

## REVIEW

# Molecular Markers in the Improvement of *Allium* Crops

LAKSHMANA REDDY DHORANALAPALLI CHINNAPPAREDDY, KIRAN KHANDAGALE,  
ASWATH CHENNAREDDY and VEERE GOWDA RAMAPPA

*Indian Institute of Horticultural Research, Bangalore, India*

### Abstract

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The genus *Allium* (Family: *Alliaceae*) is the most important among the bulbous vegetable crops. Characterization of *Alliums* based on phenotypic traits is influenced by the environment and leads to biased diversity estimates. Recognizing the potential of DNA markers in plant breeding, researchers have adopted the molecular markers for marker-assisted selection (MAS), quantitative trait loci (QTL) mapping and characterization of different quality traits in *Alliums*. This review presents details about the use of DNA markers in *Alliums* for cultivar identification, diversity studies, SSR development, colour improvement, total soluble solids (TSS), cytoplasmic male sterility (CMS) and efforts of DNA sequencing. As there are no such reports to describe the above work under a single heading, we decided to mine literature for those who are working in onion, garlic, chives and leek improvement to generate new insights in the subject.

**Keywords:** cytoplasmic male sterility; diversity; garlic; onion; quantitative trait loci; simple sequence repeat

The genus *Allium* comprises more than 750 crop species; notably onion, garlic, chives, and leek are widely distributed in the northern temperate and Alpine regions of the world. Historical and cultural significance of *Alliums* has been well documented in an ancient scripture in *Garuda Purana*, where it is regarded as aphrodisiac food (BASAK 1987) in India.

The cultivated *Alliums* e.g. bulb onion (*A. cepa* L.), Japanese bunching onion (*A. fistulosum* L.), leek (*A. ampeloprasum* L.), chives (*A. schoenoprasum* L.) are seed propagated. Over 90% of the species from Eurasia and Mediterranean region have the basic chromosome number eight (diploid bulb onion has  $2n = 2x = 16$  and tetraploid leek  $2n = 4x = 32$ ) and over 95% of the species from North America have

the basic chromosome number 7 ( $2n = 14$ ) (VED BRAT 1965). Almost all *Allium* species possess a symmetrical median to submedian centromeric chromosomes with relatively small size differences, although a few species possess a telocentric chromosomes. The guanine plus cytosine (GC) content of onion DNA is 32%, the lowest known for any angiosperm (STACK & COMMINGS 1979).

According to the United Nations Food and Agriculture Organization (UNFAO, <http://faostat3.fao.org/faostat-gateway/go/to/download/Q/QC/E>), 175 countries grow onions on estimated 6.7 million acres with production of 47.5 billion tonnes. The leading onion production countries are China, India, United States, Turkey and Pakistan. The advantage

of onion diversity has not been fully exploited but with the liberalisation of policies multinational companies have entered in hybrid onion development (KHAR *et al.* 2010). Here we have reviewed the use of molecular markers for diversity analysis, varietal identification, colour and quality improvement, male sterility analysis and recent efforts of genome sequencing in *Alliums*.

### Need of DNA marker technology

Characterization and grouping based on phenotype are influenced by environmental variations; molecular markers are preferred because of polymorphic nature, co-dominance, selective neutral behaviour, easy and fast assay, high reproducibility and easy exchange of data between laboratories (JOSHI *et al.* 1999). A molecular marker is a DNA sequence that is readily detected and whose inheritance can easily be monitored. There are different marker systems available for crop plants such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), microsatellite or simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), sequence characterized amplified region (SCAR), cleaved amplified polymorphic sequences (CAPS) and single nucleotide polymorphism (SNP) etc. (SEMAGN *et al.* 2006).

In spite of advances in sequencing technologies, sequencing of onion remains a huge challenge because of its 16.4 giga base genome size. It has one of the largest nuclear genomes among all diploid eukaryotes (ARUMUGANATHAN & EARLE 1991). The use of genetic markers in *Alliums* has increased several-fold with the development of different marker systems mentioned briefly above.

### Diversity analysis and varietal identification of *Alliums*

For diversity analysis, RAPD and ISSR markers are widely used by horticulturists due to their low cost, simplicity and no need of prior sequence information. AFLP is one of the powerful techniques used for diversity analysis. It combines both RFLP and PCR, therefore it is more specific, gives a large number of bands and allows higher genome coverage.

