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Training Manual

on

Application of Cellular, Molecular and Genomics tools in Crop Improvement

(October 07-16, 2014)

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Potato scenario- present and future

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Potato (*Solanum tuberosum* L.) is one of the most important food crops after wheat, maize and rice, contributing to food and nutritional security in the world. This tuber crop of the family solanaceae has about 200 wild species. It originated in the high Andean hills of South America, from where it was first introduced into Europe towards the end of 16th century through Spanish conquerors. There the potato developed as a temperate crop and was later distributed throughout the world largely as a consequence of the colonial expansion of European countries. It was introduced to India by early 17th century probably through British missionaries or Portuguese traders.

Potato: The Crop and the Food

Potato is an annual, herbaceous, dicotyledonous and vegetatively propagated plant. It can also be propagated through botanical seed known as True Potato Seed (TPS). The potato tuber is a modified stem developed underground on a specialized structure called stolon. It contains all the characteristics of a normal stem like dormant bud (eye) and scaly leaf (eyebrow). Potato tuber is a bulky commodity which responds strongly to its prevailing environment thus needs proper storage.

Potato is a highly nutritious, easily digestible, wholesome food containing carbohydrates, proteins, minerals, vitamins and high quality dietary fibre. A potato tuber contains 80 per cent water and 20 per cent dry matter consisting of 14 per cent starch, 2 per cent sugar, 2 per cent protein, 1 per cent minerals, 0.6 per cent fibre, 0.1 per cent fat, and vitamins B and C in adequate amount. Thus, potato provides more nutrition than cereals and vegetables. Keeping in view the shrinking cultivable land and burgeoning population in India, potato is a better alternative to deal with the situation.

Potato in India

In Europe the potato crop is grown in summer having long photoperiod of up to 14 hours and the crop duration of 140-180 days. The potato in Indian plains is, however, grown in completely contrasting situations. Nearly 85 per cent of the crop is grown during winters having short photoperiod (with about 10-11 hours sunshine) and the crop duration is also limited to 90-100 days because of short and mild winter. The mornings usually have fog, which further reduces the sunshine hours posing severe constraints on photosynthetic activity. Besides, the post-harvest period consists of long hot summer, which creates storage problems.

All these problems called for suitable varieties and technologies for growing potatoes under the sub-tropical conditions of India. This necessitated to initiate indigenous potato research and development programmes, and accordingly the Central Potato Research Institute

(CPRI) came up in 1949 at Patna. The headquarters was later on shifted to Shimla in order to facilitate hybridization and maintenance of seed health. In 1971 the All India Coordinated Research Project (AICRP) on potato was initiated under the aegis of the Indian Council of Agricultural Research (ICAR) at the CPRI with an objective to coordinate potato research and development in the country across diverse agro-ecological regions. The success story of over five decades of potato research in India is phenomenal. Compared to the area, production and productivity in 1949-50, the increase over this period is 550 per cent, 1745 per cent and 178 per cent, respectively (Table 1). India now ranks fourth in potato area (1.48 million ha) and third in production (28.47 million tonnes) in the world with an average yield of 183,3q/ha.

Table 1: Area, Production & Yield of Potato in India

Year	*Area (million ha)*	*Production (million tones)*	*Yield (q/ha)*
1949-50	0.239	1.54	65.9
1959-60	0.362	2.73	75.5
1969-70	0.496	3.91	78.9
1979-80	0.685	8.33	121.5
1989-90	0.940	14.77	157.1
1999-00	1.340	24.71	184.4
2003-04	1.270	23.12	182.0
2005-06	1.400	23.90	170.6
2006-07	1.482	22.09	149.0
2007-08	1.553	28.47	183.3
2008-09	1.810	28.58	157.8
2009-10	1.840	36.58	199.2
2010-11	1.860	42.34	227.2
2011-12	1.910	41.48	217.5
2012-13	1.992	45.34	227.8

It was only because of indigenously developed technologies that potato in India has shown spectacular growth in area, production and productivity during the last five decades. The major achievements of potato research in India are as under:

Varietal Improvement

So far 47 potato varieties have been bred for different agro-climatic regions of the country with 28 varieties alone for north Indian plains. Varieties have also been developed for north Indian hills and other special problem areas viz. Sikkim, north Bengal hills and south Indian hills. Of the 47 varieties developed, 19 possess multiple resistance to different biotic and abiotic stresses. Besides, nine varieties are suitable for processing purposes. These are Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chipsona-3, Kufri Himsona, Kufri Frysona, Kufri Jyoti, Kufri Chandramukhi, Kufri Lauvkar and Kufri Surya. All these varieties fall in three maturity groups, i.e. early (70-80 days), medium (90-100 days) and late (110-120 days).

The potato varieties developed by CPRI are grown not only in India but also in several neighbouring countries. The variety Kufri Chandramukhi is grown in Afghanistan, Kufri Jyoti in Nepal and Bhutan, and Kufri Sindhuri in Bangladesh and Nepal. Besides, five Indian hybrids are also commercially grown in Sri Lanka, Madagascar, Mexico and Philippines.

