Trans-activation of small EDRK-rich factor 2 (SERF2) promoter by Heat Shock Factor 1

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**Abstract**

Heat shock response is an adaptive mechanism of cells characterized by rapid synthesis of a class of proteins popularly known as heat shock proteins (HSPs) by heat-induced activation of Heat Shock Factor 1 (HSF1). In course of our earlier study to show that HSF1 regulates transcription of HYPK (Huntingtin Yeast two-hybrid protein K), a chaperone-like protein, we observed presence of few other genes within 10 kb of HYPK promoter. In an attempt to understand whether adjacent genes of HYPK are co-regulated, we identified that SERF2 (small EDRK-rich factor 2), an upstream neighboring gene of HYPK, is also regulated by heat stress and HSF1. We also showed that SERF2 promoter can be trans-activated by HSF1 due to the presence of functional heat shock element (HSE). Strikingly, HYPK is linked with SERF2 through a Conjoined Gene (CG) albeit the respective proteins have opposite effect on mutant Huntingtin aggregates and subsequent toxicity. Our study provides the first report on regulation of SERF2 expression and thereby depicts a paradigm where two parent genes of a CG are regulated by a common transcription factor despite the fact that they code for proteins having opposite cellular function in a given context.

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1. Introduction

Heat Shock Factor 1 (HSF1) is an evolutionary conserved transcription factor capable of inducing transcription of heat shock protein (HSP)-coding genes in response to stress by directly binding to specific DNA sequences (heat shock elements)-a process broadly known as heat shock response [1,2]. We earlier showed that Huntingtin (HTT) interacting protein HYPK is induced by heat shock and transcriptionally regulated by HSF1 [3]. HYPK is an intrinsically unstructured protein [4] and can suppress aggregates of mutant HTT and subsequent toxicity by virtue of its chaperone-like activity [5,6].

The genomic organization of HYPK revealed presence of putative promoter region of two other protein-coding genes viz. SERF2 and SERINC4 within 10 kb upstream or downstream of human HYPK promoter. Intriguingly, a Conjoined Gene (CG) containing exons of SERF2 and HYPK was also identified in the vicinity of HYPK promoter. We attempted to understand whether any of the neighboring genes of HYPK are co-regulated with heat-inducible gene HYPK and subsequently identified SERF2, an upstream neighboring gene of HYPK, as a heat-inducible gene and novel transcriptional target of HSF1. Our study shows that two neighboring genes HYPK and SERF2 are co-regulated at transcription level.

2. Materials and methods

2.1. Antibodies

Anti-HSF1 and anti-acetylated histone H4 antibodies were obtained from Abcam. Anti-\(\alpha\)-RNA polymerase II antibody was purchased from Imgenex and anti-SERF2 antibody was purchased from Novus Biologicals. Anti-\(\beta\)-actin antibody was procured from Sigma. Anti-mouse and anti-rabbit secondary antibodies conjugated with hors eradish peroxidase were purchased from Bangalore Genei (India).

2.2. Cell culture and treatments

HeLa cells were obtained from National Cell Science Centre, India and grown in Minimal Essential Medium (Himedia) supplemented with 10% fetal bovine serum (BioWest) at 37\(^\circ\)C in 5% \(\text{CO}_2\) atmosphere under humidified conditions. To induce heat shock...
response, cells were subjected to standard heat shock treatment at 42 °C for 60 min and kept at 37 °C for 4 h or unless indicated otherwise. Cells were treated with 5 μM 17-AAG (Sigma Chemicals) or DMSO (control) for 24 h. Transfection of cells was performed using Lipofectamine 2000 (Invitrogen).

2.3. Construction of plasmids

Human hsfl gene cloned in pcDNA3.1 vector was kindly gifted by Dr. Richard Voellmy (HSF Pharmaceuticals, Switzerland). Empty pSUPER vector and pSUPER constructs for HSF1 siRNA and scrambled RNA were provided by Dr. L. Sistonen (Åbo Akademi University, Finland).