The knowledge of genetic diversity helps in the efficient management of germplasm and selection

of parents for crossing. The integrity of inbred lines was studied using RAPD (BRADEEN & HAVEY 1995). Diversity analysis of seven cultivars of *A. cepa* and single cultivar of Japanese bunching onion, chive, leek and a wild relative of onion (*A. roylei*) by RAPD showed *A. roylei* as the closest relative of *A. cepa*, questioning the current classification of *A. cepa* in the section *Rhizideum* (SUSAN *et al.* 1993). 90 RAPD primers grouped 24 onion cultivars into northern and southern regions of India (SANGEETA *et al.* 2006). Ten varieties of onion (*A. cepa* L.) were analysed, Bermis and India-2 were more dissimilar and Faridpuri and Bhati were the most genetically similar (MANIRUZZAMAN *et al.* 2010).

XU *et al.* (2001) used five RAPD for classification and identification of thirty-one garlic (*A. sativum* L.) cultivars. Somaclonal variation in plants regenerated from long-term callus cultures of garlic was detected using RAPD (AL-ZAHIM *et al.* 2005). Several locus-specific RAPD and AFLP markers were developed and used as a tool for the rapid characterization of garlic germplasm collections (IPEK *et al.* 2008). ABDOLI *et al.* (2009) found the paradox in genetic diversity detected by RAPD technique and geographical origins. This may be due to limited genome coverage and poor reproducibility of RAPDs; it showed the need of an alternative more efficient marker system.

ISSRs exhibit the specificity of microsatellite markers, but they need no sequence information for primer synthesis enjoying the advantage of random markers (JOSHI *et al.* 2000). Thirty-two onion germplasm resources analysed using ISSR were divided into five groups; the first group Yellow Sweet Spanish system, the second Bejo Daytona cultivar, the third Yellow Globe system, the fourth Yellow Globe Danvers system and the fifth group of Yellow Danvers system (QIJIANG & JIA 2007). JABBES *et al.* (2011) studied diversity in Tunisian garlic genotypes with seven polymorphic ISSR markers. The exotic cultivars Alisa Craig and Brigham Yellow Globe were different compared to the Indian cultivars and Nashik Red and Poona Red were indistinguishable, and similarly N-53 and Bombay Red were quite close (MAHAJAN *et al.* 2009). The genetic fidelity of *A. ampeloprasum* L. and *A. sativum* *in vitro* regenerated clones was studied by GANTAIT *et al.* (2010) using 10 ISSR primers. JAKSE *et al.* (2005) identified 398 SNP, indels and SSRs which distinguished 35 elite onion populations. The diversity assessment of tropical Indian onion and cross amplification of genomic

Table 1. Diversity analysis and fingerprinting of *Alliums* using molecular markers

Marker	Population	References
RAPD	cultivars of onion, chives, bunching onion and leek	SUSAN <i>et al.</i> (1993)
RAPD	31 garlic cultivars	XU <i>et al.</i> (2001)
RAPD	24 cultivars of short-day onions	SANGEETA <i>et al.</i> (2006)
ISSR	32 onion cultivars	QIJIANG & JIA (2007)
SSR	14 short day and 2 long day onion cultivars	MAHAJAN <i>et al.</i> (2009)
EST SSR	tropical Indian onion	KHAR <i>et al.</i> (2010)
ISSR	<i>In vitro</i> regenerated clones for fidelity test	GANTAIT <i>et al.</i> (2010)
RAPD	10 varieties	MANIRUZZAMAN <i>et al.</i> (2010)
ISSR	31 Tunisian garlic genotypes	JABBES <i>et al.</i> (2011)
AFLP	135 garlic accessions – genbank collection	OVESNÁ <i>et al.</i> (2011)
AFLP	Argentinean collection	LAMPASONA <i>et al.</i> (2012)

RAPD – random amplified polymorphic DNA; SSR – simple sequence repeat; ISSR – inter-simple sequence repeat; EST SSR – expressed sequence tag SSR; AFLP – amplified fragment length polymorphism

and expressed sequence tag (EST)-SSR markers in distantly related native wild species were estimated (KHAR *et al.* 2010) (Table 1).