Seed Plot Technique

This technique was developed in 1970s to enable healthy seed potato production in the sub-tropical Indian plains under low aphid period. This technique aided by bio-technological approaches for virus elimination, micro-propagation and effective viral diagnostics has sustained the National Potato Seed Production Programme by producing about 2600 tonnes of breeder's seed annually. This breeder's seed is further multiplied to about 4,32,000 tonnes of certified seed by the State Departments of Agriculture/ Horticulture. Thus, the country saves about 484 million US dollars because most Asian countries like Pakistan, Bangladesh and even China continue to import seed potatoes from Europe.

The decentralization of potato breeding from hills to plains in India through the seed plot technique enabled the development of varieties suited to different agro-climatic regions of the country. The area under seed potato production also increased by 12 times and enabled the availability of seed potato throughout the country in proper physiological state.

Tissue Culture

Efforts are being made to improve seed health standards and reduce the time required for production of breeder's seed by employing *in vitro* techniques of meristem culture and micro-propagation. Presently, about 5 per cent of Breeder's seed production programme is fed annually by microtubers produced through tissue culture. It is proposed to produce 100 per cent of breeder's seed through tissue culture propagated material in the years to come.

Agro-techniques

The development of package of practices for potato production in different agro-climatic zones has helped in improving potato productivity in these zones. The potato crop is input intensive and requires optimum cultural practices for achieving higher productivity. Optimum cultural practices depend on delineated phenological phases of crop growth and development viz. pre-emergence, emergence to tuber initiation, tuber initiation to tuber bulking and tuber bulking to termination of bulking.

The cultural practices are adjusted in the Indian plains in a way so that tuber initiation and development coincide with the period when night temperature is less than 20^{o}C and day temperature is below 30^{o}C. The phenological phase of tuber initiation to tuber bulking is mainly conditioned by nutrition and moisture. For this purpose, fertilizer and irrigation requirement in different agro-climatic zones have been worked out through multi-locational trials under AICRP (Potato). Termination of tuber bulking coincides with onset of foliage senescence. By manipulating the nutrition and moisture, the foliage senescence is delayed for ensuring continuation of linear tuber bulking phase resulting in higher yield.

Several profitable potato-based inter-cropping and crop rotations have also been identified for different regions of the country. Potato can be profitably intercropped with wheat, mustard and sugarcane. These cropping systems have helped in the maintenance of soil fertility and have improved the fertilizer economy, crop yield and gross returns. Besides, potato cultivation has also been mechanized in selected regions through the fabrication and development of cost-effective tools and implements.

Plant Protection

Effective management practices have been devised for the major potato diseases and insect-pests in India. Late blight is the most notorious disease of potato which occurs almost every year in the hills and plains. Besides chemical control measures, several late blight resistant varieties have been developed. Potato varieties have also been bred which possess resistance to wart and cyst nematodes. Cultural and biological control measures have also been developed to control the diseases and insect-pests. The development of late blight forecasting systems for hills and plains has enabled the early warning mechanism for the appearance of late blight disease.

Storage

In European countries, the potato crop is grown in summer and the main storage season is the cold winter. However, in India, 85 per cent of potato is produced in winter and stored during long hot summer. This requires storage of potatoes in cold stores at 2-4^{0}C, which involves substantial cost. It also leads to accumulation of reducing sugar in the potato tubers resulting in sweetening of potatoes.

However, there are a number of traditional low-cost and non-refrigerated storage structures (essentially based on evaporative or passive evaporative cooling) in use in India with varying degrees of success. These traditional structures have been studied, validated and popularized for particular regions. In non-refrigerated storages, use of sprout suppressants have also been popularized to prevent excessive weight loss and shrinkage due to sprouting. The CIPC (isopropyl-N-chlorophenyl carbamate) is the most effective sprout inhibitor when applied @ 25 mg a.i. per kg tubers.

Processing and Value Addition

In addition to raw consumption, potatoes can be processed into several products like chips, French fries, cubes, granules and canned products. The primary determinants for potato processing include high dry matter and low reducing sugar content. A dry matter content of more than 20 per cent is desirable for chips, French fries and dehydrated products. Similarly, a reducing sugar content in tubers up to 100 mg/100g fresh weight is considered acceptable for processing. Nine varieties viz. Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chipsona-3, Kufri Jyoti, Kufri Chandramukhi, Kufri Lauvkar, Kufri Surya and Kufri Himsona, Kufri Frysona have been developed for processing purposes. In India, potato processing in organised sector started about a decade ago, and the recent proliferation of this sector mainly results from the development of three indigenous potato processing varieties, viz. Kufri Chipsona-1 and Kufri Chipsona-3 by CPRI. These two varieties are now being used by the industries for processing into chips and French fries.

Computer Applications

Simulation modelling is now widely used in various disciplines to work out tactical decisions. CPRI has developed INFOCROP-POTATO model to simulate the potato growth and development, to determine the best growing period, to optimise management practices under different agro-ecological regions, and to forecast the accurate yield estimates. An expert system (Potato Pest Manager) has also been developed for decision support with respect to identification and management of diseases and insect-pests.

Transfer of Technology

Research achievements alone are not adequate to gauge the success of an agricultural system. The research information needs to be assessed and refined under various bio-physical and socio-economic situations through adaptive research before it is labelled as a technology. In this regard, the multi-locational trials under AICRP (Potato) and the TOT projects undertaken by CPRI such as Operational Research Project (ORP), Lab-to-Land Programme (LLP), Tribal Area Development (TAD) programme and Institution-Village Linkage Programme (IVLP) proved landmark in getting feedback from the field and development of appropriate technologies.

