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Establishment of marker-recycling method in filamentous fungi and development of an *Aspergillus aculeatus* multi-auxotrophic host

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Research aims

Bioprocess for converting cellulosic substances to liquid fuels, so-called 'bioethanol', is becoming more important and getting closer to the stage for industrial use, even though saccharification of cellulosic substances is still mostly achieved by sulfate treatment. Since enzymatic saccharification is more desirable than the acid degradation procedure from the viewpoint of environmental advantage, it is expected that enzymatic saccharification at industrial scale will be put into practice in future. However, the economical cost for cellulase production prevents the industrialization of enzymatic saccharification because their activity is not sufficiently high yet. To overcome this problem, we need to establish a high-production system for cellulases, as well as to improve properties of cellulases through protein engineering and to elucidate the synergistic effects among cellulases with different properties.

A. aculeatus, a filamentous fungus isolated from soil, produces a number of extracellular cellulose- and hemicellulose-degrading enzymes which shows synergism with *Trichoderma* cellulases. It was shown that alkali-treated rice straw was hydrolyzed almost completely into monosaccharide by the mixed culture filtrate of these two strains[1,2]. Meanwhile, we constructed a host-vector system of *A. aculeatus*, and the *A. aculeatus* β-mannosidase gene was expressed by the system. The *A. aculeatus* expression system produced a 10-fold higher level of the enzyme than the *A. oryzae* expression system, indicating the high potential of *A. aculeatus* as a factory for production of useful proteins[3,4]. On the other hand, the concerted action of several cellulases with different properties is considered to be essential for the effective hydrolysis of cellulose into monosugar. Thus, we aim to breed a 'Super Mold' that simultaneously produces many types of cellulases, which should be useful for cost reduction of enzyme production. For that purpose, a host strain carrying multiple selection markers for transformation must be constructed by gene disruption. However, knock-out of a target gene is not so easy in filamentous fungi because DNA repair by nonhomologous end-joining (NHEJ) occurs prior to homologous recombination. Recently, it was demonstrated that gene disruption of Ku70/80 or Lig4 gene, which are involved in NHEJ event, resulted in the elevated frequency of homologous recombination[5–9]. In this context, the aims of this study are: i) gene disruption of Ku70/80 or Lig4 in *A. aculeatus*, ii) development of marker-recycling method in filamentous fungi, and iii) construction of a multi-auxotrophic *A. aculeatus* host strain.

Methods

A *pyrG*-deficient mutant (pyrG13, uridine auxotroph), spontaneously isolated from the *A. aculeatus* wild type by selection for resistance to 5-fluoroorotic acid (5-FOA)[3], was transformed with a DNA fragment designed for disruption of *ku80* homolog gene (*Aaku80*), and transformants grown on the uridine-free plate were obtained (Fig. 1-a).

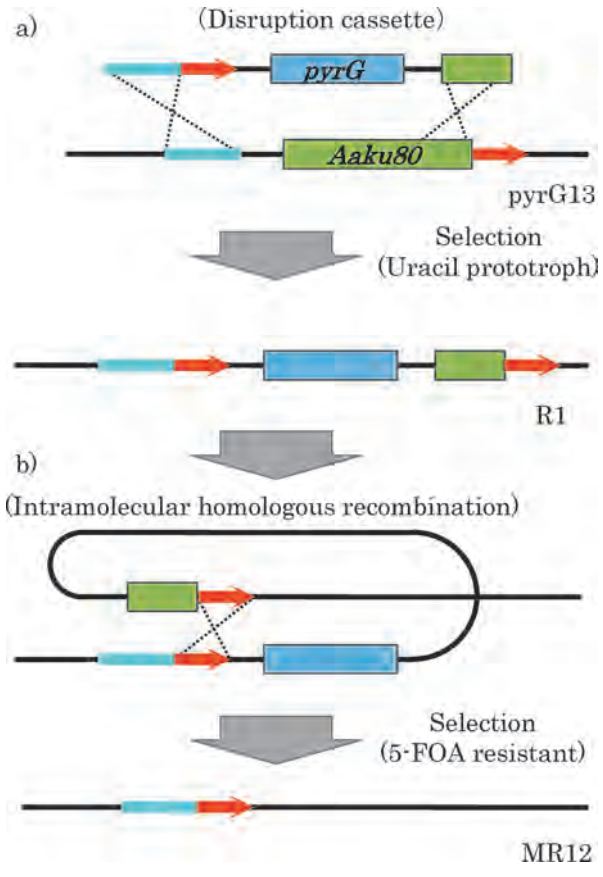

Fig. 1. Disruption of *Aaku* gene and marker-recyling method.

Out of these transformants, a strain of which the *Aaku80* locus was replaced by a single copy of the disruption cassette via double crossover recombination was identified by Southern blot analysis (R1). Then, conidia of the strain were spread on a plate containing 5-FOA, and one of 5-FOA resistant colonies obtained was confirmed as the *Aaku80* disruptant (MR12), in which the *pyrG* gene was excised by intramolecular homologous recombination between direct repeat (DR) (Fig. 1-b). The effect of the *Aaku80* disruption on the gene-targeting efficiency was assessed by performing the disruption of the *argB* locus.

