

Isolation and Characterization of the Iron-Regulated *MxbHLH01* Gene in *Malus xiaojinensis*

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Abstract Some of the genes involved in iron signaling in *Malus xiaojinensis* have been isolated and characterized; however, their regulatory control is yet to be elucidated. In this study, rapid amplification of complementary DNA ends was used to obtain a full-length cDNA fragment of the *MxbHLH01* gene encoding a basic helix-loop-helix (bHLH) protein. This protein shared 35.31% amino acid sequence identity with *AtFRU* and 31.88% amino acid sequence identity with *LeFER*. Real-time quantitative PCR revealed that *MxbHLH01* was upregulated in roots grown under iron-deficient conditions. In addition, the *MxbHLH01* protein was localized within the nuclei of plant cells and incapable of activating transcription in yeast. This indicated that *MxbHLH01* might act as an iron regulator, but requiring other proteins to form heterodimer(s) to regulate gene expression response to iron deficiency.

Keywords *Malus xiaojinensis* · RACE ·
Basic helix-loop-helix · Iron

Abbreviations

RACE Rapid amplification of complementary DNA ends
bHLH Basic helix-loop-helix
PCR Polymerase chain reaction
ORF Open reading frame
PAS Per-Ah receptor nuclear translocator/Ah receptor-Sim
AA Amino acids

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CTAB Cetyl trimethylammonium bromide
eGFP Enhanced green fluorescent protein
DAPI 4',6-Diamidino-2-phenylindole
UAS Upstream activating sequence

Introduction

During long-term evolution, plants have developed complex molecular mechanisms to survive harsh environments. Transcription factors play important roles in stress survival by serving as master regulators of sets of downstream stress-responsive genes. Important families of transcription factors include ethylene-responsive element-binding factor (Liu et al. 2011), dehydration-responsive element binding (Wang et al. 2010), and basic helix-loop-helix (bHLH) transcription factors (Meng et al. 2009), among others.

The bHLH domain-containing proteins contain several highly conserved domains, and those proteins are structurally heterogeneous. The bHLH domain, an ancient component of transcriptional regulation, typically comprises a stretch of roughly 18 hydrophilic and basic amino acids at the N-terminus of the domain, followed by two regions of hydrophobic residues predicted to form amphipathic α -helices separated by an intervening loop (Murre et al. 1994). Structural analyses of mammalian and yeast bHLH proteins show that the bHLH domain is involved in DNA binding and protein oligomerization (Jones 2004). The position of bHLH within the complete sequence of the protein varies widely between different families, and the variable pattern of domain positioning has led to the proposal that bHLH proteins have undergone modular evolution by domain shuffling, a process that involves

domain insertion and rearrangement (Morgenstern and Atchley 1999). The bHLH proteins containing this domain have broad functions in the formation of root hair (Yi 2008), the control of petal size (Szécsi et al. 2006), the anthocyanin biosynthetic pathway regulation (Quattrocchio et al. 1998), and the responses to environmental factors, such as iron deficiency, in plants.

Iron is a cofactor of several ubiquitous proteins that participate in crucial metabolic pathways in all living organisms. This essential role is supported by known disorders resulting from its deficiency, including severe anemia in mammals or chlorosis in plants (Briat 1999). Plants have developed two strategies to secure iron uptake in roots (Römheld and Marschner 1986). Non-graminaceous monocots and dicots mobilize iron through iron reduction (strategy I) as opposed to graminaceous plants that utilize a phytosiderophore-based iron chelation mechanism (strategy II). Strategy I responses include rhizosphere acidification, ferric reduction, and iron transportation via divalent iron transporters. The process is tightly regulated by iron status in plants, and *Malus xiaojinensis* is no exception. *M. xiaojinensis* is the first iron-efficient genotype in the genus *Malus*. In classical breeding, the use of rootstock genotypes efficient in iron acquisition would provide a permanent and economical solution to the problem of iron deficiency chlorosis. Furthermore, transgenic approaches can also offer good opportunities to achieve this goal.