For luciferase reporter assay, SERF2 promoter encompassing putative HSF1-binding site (−535 to −526) was cloned in pGL3 vector (Promega) between the restriction sites of MilII and BglII (NEB) and designated as SERF2_ups. Primer sequences used for cloning are given in Supplementary Table S1. Promoter sequence (NEB) and designated as hsp70_ups was used as positive control as described previously [3].

2.4. Site-directed mutagenesis

The HSE present in SERF2 promoter was destroyed by mutagenesis. Three conserved nucleotides (‘GAA’ at position −534 to −532) from HSE present in SERF2 promoter were deleted from the parental (wild-type) promoter sequence (SERF2_ups) and the resulting mutated promoter was designated as SERF2_ups ΔHSE. QuickChange site-directed mutagenesis kit from Stratagene was used for mutagenesis. Primer sequences are given in Supplementary Table S2.

2.5. Luciferase Assay

Luciferase assay was performed as earlier described [7,8]. Briefly, HeLa cells expressing empty pGL3 vector or other reporter constructs (600 ng) and empty pcDNA vector or HSF1-pcDNA (400 ng) were either grown at 37 °C (control) or subjected to heat shock at 42 °C for 60 min followed by recovery at 37 °C for 4 h. Twenty four hours after transfection, luciferase assay was carried out using luciferase reporter assay system (Promega) according to manufacturer’s protocol and detected by Sirius Luminometer (Berthold Detection Systems). The experiments were carried out in triplicates.

2.6. RNA preparation

Total RNA was prepared from cultured cells using TRIzol reagent (Invitrogen) following manufacturer’s protocol.

2.7. Semi-quantitative RT-PCR (sqRT-PCR) and Quantitative Real-time PCR (qRT-PCR)

Two microgram of total RNA was reverse transcribed using random hexamer primer and MuLV-reverse transcriptase (Fermentas). Semi-quantitative RT-PCR (sqRT-PCR) was carried out using Red TaqDNA polymerase (Bioline). Quantitative Real-time PCR (qRT-PCR) was carried out using 7500 Real time PCR system (Applied Biosystems) as described in [9]. Fold changes were calculated in accordance with SDS software V2.0 (Applied Biosystems). Primer sequences used for gene expression by qRT-PCR are given in Supplementary Table S3.

2.8. Knockdown of HSF1 by siRNA

The method used for siRNA-mediated knockdown of HSF1 was as described in [10]. Knockdown of endogenous HSF1 in HeLa cells was earlier confirmed by sqRT-PCR and immunoblot [3].

2.9. Western blot

Western blot was performed as described previously [3]. Membrane was probed with anti-SERF2 antibody. β-actin was used as loading control. Integrated optical density (IOD) of each band was calculated using Image Master VDS software (Amersham Biosciences).

2.10. Chromatin immunoprecipitation (ChIP) and Transient ChIP assay

Chromatin immunoprecipitation (ChIP) was performed in HeLa cells as described earlier [3,8]. Briefly, HeLa cells expressing endogenous HSF1 were either grown unstressed (No HS) or exposed to heat shock at 42 °C for 1 h, followed by recovery at 37 °C for 4 h. Then cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped by 125 mM glycine. Cells were washed with ice-cold PBS and harvested at 300g for 3 min at 4 °C. Cytosol was extracted with cytoplasm extraction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA and 1 mM PMSF). Nuclei were harvested at 13,000g for 10 min at 4 °C, and the pellet was resuspended in breaking buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% SDS and 2% Triton-X-100) and sonicated twice (two pulses of 10 s each). Contents were then centrifuged. Triton buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 0.1% Triton-X-100) was added to the nuclear extract. The immunoprecipitation reaction was carried out using anti-HSF1, anti-RNA polymerase II and anti-acetylated histone H4 (AcH4) antibodies followed by the addition of BSA-soaked Protein G–agarose beads. The immunoprecipitated complex was washed, followed by decross-linking, phenol–chloroform extraction and ethanol precipitation of the DNA. Amplification of the eluted DNA was carried out by semi-quantitative (sqRT-PCR) and quantitative RT-PCR (qRT-PCR) using primers specific for human SERF2 promoter. A portion of the genome having no putative HSF1-binding site was amplified along with SERF2 promoter and this non-specific sequence (NS seq) was used as control. PCR amplification of input and immunoprecipitated DNA was carried out using primers flanking the putative HSF1-binding site present in SERF2 promoter. For transient ChIP assay, HeLa cells were transiently transfected with SERF2_ups, SERF2_ups ΔHSE and Hsp70_ups constructs and 24 h after transfection, cells were either kept unstressed or exposed to standard HS treatment. Cells were then cross-linked, harvested and nuclear fraction was prepared and sonicated as described for ChIP assay. Immunoprecipitation was done using anti-HSF1 antibody and protocol used for ChIP assay was followed until elution of input and immunoprecipitated DNA. Next, instead of using primers specific for upstream sequence of human SERF2 and hsp70 promoter for amplification of DNA, a special pair of primers was used. The forward primer was specific for human SERF2 or hsp70 promoter whereas the reverse primer was specific for the firefly (Photinus pyralis) luciferase gene present in the plasmid (pGL3 basic vector) backbone. PCR amplification of input and immunoprecipitated DNA using this primer pair ensures selective amplification of the synthetic or recombinant SERF2 and hsp70 promoter cloned in pGL3 basic vector, leaving native SERF2 and hsp70 promoter in the genome unamplified. Primers used for ChIP and transient ChIP assays are given in Supplementary Table S4.
2.11. Use of databases and web tools

