGCC AACAAGCAAC CGGCATGCAA
GGTGTAGTTC AGTCCCTAGC AATTGGCACT CCAGGGATCT CTGCCTCTCC TCTCCTTCAG
GAGTTTACTA GTCCTGATGG GAATATTTTA AATTCTTCGA CAATTACATC TGGAAAACCG
AGTGCTACTG AGCTGCCTAT TGAACGCCTT ATTAGAGCCG TGAAGTCCAT CTCACCACAA
GCCCTTTCTT CTGCAGTAAG TGACATCGGA TCTGTTGTAA GCATGGTTGA TCGGATAGCT
GGTTCAGCAC CAGGAAACGG TTCAAGAGCT TCCGTTGGTG AAGACTTGGT TGCAATGACT
AAGTGCCGTC TCCAAGCAAG AAACTTCATG ACCCAAGAGG GAATGATGGC GACTAAGAAA
ATGAAGCGTC ACACAACCGC AATGCCACTA AGCGTTGCTT CACTGGGAGG AAGTGTTGGT
GATAACTACA AGCAGTTTGC TGGTTCAGAA ACATCCGATC TAGAATCTAC TGCGACTTCG
GATGGCAAGA AGGCAAGAAC TGAGACCGAG CATGCCCTTT TGGAGGAAAT AAAGGAAATA
AACCAGCGGC TGATAGATAC AGTTGTTGAG ATAAGTGATG ATGAAGATGC TGCTGATCCT
AGTGAGGTTG CAATATCAAG CATAGGGTGT GAAGGAACAA CAGTTAGATT CTCGTTTATA
GCTGTTTCTC TCAGCCCAGC CTTGAAGGCT CATCTCTCAT CAACACAAAT GTCTCCTATT
CAACCATTGC GTCTGCTGGT TCCTTGTAGC TACCCCAATG GCTCTCCATC TCTTCTAGAT
AAACTCCCGG TCGAAACCAG CAAAGAAAAC GAGGACCTGT CGTCCAAAGC TATGGCAAGG
TTCAACATAT TGCTAAGAAG TTTGTCACAG CCGATGTCGC TCAAAGACAT AGCCAAGACA
TGGGACGCCT GTGCTCGGGC AGTGATCTGT GAGTACGCAC AGCAATTTGG CGGTGGAACT
TTCAGCTCAA AATACGGCAC TTGGGAGAAA TATGTAGCAG CTTCCTGA

SEQ ID NO: 2 shows amino acid sequence of *Arabidopsis thaliana* mediator complex MED15 protein (1335 aa).

MDNNNWRPSL PNGEPAMDTG DWRTQLPPDS RQKIVNKIME TLKKHLPFSG PEGINELRRI
AARFEEKIFS GALNQTDYLR KISMKMLTME TKSQNAAGSS AAIPAANNGT SIDSIPTNQG
QLLPGSLSTN QSQAPQPLLS QTMQNNTASG MTGSTALPSS MPPVSSITNN NTTSVVNQNA
NMQNVAGMLQ DSSGQHGLSS NMFSGPQRQM LGRPHAMSSQ QQQQPYLYQQ QLQQQLLKQN
FQSGNVPNPN SLLPSHIQQQ QQNVLQPNQL HSSQQPGVPT SATQPSTVNS APLQGLHTNQ
QSSPQLSSQQ TTQSMLRQHQ SSMLRQHPQS QQASGIHQQQ SSLPQQSISP LQQQPTQLMR
QQAANSSGIQ QKQMMGQHVV GDMQQQHQQR LLNQQNNVMN IQQQQSQQQP LQQPQQQQKQ
QPPAQQQLMS QQNSLQATHQ NPLGTQSNVA GLQQPQQQML NSQVGNSSLQ NNQHSVHMLS
QPTVGLQRTH QAGHGLYSSQ GQQSQNQPSQ QQMMPQLQSH HQQLGLQQQP NLLQQDVQQR
LQASGQVTGS LLPPQNVVDQ QRQLYQSQRT LPEMPSSSLD STAQTESANG GDWQEEVYQK

IKSMKETYLP DLNEIYQRVA AKLQQDSMPQ QQRSDQLEKL RQFKTMLERM IQFLSVSKSN
IMPALKDKVA YYEKQIIGFL NMHRPRKPVQ QGQLPQSQMQ PMQQPQSQTV QDQSHDNQTN
PQMQSMSMQG AGPRAQQSSM TNMQSNVLSS RPGVSAPQQN IPSSIPASSL ESGQGNTLNN
GQQVAMGSMQ QNTSQLVNNS SASAQSGLST LQSNVNQPQL SSSLLQHQHL KQQQDQQMQL
KQQFQQRQMQ QQQLQARQQQ QQQQLQARQQ AAQLQQMNDM NDLTSRQGMN VSRGMFQQHS
MQGQRANYPL QQLKPGAVSS PQLLQGASPQ MSQHLSPQVD QKNTVNKMGT PLQPANSPFV
VPSPSSTPLA PSPMQVDSEK PGSSSLSMGN IARQQATGMQ GVVQSLAIGT PGISASPLLQ
EFTSPDGNIL NSSTITSGKP SATELPIERL IRAVKSISPQ ALSSAVSDIG SVVSMVDRIA
GSAPGNGSRA SVGEDLVAMT KCRLQARNFM TQEGMMATKK MKRHTTAMPL SVASLGGSVG
DNYKQFAGSE TSDLESTATS DGKKARTETE HALLEEIKEI NQRLIDTVVE ISDDEDAADP
SEVAISSIGC EGTTVRFSFI AVSLSPALKA HLSSTQMSPI QPLRLLVPCS YPNGSPSLLD
KLPVETSKEN EDLSSKAMAR FNILLRSLSQ PMSLKDIAKT WDACARAVIC EYAQQFGGGT
FSSKYGTWEK YVAAS



Dated this 3rd day of December, 2012

RAJANI JAISWAL
IN/PA-1463
AGENT FOR THE APPLICANT

To,
The Controller of Patents,
The Patent Office, at New Delhi