Similar to many other complex biological processes, the attack responses to iron deficiency involve transcriptional activation or repression of a large number of genes in *M. xiaojinensis*. Our group has cloned and studied some important genes to elucidate iron deficiency response. Gene encoding iron transporter gene *MxIRT1* (Qi 2003) has been obtained from *M. xiaojinensis*, and the transcription of the *MxIRT1* gene is induced and strengthened by iron stress in the roots. Additionally, the high-affinity iron transporter gene *MxNRAMP1* (Qi 2003), which shares significant similarity with the counterparts of *Arabidopsis thaliana*, has been cloned from *M. xiaojinensis*. The *MxMyb1* gene involved in iron metabolism has also been isolated from the Fe-deficient root cDNA expression library of *M. xiaojinensis* (Cao 2003). To date, although some important genes involved in iron acquisition and uptake have been characterized at the molecular level, little is known about the transcriptional control mechanisms of these iron-responsive genes in *M. xiaojinensis*.

The first description of a putative transcription factor involved in iron acquisition in plants is the LeFER protein in tomato (*Lycopersicon esculentum*). Cloning of the *LeFER* gene reveals that it encodes a bHLH putative transcription factor (Ling et al. 2002). Both *LeFER* and its orthologue of *AtFRU* from *A. thaliana* are expressed in the

roots in iron deficiency signaling pathways (Jakoby et al. 2004). Cloning of these key genes with putative roles in iron signaling can be important clues to unravel the nature of the sensor(s) and downstream targets at the molecular level.

In this study, a novel *MxbHLH01* gene is isolated from *M. xiaojinensis* using rapid amplification of complementary DNA ends (RACE), and mRNA levels of this gene have been determined. Moreover, transactivation of this gene is investigated in yeast. The *MxbHLH01* gene is found to be regulated at the transcriptional level, but depending on the iron nutritional status. The *MxbHLH01* protein is a nuclear protein; however, this protein alone has no transcriptional activity in yeast cells. Thus, its action as a transcription factor may require an additional protein-binding partner.

Materials and Methods

Plant Material and Growth Conditions

The plants were grown hydroponically, as described previously (Han et al. 1994). The plants were incubated at a light intensity of 1,500 lx and 85% relative humidity, and the culture condition was set to 16-h light/8-h dark and 22°C/20°C cycles. At the 10- to 12-leaf stage, the plants were transferred into solution with different iron concentrations: 0 μM FeNaEDTA for limiting iron supply conditions and 40 μM FeNaEDTA for sufficient iron supply. The roots were collected after 12 h (0.5 day), 1-, 3-, 6-, and 9-day treatment, immediately frozen in liquid nitrogen, and stored at -70°C until needed.

RNA Sample Preparation and First-Strand cDNA Synthesis

Total RNA samples, free from DNA contamination, were extracted from the roots via the modified CTAB method (Gasic et al. 2004), and the integrity of each total RNA sample was verified by running samples on 1% agarose gels. The concentration of each RNA sample was checked using a UV spectrophotometer. Total RNA was reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, WI, USA) using oligo(dT) 18-containing primer to obtain the first-strand cDNA.

Gene Isolation

Three pairs of gene-specific primers were used to obtain the fragments of *MxbHLH* cDNA, and F1 and R1 were used to amplify the entire coding sequence. The sequences of primers were shown in Table 1. 3'- and 5'-RACE methods were performed according to the kit (Takara, Japan). The polymerase chain reaction (PCR) products were purified

Table 1 Primers used in *MxbHLH* gene isolation

Primer name	Sequence (5'–3')
P1	GACTGAYCGGTCYAGRACTTTGAT
P2	TGCATCTCCAAYRATGGA
(3'-RACE GSP1)	AGAGAGGAAGAGGAGG GGGAGAATG
(3'-RACE GSP2)	TTGATGTCAGAGAGGAAGAGGAGGG
(5'-RACE GSP1)	GATGCTTCAAGGTTTGCAATCTCAC
(5'-RACE GSP2)	GTACCTCTACTTGTCCCTTGTTC
F1	GAAAGTCATAACATCGACAGCTCTG
R1	TCACTGAAGGGTAATTAGGATTGTA

and cloned into the Peasy-T1 simple vector (Transgen, China), followed by sequencing (Sangon, China). The entire coding sequence named *MxbHLH01* was then deposited at GenBank and analyzed for further study.

