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Review Article

Application of PCR and MAS: Potential Use for Assessment of Genetic Diversity of Rice Germplasm in Breeding Programmes in Developing Countries

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ABSTRACT

Molecular characterisations of genotypes give precise information about the extent of genetic diversity, which assists in the development of an appropriate rice breeding programme. The latest approach in plant biotechnology and molecular breeding, which is the development of the polymerase chain reaction (PCR) for amplifying DNA, DNA sequencing and data analysis, is an effective technique that can be used for the screening, characterisation and evaluation of genetic diversity. Traits that serve as genetic markers are by definition polymorphic; the more polymorphic the trait, the greater its potential value to germplasm management. The issue of homology may seem insignificant for morphological markers, but the increasing use of molecular markers has heightened its importance. Application of molecular markers is still prohibitively expensive for most large-scale applications in rice breeding programmes, where performance parameters such as yield, quality, disease resistance and other desirable growth characteristics are upgraded. Therefore, marker assisted selection (MAS) methods are currently used for more targeted applications in order to keep up with the rising demand for rice consumption. Since conventional breeding methods will not be able to meet the satisfactory harvest, the application of biotechnological tools is one plausible option to tap into the significant yield potential of rice.

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INTRODUCTION

Rice is the most important staple food in Asia. More than 90% of the world's rice is grown and consumed in Asia, where 60% of the world's population live. Rice accounts for between 35-60% of the caloric intake of three billion Asians (Guyer *et al*., 1998). The two cultivated rice species is Oryza sativa, which is widely grown in Asia and other countries, and O. glaberrima, which is grown only in Africa. O. sativa evolved from perennial or annual type of O. rufipogon and diversified into two subspecies; indica and japonica (Oka, 1974; Chang, 1985). Ancient indica and japonica diversified at approximately 200,000~440,000 and 86,000~200,000 years ago, according to the nuclear genome and chloroplast DNA sequence, respectively (Ma & Bennetzen, 2004; Vitte *et al*., 2004). Over 150 million hectares of rice are planted annually, covering about 10% of the world's arable land. In 1999/2000, this amounted to some 600 million tonnes of rice seeds, equal to 386 million tonnes of milled rice. With the world's population estimated to increase from 6.2 billion in the year 2000 to about 8.2 billion in the year 2030, the global rice demand will rise to about 765 million tonnes, or 533 million tonnes of milled rice (FAO, 2002). For almost three decades since the Green Revolution, the rice yield growth rate was approximately 2.5% per year. During the 1990s, however, this decreased to only 1.1% (Riveros & Figures, 2000).

The aims of this review are to summarise the basic knowledge concerning the PCR based molecular markers to rice breeding and to explore the use of MAS in rice breeding programmes aimed at improving new varieties in this species. Various advantages and disadvantages, as well as uses of the molecular markers relative to other molecular marker types and importance of MAS for developing countries like Pakistan, Vietnam, Sri Lanka and Indonesia where rice is consumed as staple food are also discussed.

Rapid application of molecular markers has played an increasing role in rice breeding and genetics during last few decades. Of the different types of molecular markers, microsatellites have been utilized most extensively because they can be readily amplified by PCR and the large amount of allelic variation at each locus. Diversified molecular markers have been used to classify DNA polymorphism and are generally categorised as hybridization based markers and PCR based markers. DNA profiles are visualised by hybridising the restriction enzyme-digested DNA to a labelled probe, which is a DNA fragment of known origin or sequence. PCR-based markers engage in vitro amplification of particular DNA sequences or loci by specifically or arbitrarily chosen primers and a DNA polymerase enzyme which is thermostable. The amplified fragments are separated electrophoretically and banding patterns are visualised by using various methods viz. ethidium bromide or silver staining and autoradiography (Collard *et al*., 2005).

The application of molecular markers has risen as a productive and dominant access for augmenting traditional plant breeding techniques for the present day rice breeding programmes in China, Thailand and Japan. Series of molecular markers are now accessible viz. Restriction fragment length polymorphism (RFLP) which is based on Southern blot hybridization and Random amplified polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR)/ Microsatellites, Amplified Fragment Length Polymorphism (AFLP) and Cleaved amplified polymorphic sequence (CAPS) markers, which are based on PCR. The AFLP and CAPS markers comprise pre- and post-amplification restriction digestion. Single nucleotide polymorphism (SNP) is the latest marker system that utilises the vast DNA sequence resources available in different rice varieties. The most important characters for an appropriate molecular marker should be: a) polymorphism, b) co-dominant inheritance, c) random and frequent distribution throughout the whole genome, d) easy and cheap to detect, and e) reproducibility.