Transfer of technology to the end users is a complex task which consists of a number of components and dimensions. One of the important components is proper linkage between technology generating system and the client system. In this regard, innovative approaches like need assessment, participatory planning and implementation, and direct scientist-farmer interface facilitated faster dissemination of technologies and consequent adoption by the farmers/clients. The CPRI has build up linkages with farmers through demonstrations, trainings, Kisan Melas, potato school on All India Radio, supply of literatures and other extension activities. Besides, studies have been conducted to measure the socio-economic impact and constraints in transfer of potato technology.

Potato Export

Although India contributes 7.55% to the total world potato production, its 0.7% share in world's potato export is quite insignificant. Indian potatoes are truly free from the prohibited disease like wart, black scruf, and pests like tuber moth and nematodes, which are the barometer for phytosanitary standards. India has also the natural advantage of exporting fresh table potatoes during January to June when supply from European countries dwindles. It can also supply fresh potatoes round the year because India has diverse agro-climates and potato is grown throughout the year in one or the other part of the country.

Potato has a good future in India under the changed scenario of global economy. Globalisation has resulted in many developing countries becoming much more integrated into the international potato trade. With the phasing out of quantitative restrictions on agricultural commodities, the imports and exports of potato would be based on the differences in price and production cost between the importing and exporting countries involved. Due to low production cost in the country as a result of availability of cheap labour, India will have competitive advantage in the international potato trade.

Potato in the New Millennium

With the improvement in the living standard of people in India, the dietary habits will shift from cereals to vegetables. Under such a situation it is estimated that India will have to produce 49 million tonnes of potato by 2020. This target could be achieved only by improving the productivity level. The productivity of potato in India is quite low (183.3q/ha) as compared to that of Belgium (490q/ha), New zealand (450q/ha), UK (397q/ha) and USA (383q/ha). This is due to shorter crop duration in India. There is a wide ranging variations in the agro-ecological setting of different parts of the country, which results in wide variations in the productivity levels of different states (Table 2). Therefore, all our efforts may be put in to develop location-specific and problem-specific varieties and technologies.

Table 2: Statewise Area, Production & Yield of Potato in India during 2012-13

States	Area (,000 Hectares)	Production (' 000 Tonnes)	Yield (q/ha)
Uttar Pradesh	603.76	14430.28	239.0
West Bengal	386.61	11591.30	299.8
Bihar	322.50	6640.60	205.9
Assam	99.77	975.27	97.7
Madhya Pradesh	108.87	2299.00	211.2
Punjab	85.25	2132.31	250.1
Gujarat	81.27	2499.73	307.6
Jharkhand	47.21	659.61	139.7
Karnataka	44.40	698.30	157.3
Haryana	29.47	676.01	229.4
Others	183.2	2741.20	149.7
Total	1992.2	45343.60	227.6

Source: Directorate of Economics & Statistics, Govt. of India.

Most of the people in India have either no knowledge or wrong notions about the nutritive value of potato. With low fat (0.1 per cent) and calorie contents, it does not cause obesity. Due to misconception the potato consumption, the per capita consumption of potato in India is only about 16 kg/year. On the other hand, the per capita consumption in Europe is 121 kg/year and as high as 136 kg/year in Poland. Hence, there is ample scope for improving the consumption of potatoes in India. For this purpose, a publicity compaign like eggs and milk needs to be launched through mass media such as television, radio and newspapers highlighting its nutritional value. Moreover, the possibility of using surplus potatoes as animal feed also needs to be explored.

The surplus potatoes in a season are stored in cold stores at 2-4^{o}C in the country. This makes stored potatoes just unfit for processing and loses preference for table purposes due to accumulation of sugar content. To avoid sweetening potato are required to be stored at

10-12^{o}C. Only seed potatoes should be cold stored at 2-4^{o}C. This would release atleast 60 per cent of cold storage space that can be converted to store potatoes for processing and table purposes at 10-12^{o}C with CIPC treatment leading to considerable savings on energy and storage costs.

Processing is a fast growing sector in the potato world economy. Due to increased urbanization, rise in per capita income and expanding tourism, the demand for processed potato products in India and international market has risen at a fast pace. However, in India, processing of potatoes constitutes less than 2 per cent of the total annual production as compared to 60 per cent in USA, 47 per cent in the Netherlands and 22 per cent in China. Hence, there is great scope to expand the potato processing industries in India and also to diversify the processing to produce flour, cubes, granules, flakes and starch.

Under the changed global scenario, the potato production and utilisation pattern is changing very fast. These changes harbour many opportunities which could be tapped through effective extension system. The use of modern information and communication technologies (ICT) to create awareness is highly pertinent in the contemporary times. This would enable us to reach directly to the end users by eliminating the intermediate channels which create distortion of information. Efforts are also needed to devise market-based extension strategies in order to promote entrepreneurship among potato growers with regard to potato production and marketing.