Bioinformatic Analyses

Sequence alignment and analyses were carried out with the BLAST network service of NCBI (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic tree of the alignment sequences was constructed via the DNAMAN program, version 5.2.2. The amino acid sequences of other bHLH proteins used for comparison were downloaded from GenBank: LeFER (AAN39037), AtbHLH029 (FIT1/FRU, ABH04553), OsIRO2 (FAA00382), AtbHLH038 (NP191256), AtbHLH039 (NP191257), AtbHLH100 (NP850349), and AtbHLH101 (NP196035).

Subcellular Localization of the MxbHLH01 Protein

Transient expression of enhanced green fluorescent protein (eGFP) was used to confirm the localization of MxbHLH01 protein. The open reading frame (ORF) of *MxbHLH01* was amplified using primers 5'-ATAGAATTCAAATGATCAATGGCTG-3' and 5'-CCGTCGACCCAGGATTGTAAAATT-3'. The amplified fragment was subcloned into the *EcoRI* and *SalI* sites of PEZS-NL vector. The resulting plasmid was introduced into onion epidermal cells with a particle bombardment device (Biolistic PDS-1000/He, Bio-Rad). Excised onion cells were microscopically detected with a confocal microscope (Nikon Eclipse TE2000-E).

Quantitative Real-Time PCR

Approximately 1 µg of total RNA was treated with Dnase and reverse-transcribed into cDNA using the oligo-(dT) primer. Quantitative measurement of *MxbHLH01* expression levels was performed using an ABI 7500 Real-Time PCR System. The samples were subjected to thermal

cycling conditions at 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The total reaction volume was 20 µL, with 0.2 µM final concentrations of primers. The primers were 5'-ATTATAACCTGTTCCGGTCCAGCTAGT-3' and 5'-TGACACCAAAGTTCGTGACCG-3'. Real-time PCR analysis was performed with two different cDNAs from the same time point (from two different RNAs), and each was carried out in triplicate. The amplification was detected using the SYBR Green I fluorescence dye (Takara). The slope of the standard curve was maintained to ensure maximum reaction efficiency for every PCR cycle. 18 s, "reference g gene," was used as a control.

Transactivation in Yeast

Expression of the MxbHLH01 protein in yeast was performed according to the manufacturer's kit (Stratagene, La Jolla, CA USA). The full-length *MxbHLH01* coding region was cleaved with *EcoRI/SalI* and subcloned into a yeast expression vector pBD-GAL4 to generate the MxbHLH01:BD plasmid. Primers were 5'-ATAGAATTCAATGGCTG-3' and 5'-CCGTCGACCCGAGGATTGTAAAATT-3'. The verified plasmid was then transformed into the yeast strain PRG-2, and colonies were selected by SD medium lacking tryptophan and then on SD medium without histidine. The obtained colonies were assayed for *LacZ* reporter gene activation using 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside. Yeast cells transformed with the empty BD vector were assayed in parallel and used as control.

Results

Isolation of the *MxbHLH01* Gene

Total RNA samples were extracted from the roots; RNA samples were hardly decomposed and had high purity. Through the 3'- and 5'-RACE experiment, two fragments, 522 and 612 bp, were respectively amplified. Using the PCR method, the full-length cDNA fragment of *MxbHLH01* gene was identified (Fig. 1). The 702-bp ORF encoded a protein of 234 AA. The full-length cDNA sequence of *MxbHLH01* was deposited to the GenBank database under accession number HQ889726.

Alignment and Phylogenetic Tree Analysis

Comparison of the full-length nucleotide sequence of *MxbHLH01* with those of *LeFER* and *AtFRU* showed 49.01% and 49.32% similarity, respectively (data not shown). At the amino acid level, the alignment of