Polymorphic DNA markers are especially useful for divulging differences between individuals of the same or different species and also interpreted as co-dominant or dominant where markers can segregate between homozygotes and heterozygotes. In SSR, co-dominant markers illustrate the differences in the size of the alleles, while dominant markers display the presence or absence of an allele. In a single individual plant, there are only two alleles per locus for codominance and when referred to population codominance, there is a possibility to have more than two alleles for each locus (Collard *et al*., 2005). When considering the RAPD marker (dominant marker), there are only two maximum alleles both for the individual and population, which are referred to as the present and absence of loci.

PCR is a highly advantageous tool due to the simplicity of the procedure to set up and run and the minimal requirement of the amount of genomic DNA samples. This DNA can be isolated from the early stage of rice leaf tissue. PCR is a very time efficient procedure where millions of DNA copies can be made in a minimum of two hours. In particular, PCR allows the processing of many samples in a very short time with automated and robotic assistance where many small leaf tissue samples are collected from different individual plants. Finally, amplified products can be easily visualised on an agarose or acrylamide gel. These sample DNA can be PCR to determine the presence or absence of a particular marker of interest for interested trait. Only those tested positive would be kept for the next plant breeding stage. PCR based markers may either be allele specific (SSR, SNP) or allele unspecific (RAPD, AFLP). RAPD and AFLP are multi-loci markers that use one random primer (RAPD) or primer pairs (AFLP) and generate a number of marker loci in one PCR reaction. It can be used to screen and select plants for the required characteristics in any plant breeding programmes. Plants can be screened using one of the most suitable

PCR based methods for the presence of a particular gene of interest, and only those tested positive would be kept for the next plant breeding stage. At any given stage of the breeding programme, the testing can be repeated for screening purposes.

Plant breeders have been instrumental in the process of domestication and improvement of rice. After pollination, screening of the new genetic variant is the success of plant breeding. Today, rice plant breeders have the capabilities of altering the performances of some existing crop varieties by using targeted approaches of genomic research. To date, there is an urgent need to utilise new tools to assist in the screening of new genotype efficiently.

Available rice genomic information on genes and gene functions is very important for plant improvement through biotechnological applications. The two major biotechnological applications improve rice genome, viz. (a) application of molecular markers to screen and select favourable genetic combinations, (b) application of genetic engineering (cisgenics/transgenics) to introduce interested foreign genes. These molecular markers help to screen the naturally occurring genetic variation within a species more efficiently. The association among the markers and phenotype needs to be established first and these markers can be used for indirect selection thereafter. MAS in a plant breeding context involves scoring indirectly for the presence or absence of a desired plant phenotype or phenotypic component based on the sequences or banding patterns of molecular markers located in or near the genes controlling the phenotype.

Breeders attempt to overcome limitations to rice yield by improving yield, resistance to pests and diseases, and adaptability to diverse growing conditions through breeding programmes and development of new rice varieties. MAS is an indirect selection process where a trait of interest is selected not based on the trait itself but on a marker linked to it (Ribaut & Hoisington, 1998).

MAS (also 'marker-assisted breeding' or 'marker-aided selection') is based on the concept that it is possible to infer the presence of a gene from the presence of a marker that is tightly linked to the gene. The use of genetic markers as a reliable tool for the plant breeder was recognized by Sax in 1923. However, its application was largely hindered by the lack of suitable markers and the non-availability of detailed genetic linkage maps. The rapid development of molecular techniques has opened up sources of genes to plant breeding that were not available previously through conventional breeding (Allen, 1994). With the help of molecular markers, plants can be screened at seedling stage to screen out multiple traits that would ultimately be epistatic with one another, and minimise linkage drag and rapidly recover the recurrent parent genotype; these are just a few attractions of MAS in plant breeding (Tanksley *et al*., 1989).