Application of modern biotechnological tools in crop improvement: present status and future prospects

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The number of people on Earth is expected to increase from the current 6.7 billion to 9 billion by 2050. To feed this burgeoning population the world agricultural production needs to rise by 50% by 2030 (Royal Society 2009). Because the amount of arable land is limited and what is left is being lost to urbanization, salinization, desertification, and environmental degradation, it no longer possible to simply open up more undeveloped land for cultivation to meet production needs. Another challenge is that water systems are under severe strain in many parts of the world. The fresh water available per person has decreased fourfold in the past 60 years (United Nations Environmental Programme 2002). Of the water that is available for use, approximately 70% is already used for agriculture (Vorosmarty et al. 2000). The world's 50% wetlands have disappeared, and major groundwater aquifers are being mined unsustainably, with water tables in parts of Mexico, India, China, and North Africa declining by as much as 1 m/year (Somerville and Briscoe 2001). Thus, increased food production must largely take place on the same land area while using less water. Compounding the challenges facing agricultural production are the predicted effects of climate change (Lobell et al. 2008). As the sea level rises and glaciers melt, low-lying croplands will be submerged and river systems will experience shorter and more intense seasonal flows, as well as more flooding (Intergovernmental Panel on Climate Change 2007). Yields of our most important food, feed, and fiber crops decline precipitously at temperatures more than 30^{o}C, so heat and drought will increasingly limit crop production (Schlenker and Roberts 2009). In addition to these environmental stresses, losses to pests and diseases are also expected to increase. Much of the losses caused by these abiotic and biotic stresses, which already result in 30–60% yield reductions globally each year, occur after the plants are fully grown: a point at which most or all of the land and water required to grow a crop has been invested (Dhlamini et al. 2005). For this reason, a reduction in losses to pests, pathogens, and environmental stresses is equivalent to creating more land and more water. Thus, an important goal for genetic improvement of agricultural crops is to adapt our existing food crops to increasing temperatures, decreased water availability in some places and flooding in others, rising salinity, and changing pathogen and insect threats (World Bank 2007; Gregory et al. 2009; Royal Society 2009). Such improvements will require diverse approaches that will enhance the sustainability of our farms. These include more effective land and water use policies, integrated pest management approaches, reduction in harmful inputs, and the development of a new generation of agricultural crops tolerant of diverse stresses (Somerville and Briscoe 2001). Here come in picture the modern biotechnological tools to play a significant role in complimenting the present techniques in crop improvement to hasten the improvement process for sustainable agriculture. There are many modern biotechnological tools available at dispense of modern biologist for crop improvement and are being used exclusively in combination with other basic sciences to hasten the process (fig 1.)

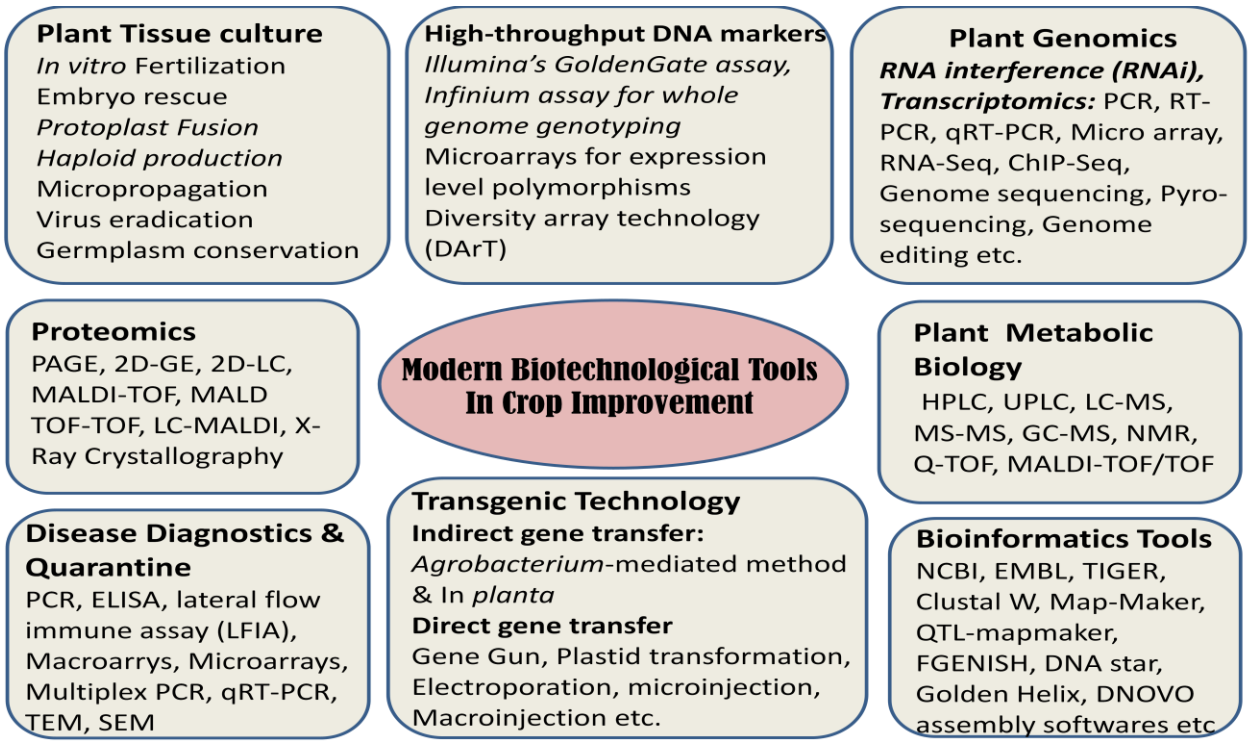

Fig1. A snapshot of modern biotechnological tools available for crop improvement

A] Plant Tissue Culture:

Plant tissue-culture technology is playing an increasingly important role in basic and applied studies, including crop improvement (Thompson & Thorpe 1990; Thorpe 1990; Vasil & Thorpe 1994). In modern agriculture, only about 150 plant species are extensively cultivated. Many of these are reaching the limits of their improvement by traditional methods. The application of tissue-culture technology, as a central tool or as an adjunct to other methods, including recombinant DNA techniques, is at the vanguard in plant modification and improvement for agriculture, horticulture and forestry. The tissue culture techniques most popularly being utilized now days for crop improvement essentially for *in vitro* fertilization, development of somaclonal variants, micro-propagation and mass multiplication, development of pathogen-free planting materials, germplasm preservation and conservation. Few successful stories of crop improvement through tissue culture techniques are being discussed in proceeding section.