An additional mutant gene (*argB*$^-$) was successfully introduced into the *argB* locus of the MR12 strain (*Aaku80*$^-$, *pyrG*$^-$) by the marker-recycling method and an *A. aculeatus* host strain carrying double selection marker for transformation was established. Also, effects of the DR length on the excision frequency were examined by comparing the occurrence rates of 5-FOA resistant derivatives from each transformant into which one of the three disruption cassettes with the different length of DR (20, 98, and 495 bp) was introduced.

Results

Disruption of *Aaku80* gene and recycling of *pyrG* marker: The pyrG13 was transformed with the *Aaku80*-disruption cassette and 40 transformants were obtained by the selection on a uridine/uracil-free selection medium. Out of these transformants, one strain (R1) in which one copy of the disruption cassette was integrated via double homologous recombination at the *Aaku80* locus was identified by Southern blot analysis. Then, we tried to excise the *pyrG*-containing fragment sandwiched by a pair of DR in order to reuse *pyrG* as a selection marker according to the method commonly used for *Saccharomyces cerevisiae*[10]. Conidia (about 10^4) were spread onto a 5-FOA-containing plate and 20 to 30 colonies, which should be 5-FOA resistant, appeared on the plate after 10 days cultivation. Southern blot analysis showed that the expected excision event through intramolecular homologous recombination occurred in the more than 90% of clones examined. Also, it was confirmed that the 5-FOA resistant clones were uridine/uracil-auxotrophs. One of these 5-FOA resistant clones (MR12) was used for further experiments.

Characteristic of *Aaku80* disruptant: Growth and sporulation of MR12 (*Aaku80*$^-$, *pyrG*$^+$) were indistinguishable from those of pyrG13 (*Aaku80*$^+$, *pyrG*$^+$). Meanwhile, growth of MR12 on 0.5% methyl methanesulfonate (MMS) was poorer than that of pyrG13. This finding was in good agreement with the previous observations that *ku* disruption increased sensitivity against the DNA-damaging reagent in *N. crassa*[5] and *A. fumigatus*[9].

Gene-targeting efficiency at the *argB* locus in *Aaku80* disruptant: The strains pyrG13 and MR12 were transformed with the *argB* disruption cassette carrying complete *pyrG* gene as a selection marker within *argB* coding region and the first selection was done on the uridine/uracil-free plate. One strain out of 32 transformants from pyrG13 found to be an arginine auxotroph (targeting frequency; 3%), while 29 out of 31 transformants from MR12 were shown to be arginine auxotrophs (94%). Southern blot analysis showed that all the arginine auxotrophic clones derived from MR12 had a single copy of the disruption cassette at the target locus, suggesting that integration of the cassette took place by homologous recombination via double crossover.

Consturuction of *pyrG, argB* double-auxotrophic host and effect of the DR length on intramolecular homologous recombination: Three types of *argB* disruption cassettes with different length of DR (20, 98, and 495 bp) and the recycling-marker of *pyrG* were constructed and introduced into MR12 to yield 15, 10, and 20 uridine/uracil-prototrophic transformants, respectively. Out of these transoformants, 11, 10, and 17 clones were arginine auxotrophs, respectively. Then, three clones carrying a single copy of each disruption cassette were chosen and named mrTA1-14, which carries 20-bp DR, mrTA2-1, which carries 98-bp DR, and mrTA3-6, which carries 495-bp DR, respectively. Firstly, 10^6 conidia of mrTA3-6 were spread on a plate containing 5-FOA, uridine, and arginine and ten colonies were obtained. All of the ten clones were found to be uridine/uracil-auxotrophs. Thus, it was concluded that an *A. aculeatus* host strain with double selection markers for transformation was successfully constructed. Moreover, the occurrence frequencies of the 5-FOA resistant clones from mrTA1-14, mrTA2-1, and mrTA3-6 were estimated to be 5.7×10^{-6}, 1.4×10^{-5}, and 1.2×10^{-4}, respectively. These results indicate that the frequency of precise excision event increases as the length of DR increases.

Conclusion

In this study, it was shown that the gene targeting efficiency was drastically elevated by the *ku* disruption in *A. aculeatus*, as shown for many other filamentous fungi. Also, it was demonstrated that the marker-recycling technique commonly used in yeast can be applied to filamentous fungi. Once a mutant with a two-way (positive and negative) selectable marker like *pyrG* is isolated, marker-recycling enables consecutive gene disruption. Though it was reported that 20-bp is the sufficient length of DR for intramolecular homologous recombination in yeast, it has been supposed that longer DR is required for homologous recombination in filamentous fungi. However, this study indicated that correct excision events via homologous recombination can occur even with 20-bp DR in *A. aculeatus*, although the frequency is relatively low. Our findings that DRs as short as 20 bp were functional for promoting correct excision via homologous recombination should be useful in designing disruption cassettes for marker-recycling in filamentous fungi. We are now constructing an *A. aculeatus* host strain with multi-selectable markers by the method developed in this study and breeding the ‘Super Mold’ producing a number of different-type cellulases to hydrolyze cellulosic substances with high efficiency.

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