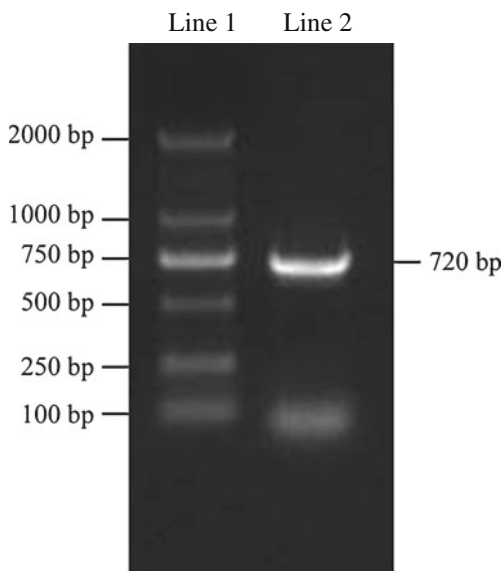


Fig. 1 Full-length PCR product of *MxbHLH01* gene. Lane 1 showed DNA marker: DL2000. Lane 2 showed the product of *MxbHLH01*

MxbHLH01 and other bHLH proteins indicated that *MxbHLH01* shared 35.31% identity with *AtFRU* and 31.88% with tomato *LeFER* (Fig. 2).

Based on the amino acid alignments of eight bHLH proteins, a phylogenetic tree was constructed. The clustering showed that there were two groups (subfamilies) of related sequences. The phylogenetic relationship set revealed that *MxbHLH01*, *AtFRU*, and *LeFER* clustered together in compact clades with high support values. Another group containing *OsIRO2*, *AtbHLH38*, *AtbHLH39*, *AtbHLH100*, and *AtbHLH101* was clearly distinct from the group containing *MxbHLH01*, *AtFRU*, and *LeFER* (Fig. 3).

Subcellular Localization of the *MxbHLH01* Protein

Images were captured 24 h after transient expression in onion cells under a confocal microscope. Either empty

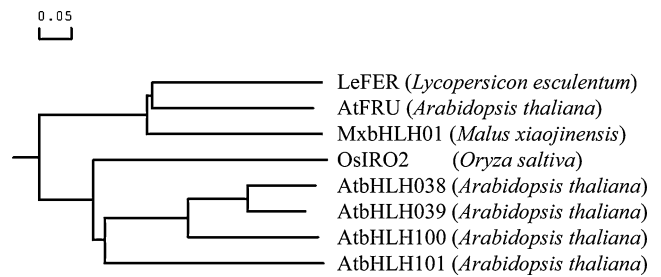


Fig. 3 Phylogenetic tree for *MxbHLH01* and other bHLH proteins in plants. The studied proteins had the following accession numbers: *LeFER* (AAN39037), *AtbHLH029* (FIT1/FRU,ABH04553), *OsIRO2* (FAA00382), *AtbHLH038* (NP191256), *AtbHLH039* (NP191257), *AtbHLH100* (NP850349), and *AtbHLH101* (NP196035)

PEZS-NL or *MxbHLH*:PEZS-NL fusion protein under the control of the 35S promoter of cauliflower mosaic virus was transformed into onion epidermis cells. The fluorescence of empty PEZS-NL accumulated in the plasma membrane (Fig. 4a), whereas the *MxbHLH01*:PEZS-NL fusion protein was expressed in the nucleus (Fig. 4b). Finally, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nucleus (Fig. 4c, blue).

Expression Pattern of the *MxbHLH01* Gene

To investigate the relationship between transcription regulation of *MxbHLH01* and iron availability, real-time quantitative PCR was conducted. Using a time course study, wherein roots were transferred to an iron-deficient medium for 0, 0.5, 1, 3, 6, and 9 days, induction of *MxbHLH01* was observed in these roots (data not shown); moreover, an upregulated expression pattern of *MxbHLH01* was detected in roots over different periods of time (Fig. 5). Transcripts of the *MxbHLH01* gene increased at 12 h (0.5 day), then decreased, and subsequently began to increase and reached peak levels on day 6, and then dropped once again by day 9 (Fig. 5).