AFLP has been employed to describe genetic diversity in cultivated *Allium* species. OVESNA *et al.* (2011) found a correlation between the genetic basis of garlic clones as identified by AFLP and cysteine sulphoxide content across 135 genotypes. 286 informative AFLP fragments grouped 135 accessions in eight clusters, two clusters contained only *A. sativum* L. var. *ophioscorodon* clones; four clusters grouped together both non-bolting and semi-bolting clones whereas the remaining two contained only non-bolting types. These AFLP markers efficiently differentiated var. *ophioscorodon* from var. *sativum* clones. Further, LAMPASONA *et al.* (2012) analysed the diversity of garlic germplasm from Argentina and stressed the importance of well characterized collections for future breeding and showed the potential of AFLP in genetic diversity analysis of *Alliums*.

#### Development of SSRs in *Alliums*

With the development of different tools and techniques SSR development has been initiated in *Alliums*. To establish a genetic basis for the breeding of Japanese bunching onion, TSUKAZAKI *et al.* (2008a) isolated more than hundreds of SSR clones from size-fractionated genomic DNA libraries. They were highly polymorphic and applicable to the related species such as bulb onion and these can be used for cultivar identification and hybrid

purity determination, marker-assisted selection etc. TSUKAZAKI *et al.* (2007) showed that bunching onion SSRs are very rich sources of highly informative genetic markers by isolating 1796 SSR clones by large-scale sequencing of SSR-enriched genomic DNA libraries. Of these, 74.1% contained  $(GT)_n$  repeats ( $n > 5$ ), while 17.5% were  $(GA)_n$ -containing clones. The average number of SSR repeats was 10.5 and 10.4 in the  $(GT)_n$  and  $(GA)_n$ -containing clones, respectively. The MISA (MicroSatellite identification tool) program revealed 336 dinucleotide to hexa-nucleotide SSRs among 313 unique onion ESTs, representing a frequency of 1 SSR/25 kb similar to 1 SSR/27.2 kb observed in a survey of higher plants (CARDLE *et al.* 2000). JOSEPH *et al.* (2004) generated 11 008 unique ESTs from a normalized cDNA library of onion to assess the genomic differences between the *Asparagales* and *Poales*, sequence analyses of these ESTs revealed microsatellite markers, single nucleotide polymorphisms and homologs of transposable elements. Molecular marker development in *Allium* was previously restricted due to the giga base genome size and costs of sequencing. Expressed sequence tags (ESTs) from onion that showed significant similarities (70–80%) to single positions in the rice genome revealed scant collinearity demonstrating that the grasses are not appropriate genomic models for the *Asparagales*; this makes it necessary to develop genomic resources for these important plants (MARTIN *et al.* 2005). Eight novel polymorphic SSR markers developed from an enriched genomic library of garlic (*A. sativum* L.), were used to understand the relationships among 90 garlic

accessions (KYUNG-HO *et al.* 2009). TSUKAZAKI *et al.* (2009) developed an SSR tagged breeding scheme for bunching onion (*A. fistulosum*). BALDWIN *et al.* (2010) reported the application of Roche 454 technology to develop sequence resources for population analyses and genetic mapping. So far 170 genomic SSRs, 9 EST-SSRs, 31 Indels and 156 CAPS markers have been developed and tested (Table 2).

### Linkage and QTL mapping

In onion breeding, the traits such as size, shape, colour, pungency, soluble solids and disease resistance were targeted. Quantitative trait loci (QTL) analysis based on a genetic linkage map would be efficient for revealing the mode of inheritance of these traits.

The first genetic linkage map of Japanese bunching onion with AFLP markers was constructed using reciprocally backcrossed progenies (TAKAYOSHI *et al.* 2005). TSUKAZAKI *et al.* (2008b) built a P<sub>1</sub> linkage map that comprises 149 AFLPs, 2 CAPSs, and 12 SSRs from Japanese bunching onion, and 1 SSR from bulb onion (*A. cepa*) on 15 linkage groups covering 947 cM. The P<sub>2</sub> linkage map composed of 105 AFLPs, 1 CAPS, and 13 SSRs was developed from Japanese bunching onion and 1 SSR from bulb onion on 14 linkage groups covering 775 cM.