Primers used for cloning were designed using Primer3 software (http://primer3.ut.ee/), whereas primers used for gene expression study (semiquantitative RT-PCR and quantitative Real-time PCR) and Chromatin Immunoprecipitation or Transient Chromatin Immunoprecipitation were designed by online software Primer Express (http://home.appliedbiosystems.com/support/tutorials/taqman/taqman_probes_121502.cfm). Primers used in site-directed mutagenesis were designed by Primer X software (http://www.bioinformatics.org/primexr/). UCSC Genome Browser (https://genome.ucsc.edu/) was used for identifying promoter or other regulatory regions of respective genes. In-house search tool (http://www.bioinformatics.org/grn/npb1) was used for identifying putative HSF1-binding site in SERF2 promoter. All information about conjoined gene was retrieved from ConjoinG database (http://metasystems.riken.jp/conjoing/index).

2.12. Statistical analysis

For statistical analysis, unpaired t test was performed to compare the means of two experimental groups using the software GraphPad QuickCalcs. Error bars indicate ± SD. The statistical significance level between various experimental pairs is indicated (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

3. Results and discussion

3.1. HYPK and its adjacent genes in human genome

In an interesting study conducted on yeast, Arnone et al. observed that among the heat shock genes, immediately adjacent gene pairs often exhibit a tighter degree of transcriptional co-regulation than the genes distantly located across the genome [11]. HYPK is induced by heat shock and transcriptionally regulated by HSF1 [3]. To investigate whether regulation of adjacent genes of HYPK are similar to that of HYPK, we used UCSC Genome Browser (https://genome.ucsc.edu/) and observed presence of three protein-coding genes, namely SERF2, SERINC4 and MFAPI1 within 10 kb of HYPK promoter (Supplementary Figs. S1 & S2). Of these, the putative promoter of MFAPI1, as revealed by UCSC, is beyond 10 kb of HYPK promoter (Supplementary Table S5) and hence it was excluded from further analysis. In addition to these protein-coding genes, one Conjoined Gene (CG) (SERF2-C15orf63) and microRNA (miR-1282) gene were also present in the vicinity (10 kb) of HYPK promoter (Supplementary Figs. S1 & S2). Interestingly, apart from being a neighboring gene of HYPK, SERF2 is also linked to HYPK through the CG SERF2-C15orf53 (Supplementary Figs. S1 & S2). A CG is defined as a gene which gives rise to transcripts by combining at least part of one exon from each of two or more parent genes, which lie in same orientation on the same chromosome and may translate independently into different proteins [12] (Supplementary Fig. S3).