Genetic diversity estimation, high density genome maps construction, mapping and tagging of genes, map-based isolation

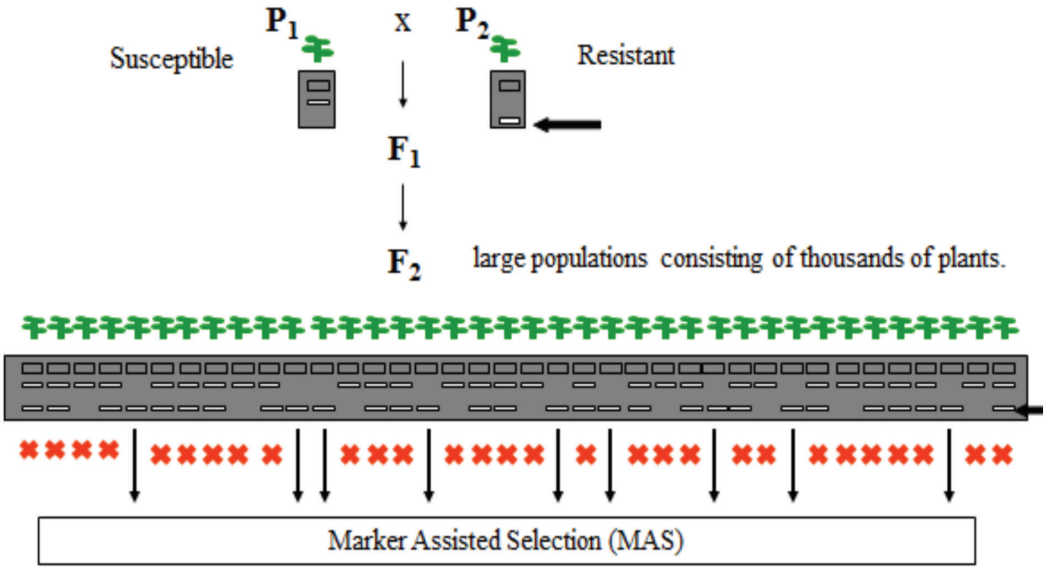
of genes and MAS are some aspects of molecular crop breeding. Genes that have been closely linked to molecular markers in rice are able to combine with conventional breeding approaches where MAS can be used to scan the existence or non-existence of these genes in breeding populations. MAS is done to quickly recover recurrent parent genome in backcross breeding and transfer the gene/s of interest from one genetic background to another using the closely linked markers.

Selecting plants in a segregating progeny that contains appropriate combinations of genes is a critical component of plant breeding (Ribaut & Betran, 1999; Weeden *et al*., 1994). Crop breeders work with huge populations, specifically ones that contain large number or hundreds or even thousands of crop plants (Ribaut & Betran, 1999; Witcombe & Virk, 2001). Thus, MAS has a great influence in plant breeding compared to conventional breeding methods that increase efficiency and effectiveness. Once markers that are closely linked to genes or quantitative trait loci (QTL) of interest have been identified prior to field evaluation of a large number of plants, breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTLs (Michelmore, 1995; Ribaut *et al*., 1997). The presence or absence of a molecular marker is used as a substitute for or to assist in phenotypic selection in MAS (see Fig.1) (Collard *et al*., 2005).

In summary, there are four major schemes of MAS: a) Marker-assisted backcrossing (MAB), b) pyramiding, c) early generation selection, and d) combined approaches.

MAB has several advantages over conventional backcrossing that include effective selection of target loci, minimising linkage drag and accelerated recovery of recurrent parent. Gene pyramiding is widely used for combining multiple disease resistance genes for specific races of a pathogen. It is important to note that gene pyramiding is extremely difficult to be achieved using conventional methods which consider phenotyping a single plant for multiple forms of seedling resistance. Gene pyramiding is almost impossible but it is also important to develop 'durable' disease resistance against different races. In early generation of MAS, it conducted at F2 or F3 generations and plants with desirable genes/QTLs were selected and alleles could be 'fixed' in the homozygous state, while plants with undesirable gene combinations could be eliminated. This is an advantage for later stages of breeding programme because resources can be used to focus on fewer lines (Fig.1). In some cases, a combination of phenotypic screening and MAS approach may be useful to maximise genetic gain when some QTLs have been unidentified from QTL mapping and level of recombination between marker and QTL and to reduce population sizes for the traits where marker genotyping is cheaper or easier than phenotypic screening.