Large genome size, clonal propagation and lack of flowering in some clones are the problems of garlic mapping population development but with the availability of self-pollinating and male sterile lines mapping has been initiated. IPEK *et al.* (2005) constructed two low-density genetic maps of gar-

lic, consisting of AFLP and gene-specific markers (*alliinase*, *chitinase*, *sucrose 1-fructosyl transferase* and *chalcone synthase*). The map for the MP1 family with 216 markers spanned 1166 cM with 5.4 cM average, while 143 markers of MP2 spanned 862 cM with 6.0 cM average. Two partial bacterial artificial chromosome (BAC) libraries of the garlic were constructed. The sequence compositions of the BAC clones were characterized by southern hybridization and localized these BAC clones by FISH (HYE-RAN *et al.* 2003).

### Molecular markers for colour improvement in onion

Bulb colour is one of the important traits in onion (*A. cepa* L.). Three major colours of white, yellow, red and a variety of other bulb colours such as chartreuse and gold exist in onion germplasm. The bulb colour is due to flavonoid compounds and 54 kinds of flavonoids have been reported in onion (SLIMESTAD *et al.* 2007). Flavonoids are involved in UV protection, fertility and pigmentation in plants (SHIRLEY 1996) and act as antioxidants (LOTITO & FREI 2006). Onion bulb colours are inherited in a complex manner and involve epistatic interaction and the loci might code for enzymes involved in the anthocyanin synthesis (EL-SHAFIE & DAVIS 1967; KOOPS *et al.* 1991).

Unusual gold-coloured onions showed a reduced amount of quercetin, the most abundant flavonoid in onions. KIM *et al.* (2004) identified critical mutations in the chalcone isomerase (CHI) gene causing gold onions. The colour difference between yellow

Table 2. Development of simple sequence repeat (SSR) in *Alliums*

Method	Marker developed	References
MISA programme	revealed 336 SSRs, 1 SSR/25kb	CARDLE <i>et al.</i> (2000)
Normalized cDNA library of onion	11 008 unique ESTs generated	JOSEPH <i>et al.</i> (2004)
Size fractionated genomic library	32 SSRs and 18 bulb onion expressed sequence tags (EST)	TSUKAZAKI <i>et al.</i> (2008a)
Large scale sequencing of SSR-enriched genomic DNA libraries	1796 SSRs isolated	TSUKAZAKI <i>et al.</i> (2007)
Enriched genomic library of garlic	8 novel polymorphic SSRs	KYUNG-HO MA <i>et al.</i> (2009)
Roche 454 technology	170 genomic SSRs, 9 EST SSRs, 31 InDels and 156 CAPS markers developed	BALDWIN <i>et al.</i> (2010)

EST – expressed sequence tag



and red onions was revealed suggesting the involvement of two complementary genes in anthocyanin production in the  $F_1$  hybrids (KIM *et al.* 2005a).

The inactivation of dihydroflavonol 4-reductase (DFR) in the anthocyanin synthesis pathway was responsible for colour differences between yellow and red onions, and two recessive alleles of the anthocyanidin synthase (ANS) gene were responsible for a pink bulb colour (KIM *et al.* 2005b). Based on mutations in recessive alleles of these two genes KIM *et al.* (2007) developed PCR based markers for identification of polymorphisms between pink and red alleles of the ANS gene. Most pink onions were homozygous recessive for the ANS gene indicating the homozygous recessive. The two pink onions, heterozygous for the ANS gene, were also heterozygous for the dihydroflavonol 4-reductase (DFR) gene indicating that the pink colour was produced by incomplete dominance of a red colour gene over that of yellow colour. KIM *et al.* (2006) identified the allele of ANS, ANS-h1, in a dark red doubled haploid line.  $F_2$  populations originating from the crosses between wild-type (ANS-L) allele-containing red and pink (ANS-p) allele-containing white or yellow parents show a discrete segregation ratio of 3 red to 1 light pink, indicating that the wild-type allele is completely dominant over the pink allele.