***In vitro* Fertilization:** IVF has been used to facilitate both interspecific and intergeneric crosses, to overcome physiological-based self incompatibility and to produce hybrids. A wide range of plant species has been recovered through IVF via pollination of pistils and self- and cross-pollination of ovules (Yeung *et al.* 1981; Zenkteler 1990; Raghavan 1994). This range includes agricultural crops, such as tobacco, clover, com, rice, cole, canola, poppy and cotton. The use of delayed pollination, distant hydridization, pollination with abortive or irradiated pollen, and physical and chemical treatment of the host ovary have been used to induce haploidy (Maheshwari & Rangaswamy 1965; Zenkteler 1984).

Embryo rescue: It is one of the earliest and successful forms of in-vitro culture techniques used to assist in the development of plant embryos that might not survive to become viable plants (Sage et al, 2010). Embryo rescue played an important role in modern plant breeding, allowing the development of many interspecific and intergeneric food and ornamental plant crop hybrids. Embryo culture has been successful in overcoming this major barrier as well as solving the problems of low seed set, seed dormancy, slow seed germination, inducing embryo growth in the absence of a symbiotic partner, and the production of monoploids of barley (Raghavan 1980, 1994; Yeung *et al.* 1981; Collins & Grosser 1984; Zenkteler 1990). The breeding cycle of *Iris* was shortened from 2 to 3 years to a few months by employing embryo rescue technology (Randolph 1945). Interspecific and intergeneric hybrids of a number of agriculturally important crops have been successfully produced, including cotton, barley, tomato, rice, jute, *Hordeum* X *Secale,Triticum* x *Secale,Tripsacum*x *lea* and some Brassicas (Collins & Grosser 1984; Palmer & Keller 1994; Zapata-Arias *et al.* 1995).

Protoplast fusion: It has often been suggested as a means of developing unique hybrid plants which cannot be produced by conventional sexual hybridization. Protoplasts can be produced from many plants, including most crop species (Gamborg et al. 1981; Evans & Bravo 1983; Lal & Lal *1990;* Feher & Dudits 1994). Perhaps the best example of the use of protoplasts to improve crop production is that of *Nicotiana,* where the somatic hybrid products of a chemical fusion of protoplasts have been used to modify the alkaloid and disease-resistant traits of commercial tobacco cultivars (Pandeya et al. 1986).

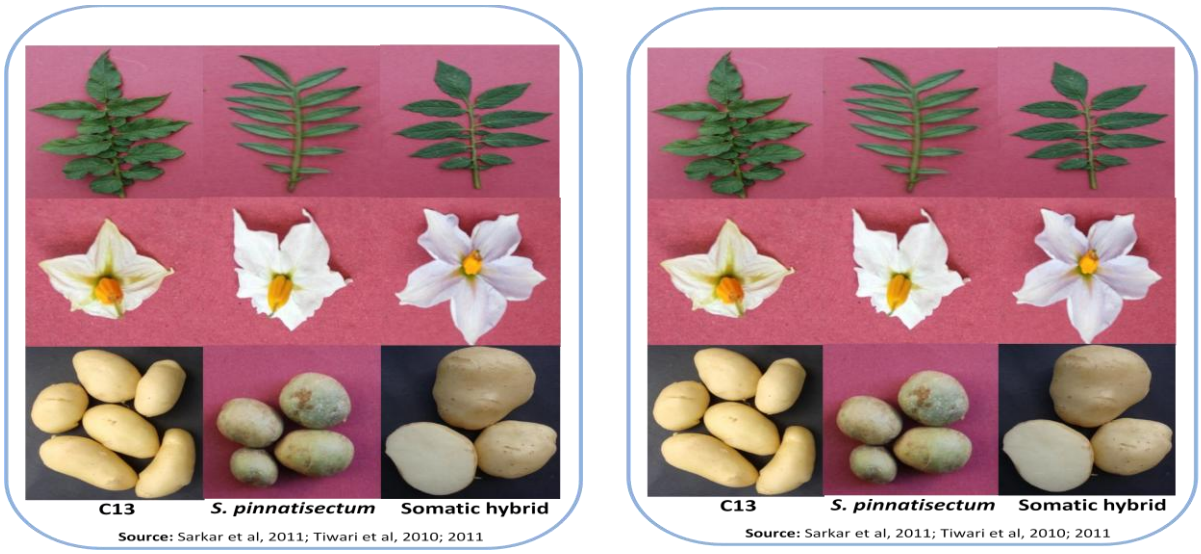

Fig. 2 Somatic hybridization for potato improvement for LB and PVY resistance

At CPRI, symmetric protoplast fusion approaches have been successfully utilized to develop interspecific potato hybrids between 1 EBN wild *Solanum* Sp. *S. pinnatisectum* (+) dihaploid *S. tuberosum*, and *S. etuberosum* (+) dihaploid *S. tuberosum* for late blight and potato virus Y respectively (Sarkar et al, 2011; Tiwari et al, 2010; 2011; Fig 2).