For example, marker-assisted backcross breeding can be used to integrate crucial genes with significant biological effects into a number of commonly grown rice varieties.



A susceptible (S) parent is crossed with a resistant (R) parent and the F1 plant is self-pollinated to produce a F2 population. In this diagram, a robust marker has been developed for a major QTL controlling disease resistance (indicated by the arrow). By using a marker to assist selection, plant breeders may substitute large field trials and eliminate many unwanted genotypes (indicated by crosses) and retain only those plants possessing the desirable genotypes (indicated by arrows). Note that 75% of the plants may be eliminated after one cycle of MAS. This is important because plant breeders typically use very large populations (e.g., 2000 F2 plants) derived from a single cross and may use populations derived from hundreds or even thousands of crosses in a single year.

Fig.1: MAS scheme for early generation selection in a typical breeding programme for disease resistance (Adapted from Ribaut & Betran, 1999)

The use of cost-effective, MAS strategies and finely mapped microsatellite markers should provide different opportunities for breeders to develop high-yield, selected trait rice cultivars.

Genetic diversity assessments of the traditional rice varieties, landraces are very essential component in germplasm characterization and conservation to identify potential parents for future breeding perspective. Morphological and seed traits have long been the means of studying taxonomy and variability among plant species. Microsatellites/ SSR are the most widely used DNA marker for many purposes such as diversity studies, genome mapping, varietal identification, etc. (Teixeira da Silva, 2005). Molecular markers are not stressed by environmental factors and growth practices as the morphological and biochemical markers (Ovesna *et al*., 2002). Application of these markers to investigate genotypic variations among different cultivars has previously been reported by some researchers (Singh *et al*., 2004; Joshi & Behera, 2006).

The knowledge of cereal genetics figures out of the structure and behaviour

of cereal genomics which are influenced rapidly with the advancement of molecular techniques. The present molecular genetics techniques and especially utilization of molecular markers help to scan the DNA sequence variation in and among the species and create new sources of genetic variation by introducing new favourable traits from landraces and related grass species. Markers linked to useful traits have enabled great advances for crop molecular breeding during recent years with the improvement of marker detection systems and in the techniques (Korzun *et al*., 2001).

Time consuming and environmental conditions are the main constraints of conventional cereal breeding. It usually takes between eight to twelve years for conventional rice breeding to take place, and even then, the release of an improved variety cannot be guaranteed within that time period.

Hence, molecular marker technology offers such a possibility by adopting a wide range of novel approaches to improving the selection strategies in cereal breeding (Korzun *et al*., 2001).

The genetic diversity of rice germplasm was assessed by the use of RFLP, RAPD and SSRs molecular markers. SSRs have been frequently used in genetic and breeding research because of the relatively high allelic polymorphism and easy genotyping by PCR. Moreover, approximately 20,000 SSR markers were mined from the genome sequence of japonica cv. Nipponbare and are publicly accessible (IRGSP, 2005). Mainly SSR markers have been extensively used in evaluating the genetic diversity of wild relatives, landraces, and cultivars of rice (Ram *et al*., 2007; Pusadee *et al*., 2009). However, each marker system has both advantages and disadvantages (Table 1).

Identification of markers linked to useful traits has been based on complete linkage maps and bulked segregant analysis. However, alternative methods such as construction of partial maps and the combination of pedigree and marker information have also been proven to be useful in identifying marker/trait associations. A revision of the current breeding methods by utilising molecular markers in breeding programmes is therefore crucial in this phase (Korzun *et al*., 2001).

During domestication, some key agronomy traits such as grain shattering, grain dormancy and grain size were strongly selected, which led to greatly diminished genetic diversity in rice. The rice genome encountered a severe early domestication bottleneck; thus, landraces represented only small proportion of the genetic variation of wild rice (Kovach & McCouch, 2008). Moreover, modern breeding programmes continuously select desirable characters under highly controlled conditions to achieve an ideotype which exacerbates the reduction in gene pool of cultivars (McCouch, 2004).