KIM *et al.* (2009) identified two novel inactive alleles of DFR-A in yellow onion cultivars and breeding lines from Korea and Japan. A 20 bp deletion of a simple sequence repeat in the promoter region of the DFR-APS allele was used to develop a simple PCR-based molecular marker for the selection of the DFR-APS allele. Furthermore, RT-PCR results showed that no DFR-A transcript was detected in any yellow  $F_2$  individuals. Further, PARK *et al.* (2013b) developed functional CAPS markers for two inactive DFR-A alleles, DFR-A<sup>PS</sup> and DFR-A<sup>DEL</sup>, for detection of inactive DFR-A alleles responsible for a failure of anthocyanin production in onions. Of these two alleles, DFR-A<sup>PS</sup> predominantly occurs in yellow onion cultivars.

### Markers for quality traits of onion

Onion and other *Alliums* have been valued since antiquity for their pungent flavour and aroma. Modern science has confirmed traditional beliefs that the organosulphur compounds that impart flavours have significant human health benefits. The flavour precursors of onion are 1-propenyl,

propyl and methyl cysteine sulphoxides (RANDLE & LANCASTER 2002). GALMARINI *et al.* (2001) created a genetic map to identify and estimate the effects of QTLs on phenotypic correlations among soluble solids content (SSC), total dry matter, pungency and onion-induced in vitro anti-platelet activity. MCCALLUM *et al.* (2006) found a polymorphic SSR marker which exhibited strong disequilibrium with bulb fructan content and was mapped to chromosome 8 in the interspecific population *A. cepa* × *A. roylei*. QTL analysis of total bulb fructan content in the intraspecific mapping population using a complete molecular marker map revealed only one significant QTL which may account for the major differences in bulb carbohydrate content between storage and sweet onion varieties. Candidate genes for sulphur assimilation were used to identify genomic regions affecting pungency in the W202A × Texas Grano 438 cross. Linkage mapping revealed that genes encoding chloroplast ferredoxin-sulphite reductase and chloroplast ATP sulfurylase (ATPS) are closely linked (1–2 cM) on chromosome 3. QTL analysis revealed significant associations of both pungency and bulb soluble solids content with marker intervals on chromosomes 3 and 5 (MCCALLUM *et al.* 2007). The non-structural dry matter content of onion bulbs consists principally of fructose, glucose, sucrose and fructans. RAINES *et al.* (2007) constructed a cDNA subtraction library to differentiate the high and low fructan accumulating background.

### Marker for cytoplasmic male sterility (CMS) in onion

Cytoplasmic male sterility (CMS) is associated with the mitochondrial genome. CMS exists widely in most natural populations of onion, which makes it possible to breed out many male sterile lines for heterosis utilization. The identification of male sterile lines and their maintainers is a major obstacle in the exploitation of male sterility in onion hybrid seed production.

A low-density genetic map of onion (*A. cepa* L.) was developed, comprised of RFLPs and distinguished normal fertile (N) and sterile (S) cytoplasm of onion. There was a correlation between expected and observed numbers of plants maintaining CMS (HAVEY *et al.* 2001). The RFLP approach was applied to identify the CMS genotypes using the probes for the following mitochondrial genes: *atpA*, *atp6*, *atp9*,

*cob*, *cox1*, *nad3*, *nad4* and *nad6* (SZKLARCZYK *et al.* 2002) and these markers are located in a chloroplast *psbA* gene amplicon which can distinguish male-fertile (N) and male-sterile (S) cytoplasm in onions (CHO *et al.* 2006). Genomic and mitochondrial genome diversity was evaluated by employing RAPD, SSR and RFLP markers (CHAURASIA *et al.* 2010). Specific cytoplasm types of all tested cultivars were identified. At least three restorer genes are involved in the restoration of fertility in CMS-T male-sterile while fertility restoration in CMS-S is controlled by a single gene only, rendering it suitable for the establishment of molecular breeding systems (KIM *et al.* 2009). One SCAR marker and one RAPD marker were identified, which could distinguish between N and S cytoplasm in several Welsh onion cultivars, confirmed by Southern blotting (GAI & MENG 2010).