Haploid production: Haploid plants have the gametophytic (one-half of the normal) number of chromosomes (Atanassov *et al.* 1995; Zapata- Arias *et al.* 1995). They are of interest to plant breeders because they allow the expression of simple recessive genetic traits or mutated recessive genes and, doubled haploids can be used immediately as homozygous breeding lines. The efficiency in producing homozygous breeding lines via doubled *in* vitro-produced haploids represents significant savings in both time and cost compared with other methods. At least 171 plant species have been used to produce haploid plants by pollen, microspore and anther culture (Evans *et al.* 1984; Hu & Zeng 1984; Bajaj 1990). These include cereals (barley, maize, rice, rye, triticale and wheat), forage crops (alfalfa and clover), fruits (grape and strawberry), medicinal plants *(Digitalis* and *Hyoscyamus),* ornamentals *(Gerbera* and sunflower), oil seeds (canola and rape), trees (apple, litchi, poplar and rubber), plantation crops (cotton, sugar cane and tobacco), and vegetable crops (asparagus, brussels sprouts, cabbage, carrot, pepper, potato, sugar beet, sweet potato, tomato and wing bean). Haploid wheat cultivars, derived from anther culture, have been released in France and China (Bajaj 1990). Five to 7 years were saved producing inbred lines in a Chinese maize-breeding program by using anther culture-derived haploids.

Micropropagation: During the last 30 years it has become possible to regenerate plantlets from explants and/or callus from all types of plants. As a result, laboratory-scale micropropagation protocols are available for a wide range of species (Debergh & Zimmerman 1991) and at present micropropagation is the widest use of plant tissue-culture technology.

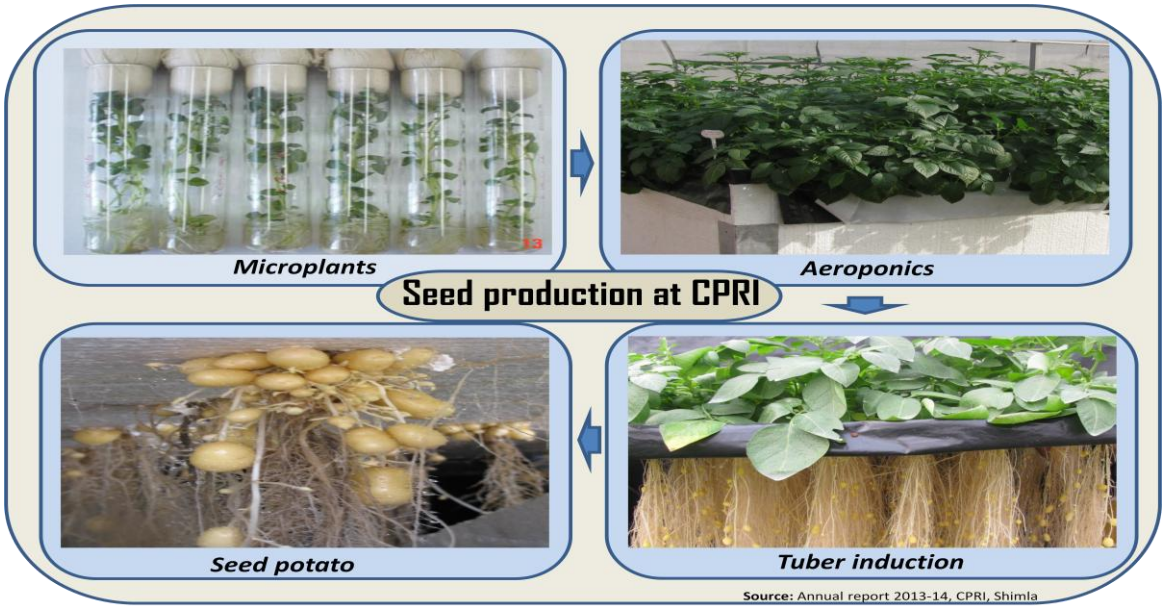

Fig. 3 Integration of micropropagation and aeroponic methods for potato seed production at CPRI

In Europe, there were 172 micropropagation firms and about 1800 different tissue lines (species and varieties) in culture amongst the 501 plant tissue-culture laboratories identified in 1993 (O'Riordain 1994). For example, of the 88 European laboratories using potato in

tissue culture, 58 were listed as using *in-vitro* multiplication, 49 were involved in the elimination of pathogens, 45 were using tissue-culture simply to store germplasm, 44 were involved in genetic modification and 26 had plant-breeding programs. CPRI has standardized the micropropagation protocol for developing tissue culture plantlets in all most all potato varieties released by the institute and successfully utilized the aeroponic system for production of quality seeds. A total number of 58,796, 71,247 and 23,349 minitubers were produced through aeroponics at Shimla, Modipuram and Patna facilities respectively using micoplants developed through micropropagation methods (fig. 3).