In order to breed new varieties that can endure the effects of global climate changes, the gene pool of cultivars must be broadened by introducing wild species, landraces and exotic germplasm into the breeding programmes. Wild species that are resistant

TABLE 1
Advantages and disadvantages of most commonly used PCR based DNA markers

Markers type	Codominant or Dominant	Advantages	Disadvantages
RAPD	Dominant	More rapid No radioactive labelling Genomic abundance high Better genome coverage / Multiple loci from a single primer possible Does not need prior sequence information Perfect for automation Requires less DNA Inexpensive	No need of probe information Dominant markers Not reproducible Not well tested
SSR	Codominant	Easy to automate Genomic abundance high Highly reproducible High polymorphism Multiple alleles Moderately genome coverage No radioactive labelling Robust and reliable Highly reproducible Transferable between populations Require low amount of DNA Does not need high quality DNA	Not well examined Cannot suitable across species Sequence information needed Development of primer pairs is considerably expensive and time consuming Require prior sequence information Usually require polyacrylamide gel electrophoresis
AFLP	Dominant	High polymorphism Multiple loci detected Does not need prior sequence information Generate high levels of polymorphism High reproducibility Genomic abundance high Can be used across species Useful in preparing counting maps Works with smaller RFLP fragments	Very tricky due to changes in materials used Not reproducible Very good primers needed Complicated procedure Require large amount of DNA

to biotic and abiotic stresses are an important genetic resource (Khush, 1997). However, the incompatibility of the wild species with cultivars delimits the introgression of wild species' genes to cultivars (Brar & Khush, 1997). Landraces whose seeds are maintained by farmers still contain useful genes (Jackson, 1997; Pusadee *et al*., 2009). Many genes conferring resistance to abiotic and biotic stresses, viz. salinity, rice stripe virus, and rice blast, are preserved and used in modern breeding programmes (Shi *et al*., 2010).

There are many advantages of MAS over other screening techniques. A concise list of the most important features of MAS has been provided by Collard et al. (2005), Xu and Crouch (2008) and Koebner and Summers (2003).

a. It saves time especially during the substitution of complex field trials (experiments conducted at specific time period of year or at specific locations) with molecular tests.

b. It eliminates phenotypic evaluation associated with field trials due to environmental effects. These molecular markers are not products of translation; therefore, they are not affected by any environment factor.

c. It has the ability to screen extremely difficult, expensive or time consuming (score phenotypic score) traits (e.g., root morphology, resistance to quarantined pests / specific races / biotypes of diseases / insects, tolerance for certain abiotic stresses such as drought, salt, mineral deficiencies and toxicities).

d. Selection of genotypes at seedling stage. It is very efficient as organogenesis does not have to be completed; traits that are expressed later in the lifecycle of a plant (e.g., grain or fruit quality, male sterility, photoperiod sensitivity).

e. Gene pyramiding or simultaneous multiple genes combine effect.

f. Escapes transferring of undesirable genes ('linkage drag'; this is of particular relevance when the introgression of genes from wild species is involved). A common problem in a conventional breeding programme is when integrated genes are often found to be linked with other undesirable gene(s). This linkage drag can be avoided in MAS programme where flanking markers closely linked to the gene of interest are used to identify crossover break points. Therefore, unwanted recombinants within the target locus can be rouged out further to reduce the chances of selecting undesirable genotypes which leads to linkage drag. This strategy effectively and efficiently reduces population size and the time required to fix the genes of interest in the desired genetic background.

g. Low heritable trait selection.

h. Possibility to diagnose specific traits when phenotypic screening is not applicable (e.g., quarantine pathogens).

i. Ability to screen homozygous and heterozygous genetic makeup of many loci in a single generation by avoiding progeny evaluations (since molecular markers are codominant).

Rapid identification of individuals that contain complementary parts of a complex character by RFLP, SSR or SNP-tagged QTLs is allowed in the marker-aided selection. These individuals often oppose accurate phenotypic identification due to the complex gene interaction that may govern the trait of interest (Yamamoto *et al*., 2000). Therefore, molecular markers are better than morphological and biochemical markers and have great use in molecular breeding.