At present, about 800 CGs have been identified in the entire human genome [12; 13; 14; 15]. Two parent genes of the CG SERF2-C15orf63 are SERF2 (‘5′ parent gene) and HYPK (previously known as C15orf63; ‘3′ parent gene). The CG is about 10 kb long and encompasses the entire genomic length of two parent genes viz., SERF2 and HYPK (Supplementary Table S5). From UCSC database, it appears that the putative promoter of the CG overlaps with SERF2 promoter (Supplementary Fig. S1, Supplementary Table S5). Notably, presence of this CG is also documented in ConjoinG, a database of around 800 CGs identified in human [12], having the symbol SERF2-HYPK and ConjoinG ID CGHSA0117 (Supplementary Fig. S5). It was first identified by Akiva and his colleagues [13] without any experimental validation. Later, one of the two transcripts (AK095876) of this CG was reported to be expressed in pancreas [12]. The function and regulation of this CG is yet unknown.

Therefore, SERF2 is an upstream neighboring gene of HYPK and also connected to HYPK via a CG. Intriguingly, an earlier study showed that overexpression of SERF2 increases aggregates of mutant HTT and cell death and knockdown of SERF2 has opposite effect in mammalian cells [16]. On the other hand, HTT-interacting protein HYPK can suppress aggregates of mutant HTT and subsequent toxicity by virtue of its chaperone-like activity [5]. Therefore, SERF2 and HYPK have opposite functional effect on mutant HTT aggregates and toxicity. We attempted to determine whether any of the two neighboring genes (SERF2 and SERINC4) are co-regulated with HYPK.

3.2. Hyperthermia increases SERF2 expression in HeLa cells

To determine the effect of thermal stress on SERF2 and SERINC4 expression, HeLa cells were either grown at 37 °C (control) or were exposed to standard HS treatment (HS at 42 °C for 1 h followed by recovery at 37 °C for different time points). Total RNA was extracted from each sample and analyzed for SERF2, SERINC4 and β-actin expression by quantitative RT-PCR (qRT-PCR).

As evident from Fig. 1A, heat shock-driven induction of SERF2 expression was first detected during 2 h recovery (p = 0.003, n = 3) and continued till 4 h recovery (p < 0.001, n = 3), followed by a significant drop during 6 h recovery (p = 0.002, n = 3). SERINC4, on the other hand, showed no induction in response to HS treatment (Fig. 1B). We used hsp70, a bona fide transcriptional target of HSF1, as a marker of effective induction of heat shock response pathway. As expected, HS treatment resulted in robust induction of hsp70 in HeLa cells (Fig. 1C), suggesting that the treatment was adequate to induce heat shock response in host cells. Notably, upon same HS treatment, HYPK expression was significantly induced in HeLa cells [3]. Perplexingly, SERF2 and HYPK exhibited similar response in heat-shocked cells in spite of the fact that the respective proteins have opposite effect on mutant HTT aggregates and toxicity. We failed to detect the expression of the CG in unstressed and stressed HeLa cells (data not shown), possibly due to its faster degradation or tissue-specific expression.

To determine whether the effect of hyperthermia on SERF2 transcript holds true also at the protein level, SERF2 expression was measured by immunoblot in all unstressed and stressed samples described above. As presented in Fig. 1D, no significant change in SERF2 protein level was observed during heat shock and subsequent recovery time. It is difficult to explain this observation at this stage of our knowledge. We speculate that the basal SERF2 level might be sufficient for its yet unexplored function(s) in stressed cells. Further investigation on in vivo functions of SERF2 and regulation of its metabolism in cells is required to draw definitive conclusions.

3.3. Identification and functional validation of Heat Shock Element (HSE) present in SERF2 promoter

Our observation that SERF2 is transcriptionally induced by heat shock motivated us to investigate the molecular mechanism underlying such induction. To understand whether HSF1, the evolutionary conserved transcription factor and master regulator of heat shock proteins, has any effect on SERF2 expression, SERF2 promoter was searched for presence of any putative HSE using the search tool described earlier [17]. Our search identified a putative HSE...
AGAAGGGTCG at −535 to −526 region of human SERF2 promoter. To verify the functionality of this HSE, part of SERF2 promoter encompassing the HSE was cloned in pGL3 reporter vector and designated as SERF2_ups. A mutant SERF2 promoter was also generated by deleting two consensus nucleotides of HSE (‘G’ at position −534 and ‘A’ at position −533) by mutagenesis and the mutant promoter was designated by SERF2_ups_ΔHSE. A part of hsp70 promoter carrying multiple functional HSEs (designated as Hsp70_ups) was used as a positive control as also used earlier [3,7].