Virus eradication: Crop plants, especially vegetatively propagated are generally infected with pathogens. Strawberry plants, for example, are susceptible to over 60 viruses and mycoplasms and this often necessitates the yearly replacement of mother plants (Boxus 1976). In many cases, although the presence of viruses or other pathogens may not be obvious, yield or quality may be substantially reduced as a result of the infection (Bhojwani & Razdan 1983). In China, for example, virus-free potatoes, produced by culture in vitro, gave higher yields than the normal field plants, with increases up to 150% (Singh 1992). As only about 10% of viruses are transmitted through seeds (Kartha 1981), careful propagation from seed can eliminate most viruses from plant material. The excision and culture of apical meristems, coupled with thermo- or chemo-therapy, have been successfully employed to produce virus-free and generally pathogen-free material for micropropagation (Kartha 1981; Bhojwani & Razdan 1983; Wang & Charles 1991; Singh 1992). At CPRI, by using modern tissue culture procedures 20 virus free potato varieties material, havening genetically pure and true to type with parent variety has been developed and maintained.

Germplasm Preservation & conservation: One way of conserving germplasm, an alternative to seed banks and especially to field collections of clonally propagated crops, is *in vitro* storage under slow-growth conditions (at low temperature and/or with growth-retarding compounds in the medium) or cryopreservation or as desiccated synthetic seed (Harry & Thorpe 1991; Villalobos & Engelmann 1995). The technologies are all directed towards reducing or stopping growth and metabolic activity. Techniques have been developed for a wide range of plants (Bajaj 1991b). At CPRI, approximately 2000 potato accessions collected from all over world are being preserved and are successfully being maintained using tissue culture techniques. In addition efforts are underway to develop cryopreservation and pollen storage procedures for conservation of these potato accessions.

B] Array-based high-throughput DNA markers for crop improvement:

The last two decades have witnessed a remarkable activity in the development and use of molecular markers in plant systems. This activity started with low throughput restriction fragment length polymorphisms (RFLPs) and culminated in recent years with single nucleotide polymorphisms (SNPs), which are abundant and uniformly distributed. Although the latter became the markers of choice for many, their discovery needed previous sequence information. However, with the availability of microarrays, SNP platforms have been developed, which allow genotyping of thousands of markers in parallel. Besides SNPs, some other novel marker systems, including single feature polymorphisms, diversity array technology and restriction site-associated

DNA markers have also been developed, where array based assays have been utilized to provide for the desired ultra-high throughput and low cost. In this part, we briefly discuss the characteristics of these array-based marker systems and review the work that has already been done involving development and use of these markers in a variety of seed plants with simple or complex genomes.

[I] Microarrays for detection of single nucleotide polymorphisms:

Single nucleotide polymorphisms refer to DNA polymorphisms at the level of individual base pairs and constitute approximately 90% of genetic variation in any organism. The SNPs are generally discovered *in silico* from genomic or expressed sequence tag (EST) sequences available in the databases, or through sequencing or resequencing of candidate genes/PCR products/whole genomes in more than one genotype. Once SNPs are discovered, genotyping for these markers can be done using any one of more than 30 different available methods providing the desired ultra-high throughput (Khlestkina and Salina, 2006; Kim and Misra, 2007). Among them the most popular and extensively used methods includes.

Illumina's GoldenGate assay: The assay, based on their BeadArray/BeadChip technology is now being used for several crops. At present Oligonucleotide Pool Assays are being developed for several plant systems including *Arabidopsis*, barley, wheat, maize, sugarcane, tomato and potato. In barley, a oligonucleotide pool assay for detection of SNPs in 1524 unigenes was developed at SCRI, UK. This platform is being used by US barley Coordinated Agricultural Project to allelotype 3840 genotypes and by Association Genetics of UK Elite Barley (AGOUEB) to allelotype B1500 genotypes. The genotypes will also be assessed for about 40 traits that are pertinent for barley breeding and the information may be used for a study of haplotype-trait associations (Hayes and Szucs, 2006). In soybean, recently a custom-made 384-SNP GoldenGate assay was successfully designed for genotyping of three RILs mapping populations; the above 384 SNPs were discovered through resequencing of five diverse accessions.

Infinium assay for whole genome genotyping: This genotyping system has already been used for human blood/saliva samples for forensic purposes and in several plant systems including corn, canola, cotton (Shah et al,2003) and poplar (Meirmans et al,2006). Tiling arrays developed by Affymetrix GeneChip platform on the basis of known sequences in several organisms have also been used for SNP discovery and detection. These tiling arrays may be either designed for resequencing (sequencing by hybridization or SBH) of specific genomic regions for SNP genotyping or may be designed for interrogating every individual nucleotide in a template genomic sequence by multiple probes available on the array. In rice, where genome sequence of one genotype (Nipponbare) is already available, SNP discovery in 100Mb of the rice genome has been undertaken at IRRI using tiling microarrays that are based on allele-specific oligonucleotides from the non-repetitive regions of the genome. This approach allowed identification of 260000 non-redundant SNPs by Perlegen's model-based (MB) algorithms (McNally et al, 2006; Collard et al, 2008). Affymetrix GeneChips can also be used for SNP genotyping of a number of samples for one or more genes of interest as done in Arabidopsis, where the array AT412 was used for the study of variation in several genes (for example, Eds16 (Cho et al, 1999), Rsf1 (Spiegelman et al,2000) and FRI (Nordborg et al, 2002)).