PARK *et al.* (2013a) developed a high resolution linkage map of *Ms* locus which is involved in fertility restoration in onion. Tightly linked RAPD and CAPS markers were used to construct a fine map using F<sub>2</sub> populations. So closely linked markers could be utilised in marker-assisted selection of *Ms* locus and map based cloning. Further, KIM *et al.* (2013) studied the origin and dynamics of genome rearrangements between normal and male-sterile onions. Very recently, HAVEY (2013) studied linkage disequilibrium in male-fertility restoration (*Ms*) locus in open-pollinated and inbred populations of onion using SNP. Three SNPs were identified which were tightly linked

to *Ms* locus on chromosome 2; these SNPs could be helpful in the development of maintainer lines for hybrid onion development (Table 3).

### Future trends

Besides the molecular markers and linkage mapping, the sequencing of a huge genome of onions has been initiated by development of high throughput methods but collaborative international efforts are crucial for the sequencing of this large onion genome. Initially, MCCALLUM *et al.* (2001) and KUHL *et al.* (2004) did Sanger sequencing of random cDNAs from non-normalised and normalised libraries of onion. Then JAKSE *et al.* (2008) undertook a pilot sequencing project of onion genomic DNA to estimate gene densities and investigate the nature and distribution of repetitive DNAs. Complete sequences from two onion BACs (Bacterial Artificial Chromosomes) were AT rich (64.8%) and revealed long tracts of degenerated retroviral elements and transposons, similar to other larger plant genomes. BALDWIN *et al.* (2010) reported the application of Roche 454 technology to develop sequence resources for population analyses and genetic mapping to develop SSRs and ESTs. With development of sequencing information *in silico* database and genomic resource creation was also initiated. BHASI *et al.* (2010) developed a RoBuST (<http://robust.genome.com>), integrated genomic resources for *Apiaceae* and *Alliaceae* which can be used for

Table 3. Molecular markers for cytoplasmic male sterility (CMS) analysis in *Alliums*

Marker	Application	References
RFLP	identify the cytoplasmic genotypes	SZKLARCZYK <i>et al.</i> (2002)
PCR-RFLP	distinguish male-fertile (N) and male-sterile (S) cytoplasm	CHO <i>et al.</i> (2006)
RFLP	CMS-T and CMS-S cytoplasm type identification	KIM <i>et al.</i> (2009)
RAPD, SSR, RFLP	genomic and mitochondrial genome diversity	CHAURASIA <i>et al.</i> (2010)
SCAR and RAPD	distinguish between N and S cytoplasm in welsh onion	GAI SHU-PENG <i>et al.</i> (2010)
RAPD and CAPS	high-resolution linkage map of the <i>Ms</i> locus	PARK <i>et al.</i> (2013a)
Chloroplast and mitochondrial markers	study of mitochondrial genome rearrangements	KIM <i>et al.</i> (2013)
SNP	linkage disequilibrium study in the male-fertility restoration ( <i>Ms</i> ) locus	HAVEY 2013

RFLP – restriction fragment length polymorphism; PCR – polymerase chain reaction; RAPD – random amplified polymorphic DNA; SSR – simple sequence repeat; SCAR – sequence characterized amplified region; CAPS – cleaved amplified polymorphic sequences; SNP – single nucleotide polymorphism

sequence annotations, access to traits, biosynthetic pathways, genetic linkage maps and comparative analysis of plant splicing patterns. McCallum *et al.* (2012) developed a comparative genomics resource AlliumMap for cultivated *Allium* vegetables which is the first online resource providing a genetic map and marker data from multiple *Allium* species and populations. In the future we need to exploit high throughput SNP genotyping, functional genomics using RNAi or other mutagenic methods and transcriptome mapping to know the function of each gene in *Allium* genome. Genomic resources and databases thus developed will be very useful for *Allium* genomics and improvement in the near future.

## CONCLUSION

Genetic analysis of *Alliums* germplasm by molecular markers will help in the understanding extent of genetic diversity and varietal identification. QTL detection and linkage mapping will help in marker-assisted selection for different qualitative traits in onion. Markers linked to colour, TSS, pungency and CMS will be useful in functional analysis of these traits and further improvement of the cultivars and recent sequencing efforts will definitely speedup the process. This review is a very useful source of recent information on the *Alliums* genetic studies for researchers who are working in *Alliums*.

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*Corresponding author:*

LAKSHMANA REDDY DHORANALAPALLI CHINNAPPAREDDY, Ph.D., Indian Institute of Horticultural Research, Bangalore, India  
e-mail: dclreddy@gmail.com

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