HeLa cells were transfected with empty pGL3 vector (control), SERF2_ups, SERF2_ups_ΔHSE and Hsp70_ups and cells were either grown at 37 °C or were allowed to undergo standard HS treatment. Luciferase assay performed 24 h after transfection clearly showed induced reporter activity of SERF2_ups construct (p = 0.006, n = 3) whereas SERF2_ups_ΔHSE remained unaffected by thermal stress (Fig. 2A), indicating that heat shock could specifically induce SERF2 promoter fragment. To determine the direct effect of HSF1 on SERF2 promoter, HeLa cells were transfected with aforementioned reporter constructs together with empty pcDNA vector (control) or HSF1-pcDNA and luciferase activity of reporter constructs was measured in presence of standard HS treatment. Result showed that ectopic HSF1 upon heat shock could increase reporter activity of SERF2_ups (p = 0.005, n = 3) in HeLa cells (Fig. 2B). The mutant promoter (SERF2_ups_ΔHSE) having deleted HSE showed no response to ectopic HSF1 (Fig. 2B). Not surprisingly, reporter activity of Hsp70_ups was induced in response to thermal stress as well as exogenous HSF1 (Fig. 2A and B). Our finding thus showed that SERF2 promoter fragment containing HSE is responsive to both heat shock as well as exogenous HSF1. It should be noted here that we cloned about one third of the 1600 bp long SERF2 promoter as predicted from UCSC Genome Browser. No other putative HSE was identified in the entire SERF2 promoter; however, it can be trans-activated by other transcription factors through distinct regulatory elements located anywhere in the entire SERF2 promoter.

We earlier reported that human HYPK promoter is similarly trans-activated by HSF1 in response to same HS treatment in HeLa cells [3]. However, both thermal stress and ectopic HSF1 had stronger effect on HYPK promoter (induced by ~4.6 and ~4.3 fold respectively) [3] compared to SERF2 promoter (~2.6 and ~2.4 fold respectively) (Fig. 2, A and B). This quantitative difference in the extent of induction of two promoters is possibly due to the difference in the composition of two HSEs present in respective promoters. The HSE responsible for heat-induced activation of HYPK promoter is composed of three tandem copies of the pentameric motif (nGAAn) [3], whereas the HSE present in SERF2 promoter contains two copies of the consensus motif which is considered as the minimal requirement for inducible binding of HSF1 [18, 19]. This might have contributed to the weaker induction of SERF2 promoter compared to HYPK promoter in response to thermal stress. Since expression of a single gene is often regulated...
by the interplay of many transcription factors and co-factors, the quantitative difference between induction of HYPK and SERF2 promoter could also be due to other regulatory proteins. To further understand the regulation of SERF2 promoter by HSF1, we performed chromatin immunoprecipitation (ChIP) in HeLa cells in presence or absence of HS treatment. As demonstrated in Fig. 2, C and D, heat shock increased the occupancy of HSF1 (p = 0.008, n = 3) in the HSE present in SERF2 promoter. It was accompanied by simultaneous increase in histone H4 acetylation (AcH4) at the same site. Therefore, functionality of the HSE present in SERF2 promoter is induced by HSF1 in heat shock-dependent manner and HSF1-mediated transactivation of SERF2 promoter involves chromatin remodeling.