Fig. 2 Amino acid alignment of *A. thaliana* *AtFRU*, *L. esculentum* *LeFER*, and *M. xiaojinensis* *MxbHLH01* proteins. Black shading indicated identical amino acid positions, whereas gray shading

indicated similar residues. The basic (b), helical (H), and loop (L) regions of the bHLH domain were highlighted by different boxes

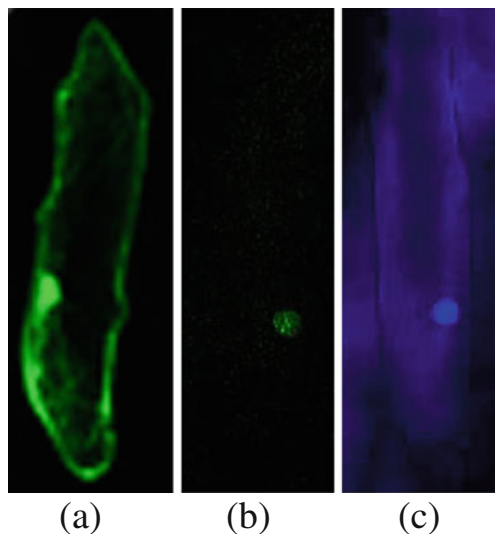


Fig. 4 Subcellular localization of MxbHLH01 protein. **a** Subcellular localization of empty PEZS-NL protein in onion cells. **b** Subcellular localization of MxbHLH01:PEZS-NL fusion protein in onion cells. **c** Onion cell stained with DAPI

Transactivation in Yeast

To test whether the MxbHLH01 protein alone initiated the expression of its target gene, we transformed yeast expression vector containing the *MxbHLH01* coding sequence under the control of GAL4 BD promoter into yeast strain PRG-2. Fusion protein of MxbHLH01:BD expressed in yeast cells on the SD medium lacking tryptophan (figure not shown) and the obtained colonies were identified by growth after 2–3 days on the SD medium without histidine (Fig. 6a). However, the filter lift assay of the X-Gal activity of yeast strains showed that the transformants of the MxbHLH01:BD fusion plasmids did not turn blue (Fig. 6b)—that is, the MxbHLH01:BD fusion protein was incapable of promoting the activity of the *LacZ* reporter gene in yeast—while the colonies of the BD+ plasmid turned blue (Fig. 6b).

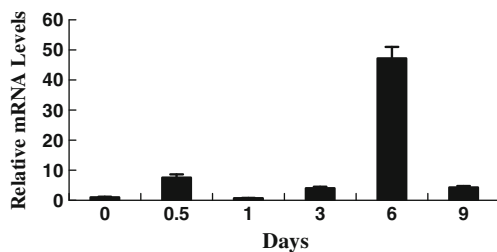


Fig. 5 Real-time quantitative PCR expression pattern of *MxbHLH01* in the roots. The vertical axis showed the relative gene expression ratio. The number of days after initiation of Fe deficiency was shown on the horizontal axis

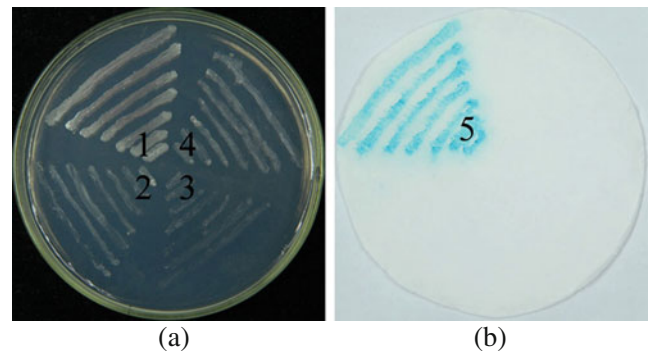


Fig. 6 Transactivation experiment in yeast. **a** Histidine prototrophy assay on histidine-free SD plate. **b** Filter lift assay of X-Gal activity of yeast strains grown on histidine-free SD plate. Numbers 1–4 indicated plasmid combinations as follows: 1 BD+, 2 BD:MxbHLH01, 3 BD–, as a negative control, 4 BD:MxbHLH01. Transcription activation was visualized by a positive *LacZ* assay (blue colonies). 5 shows BD+, as a positive control, which activated the *LacZ* reporter gene