DISCUSSION

One crucial step for successful breeding programmes is the genetic diversity of the genotypes. The information of genetic diversity can be used to devise the best strategies for logical utilisation of genetic resources within and among closely related crop varieties. The analysis of genetic diversity within and among varieties is important for breeders because it can help to assess the variation in the germplasm and also predict potential genetic gains (Chakravarthi & Naravaneni, 2009).

Rice breeding has been upgraded by using the molecular markers system. In MAS, individuals carrying target genes are selected in a segregating population based on linked markers rather than on their phenotype. In MAS, the population can be screened at any growth stage and in various environmental conditions. Efficiency of a breeding programme can be increased by selecting markers which linked to target traits or QTLs (e.g., pest and diseases resistance, high yielding). The problem in relation to QTL is crucial. Identifying QTL/s for a particular phenotype is difficult due to polygenic characters.

Thus, molecular markers are powerful tools in basic and applied research for analysing genetic diversity within and among varieties. There are different molecular markers which are based on polymorphism of protein or DNA (Schnable *et al*., 2009). Molecular markers which can show differences between accessions at DNA level are more direct and reliable, making them ideal tools for germplasm conservation and management.

The success of MAS in a plant breeding programme depends on the following important factors:

i. co-segregation or tight linkage of markers (< 5 cM) with the desired trait,

ii. efficient, user-friendly, cost effective means to screen large populations for the molecular marker(s), and

iii. high reproducibility of the screening technique across laboratories.

Rice germplasm which are linked with very important agronomic traits can be improved and upgrade by using molecular markers system in efficiently and in an accelerated development program. However, considering the effort and expense of DNA marker analysis, it is important that the MAS program itself be as efficient as possible. The foundation of any plant-breeding

program is its germplasm collection, and it is important that it be well characterized so that the breeder can improve chances of success in developing lines for commercial release. With the impetus in recent years of using exotic cultivars and landraces to broaden the breeders' germplasm base (Eizenga et al., 2006) this characterization has become even more important. Data from genotyping the parental material make the MAS program more efficient by determining not only which current cross populations would benefit from marker analysis, but also which breeding lines to use as parents in future crosses and which cross combinations to make.

There are major possible reasons to fail MAS in rice breeding in Sri Lanka, Pakistan and Vietnam. One main reason is the lack of resources (scientists, skilled labors, chemicals, laboratory, and equipment and funds). Skilled scientists migrate to developed countries for better opportunities and the remaining scientists are located near big cities where fields are too far away for easy access. Most of these markers may not be cost-effective and poor integration between molecular genetics and conventional breeding has a big impact. Essential concepts of breeding may not be understood by molecular biologists and conventional breeders are not thorough enough with newly developed molecular techniques. Therefore many unforeseen issues arise at breeding stations regarding the transition from conventional breeding and molecular breeding aspects in developing countries like Sri Lanka. Mainly analyzing of generated data and result interpretation are crucial if a rice breeder doesn't thorough with molecular genetics and statistics. Therefore existing rice breeders are extremely interested in traditional technologies that could make this procedure more in-efficient.

Cost is a major obstacle and prohibitive in MAS. MAS are more expensive than conventional methods for most traits. Detailed study of cost-efficiency has rarely been studied or calculated. Cost is mainly determined by the interest trait or traits and method of phenotypic screening cost (greenhouse, field trials), cost of genotypic methods (equipment, consumables, and other laboratory facilities) and labour costs (field technicians, research fellows). Unfortunately in most cases, funding will largely determine the extent to which markers should use in breeding. The questions that should be answered would be which traits should get the highest priority for marker development and how to explore the advantage and importance of the molecular breeding over the conventional breeding by minimizing the costs and increase efficiency of MAS furthermore.

In conclusion, it is understood that MAS allows selection of rice plants at the juvenile stage at very early generation and pyramiding of different resistance genes. In addition, MAS provides opportunities to breeders to develop broad-spectrum of very valuable and interesting new rice varieties such as pest and disease resistance, high yielding with dominant characters (colour, taste, size, shape, etc.). PCR method, which

requires small amount of DNA, is becoming a very useful technique for screening large populations of segregating progenies for simply and complexly inherited traits, where unwanted genes can be eliminated or greatly minimized at the early stages of rice breeding. This would cut down the cost, time and labour of breeding in an efficient and effective way and eventually leads to a prosperous agricultural lifestyle in developing countries.

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