3.4. In vivo interaction of HSF1 with recombinant SERF2 promoter

To further confirm the specificity of in vivo interaction of endogenous HSF1 with its cognate binding site in SERF2 promoter,
we performed transient ChIP assay. HeLa cells were transiently transfected with SERF2_ups, SERF2_ups_ΔHSE and Hsp70_ups constructs and twenty four hours after transfection, cells were harvested in absence or presence of HS treatment. Anti-HSF1 antibody was used for immunoprecipitation and the immunoprecipitated DNA was amplified using forward primer specific for 5'-end of SERF2 or hsp70 promoter and reverse primer specific for luciferase gene present in vector backbone (downstream of the multiple cloning site). This ensures selective amplification of synthetic or recombinant SERF2 promoter (SERF2 promoter cloned in pGL3 basic vector) leaving native SERF2 promoter in the genome unamplified. As presented in Fig. 3, occupancy of endogenous HSF1 in recombinant or synthetic SERF2 promoter increased significantly in response to hyperthermia in HeLa cells. As evident from Fig. 3, mutant SERF2 promoter (SERF2_ups_ΔHSE) showed no occupancy of endogenous HSF1 even in presence of heat shock whereas recombinant hsp70 promoter showed heat shock-driven increased occupancy of HSF1. Therefore endogenous HSF1 could interact in vivo with recombinant SERF2 promoter and deletion of putative HSE by mutagenesis abolished this interaction. Transient ChIP assay thus strengthened our conclusion from luciferase reporter assay by showing that the truncated SERF2 promoter cloned in reporter vector is sufficient to recruit endogenous HSF1 to its cognate binding site which subsequently leads to heat shock-dependent trans-activation of SERF2 promoter by HSF1. This result also shows that despite being composed of two pentameric (nGAAn) repeats, the HSE present in SERF2 promoter is sufficient for HSF1-mediated trans-activation of SERF2 promoter.

3.5. Effect of HSF1 loss-of-function and gain-of-function on SERF2 expression

We next attempted to determine the effect of HSF1 on SERF2 expression. To understand the effect of HSF1 knockdown, HeLa cells were transfected with HSF1-specific siRNA as described earlier [3]. Upon knockdown of endogenous HSF1, no significant change in endogenous SERF2 level was observed (Fig. 4A). We then wanted to investigate the effect of HSF1 knockdown on heat shock-driven induction of SERF2. HeLa cells transiently expressing empty pSUPER vector or HSF1-siRNA containing pSUPER vector or scrambled RNA-containing pSUPER vector were either grown at 37 °C (control) or were exposed to standard HS treatment and SERF2 and β-actin expression was measured by qRT-PCR. Unlike HeLa cells expressing empty vector and scrambled RNA, cells expressing HSF1-siRNA showed no significant induction in SERF2 expression.
level in response to HS treatment (Fig. 4B). We conclude that knockdown of HSF1 has no effect on endogenous SERF2 expression, however; it abolishes the ability of host cells to induce SERF2 expression in response to thermal stress. Importantly, the observation that endogenous SERF2 expression was not affected by knockdown of HSF1 suggests that HSF1 may not have any effect on basal SERF2 expression as we thought earlier. Therefore, the interaction of endogenous HSF1 with SERF2 promoter in unstressed HeLa cells, as depicted in Fig. 2C, possibly has no functional outcome and recapitulates earlier observation made by Trinklein and his colleagues [18].

Our final aim was to check the effect of HSF1 activation on SERF2 expression. 17-AAG is a small molecule activator of HSF1 and has been shown beneficial in the context of many proteinopathies primarily because of its ability to boost the expression of multiple heat shock proteins [21]. HeLa cells were either treated with DMSO (control) or 17-AAG and SERF2 expression was measured by qRT-PCR. As presented in Fig. 4C, 17-AAG treatment augmented SERF2 expression significantly (p = 0.007, n = 3) in HeLa cells. Therefore, both loss-of-function and gain-of-function studies revealed that SERF2 expression is regulated by HSF1. It is noteworthy that SERF2 protein level was found unaltered in response to 17-AAG treatment in HeLa cells (data not shown) which is in line with the observation that thermal stress had no significant effect on SERF2 protein level (Fig. 1D). Therefore, both thermal stress and 17-AAG were effective in upregulating SERF2 expression only at transcription level.

To conclude, this is the first report focusing on regulatory mechanism of poorly characterized protein SERF2. Our findings clearly show that SERF2 expression is transcriptionally regulated by HSF1 and SERF2 promoter is trans-activated by HSF1 in heat shock-dependent manner by virtue of a functional HSE. To the best of our knowledge, this also represents existence of a unique CG whose two parent genes are regulated by a common transcription factor despite the fact that proteins translated from these two parent genes have opposite cellular function in a given context.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.04.003.

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