Discussion

Rapid amplification of cDNA ends is a technique used in molecular biology to obtain the full-length sequence of genes found within a cell in recent years (Yin et al. 2010; Duan et al. 2009). The novel *MxbHLH01* gene was obtained using the RACE method. The bHLH domain sequences of *MxbHLH01* were almost identical with that of *AtFRU* and *LeFER*, both of which were proposed as transcriptional regulators functioning in iron deficiency responses and iron uptake. The MxbHLH01 protein contained the typical T-E-R at positions 5, 9, and 13, which was similar to LeFER and AtFRU. The motif of T5-E9-R13 was bound to a variation of the E-box hexanucleotide sequence (E-box:CANNTG; Heim et al. 2003; Jakoby et al. 2004), whereas 53% of the bHLH proteins, in plants, had the characteristic structure H5-E9-R13 (Pires and Dolan 2010). Furthermore, the conclusions from phylogenetic trees showed that MxbHLH01 closely grouped together with LeFER and AtFRU. All of the information gave support to the indication that MxbHLH01 was a protein belonging to the subfamily of LeFER-related bHLH-type proteins.

The presence of the 35S promoter-MxbHLH01-eGFP signal revealed that the fusion protein was expressed in the nucleus, consistent with its role as a regulator in terms of protein localization pattern. Expression analysis of the *MxbHLH01* showed that it was restricted to the roots, similar to the *AtFRU* and *LeFER*. RT-PCR expression analysis of the *LeFER* gene showed that its action was restricted to the roots where it might control iron mobilization from the soil into the roots.

Quantitative real-time polymerase chain reaction, a precise method to measure changes in the gene transcription level, has been widely used in recent years (Remans et al. 2008; Phillips et al. 2009; Tai et al. 2009; Qi et al. 2010).

The upregulated expression pattern of the *MxbHLH01* gene was similar to that of *AtFRU* accumulating to high levels under iron-deficient conditions in roots (Jakoby et al. 2004), but this pattern was different from that of *LeFER*, which was reported to be independent of the iron supply (Ling et al. 2002). The *MxbHLH01* gene was first upregulated and then increased with a peak expression ratio on day 6. It may well be that the first increase was mainly due to the local environment of the root and not to the overall nutrient status of the plant. However, after 6 days of withholding iron from the roots, the second increase was probably the result of the root sensing fluctuations in external iron availability, so it monitored the time course of changes in gene expression under iron-deficient conditions (Wang et al. 2002). Given the unbalance between the requirement for life-sustaining processes from respiration to photosynthesis and insufficient iron supply, it seems likely that the decrease of *MxbHLH01* gene expression at day 9 was due to iron starvation. Similarly, some other genes, such as *MxIRT1* and *MxNRAMP1* (Qi 2003), were coordinately regulated in response to iron according to blotting analysis, indicating that the sixth day of iron deficiency was likely a very essential time for *M. xiaojinensis* to induce some gene response to iron deficiency. In addition, the groups of genes with similar expression patterns might be controlled by some kind of iron regulatory mechanisms present in *M. xiaojinensis*.

The bHLH proteins are members of a large family of diverse transcription regulators that form homodimers or heterodimers to regulate gene(s) expression. The structure of a dimer is stabilized by the hydrophobic amino acids isoleucine, leucine, and valine in conserved positions in the bHLH domain (Ferré-D'Amaré et al. 1993), and the positions are highly conserved in plants. For example, a leucine residue is present in site 23 in 99% of the plant proteins (Pires and Dolan 2010). Consistent with the view is the observation that the typical leucine residue in site 23 of the bHLH domain was also found in *LeFER*, *AtFRU*, and *MxbHLH01* proteins. The result of yeast one-hybrid was an indication that binding of the *MxbHLH01*:BD plasmid to the UAS was not sufficient to initiate transcription of the reporter gene; that is, *MxbHLH01* might interact with other proteins to form heterodimer(s) to regulate gene responses to iron shortage. Similarly, the interaction between *AtFRU*, *AtbHLH38*, and *AtbHLH39* directly functions in controlling the transcription of the iron uptake genes *AtFRO2* and *AtIRT1* as well as the iron homeostasis of *Arabidopsis* (Yuan et al. 2008). There is definitely a need to identify whether other proteins combine with *MxbHLH01* to regulate iron homeostasis by controlling key genes, such as *MxFRO* and *MxIRT1*, in *M. xiaojinensis*. In addition, upstream of *MxbHLH01* protein, an iron sensor

that is unaffected by iron status but is able to sense iron levels and communicate this message through the activation or repression of downstream targets may also be found in our future research.

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