[II] Microarrays for detection of SFPs/expression level polymorphisms:

Expression oligonucleotide arrays that are often used for SFP technology include Affymetrix (http://www. affymetrix.com) GeneChips or Nimblegen (http://www.nimblegen.com) arrays, which could be either the catalogue microarray or may be custom made for the intended use. The technique was used in a number of seed plants including those with moderately complex genomes (Arabidopsis and rice) and also those with large and highly complex genomes (maize, soybean, tomato, lettuce, barley and wheat). As evident from the information, SFPs have been used for a variety of purposes including detection of marker-trait associations, which sometimes involved construction of a molecular map followed by QTL interval mapping. Arabidopsis, bulk segregant analysis has been used for mapping of circadian and developmental genes (Hazen et al, 2005), ion accumulation genes (Gong et al, 2004) and light responsive QTLs (Wolyn et al, 2004). Similarly, in tomato, 17 SFPs were identified, which were tightly linked to a disease resistance locus (Zhu and Salmeron, 2007). Other important examples of the utility of SFPs are the development of a map of 34000 SFP loci representing 11000 genes in maize (Zhu et al, 2006) and that of 8500 SFP loci from 6000 genes in tomato (Salmeron and Zhu, 2007).

[III] Diversity array technology:

It is a high-throughput microarray hybridization-based technique that allows simultaneous typing of several hundred polymorphic loci spread over a genome without any previous sequence information about these loci (Jaccoud et al, 2001; Wenzl et al, 2004). The technique has also been shown to be reproducible and cost effective. DArT markers have been developed now on large scale (in wheat 43000 markers) and were extensively utilized for the study of genetic diversity, preparation of integrated framework linkage maps and association mapping (White et al,2008). DArT markers have also been used for QTL mapping for *Fusarium* head blight in wheat and for leaf pubescence in barley (Rheault et al, 2007; Wenzl et al, 2007).

Table1: Differences among different array-based techniques for detecting DNA polymorphisms

Parameter	NP	FP	ArT
Sequence information	Required	Required	Not required
Resolution	High	High	Moderate
PCR reaction	Required in some assays like MIP and Golden Gate	Not required	Required, at the time of development & hybridization
Cost	Cost effective	Cost effective	Cost effective
Data type	Presence/absence	Presence/absence	Presence/absence
larkers / assay	High	High	Moderate
rray used	Tag array on beads/ glass, oligonucleotide array/GeneChip	High-density oligonucleotide array/ GeneChip	Glass-spotted DNA microarray

C] Plant Genomics

The number of sequenced plant genomes and associated genomic resources is growing rapidly with the advent of both an increased focus on plant genomics, and the application of inexpensive next generation sequencing. Several new genomics technologies such as next generation sequencing (NGS), high-throughput marker genotyping, -omics technologies have emerged as powerful tools for understanding genome variation in crop species at DNA, RNA as well as protein level. These technologies promise to provide an insight into the way gene(s) are expressed and regulated in cell and to unveil metabolic pathways involved in trait(s) of interest for breeders not only in model-/major- but even for under-resourced crop species which were once considered "orphan" crops. In parallel, genetic variation for a species present not only in cultivated genepool but even in landraces and wild species can be harnessed by using new genetic approaches such as advanced-backcross QTL (AB-QTL) analysis, introgression libraries (ILs), multi-parent advanced generation intercross (MAGIC) population and association genetics. The gene(s) or genomic regions, responsible for trait(s) of interest, identified either through conventional linkage mapping or above mentioned approaches can be introgressed or pyramided to develop superior genotypes through molecular breeding approaches such as marker-assisted backcrossing (MABC), marker assisted recurrent selection (MARS) and genome wide selection (GWS). The cutting edge of basic plant research is rapidly evolving from an understanding of the function of single genes to studying networks of genes that control biological processes (European Commission, 2005). Genomics is the study of an organism's entire genome. Initial activity in the field of genomics has employed genome sequencing to identify genes, thus providing the foundation for functional genomics (covering a range of large-scale Omics technologies) to understand gene expression and biological activity. Genomics initiatives are rapidly generating the basic knowledge that is broadly useful for the scientific community-supporting discovery in basic research and applications in breeding and conservation. Advances in genomics and cognate biosciences are beginning to explain the fundamental elements of plant biology with regard to growth, development, reproduction, photosynthesis and the responses to environmental conditions and pathogens. Much remains to be discovered about these key processes and the integrated circuitry that links the different levels of organisation within the phenotype, and of how natural genetic variation creates biodiversity. The most popularly used genomics tools for crop improvement are briefly discussed in the following section.

RNA interference (RNAi) as reverse genetics tool

The phenomenon of interference was first studied functionally in plants (flowering in petunias in the 1990s), when it was termed post-transcriptional gene silencing. The use of RNAi to eliminate messenger RNA is a novel approach to understand gene function, as an alternative to conventional chemical mutagenesis or the production of DNA knockout mutants, and is potentially a more specific and controllable technique to tune gene function at particular developmental and physiological stages by contrast with complete gene deletion. RNAi as a tool is currently being used in a broad range of applications, and in plant science it is being used to study host plant-pest interactions ("interactomics"), tolerance to abiotic stress and determinants of growth and development. At CPRI by using Avr3a-siRNA construct late blight transgenic lines has been develoed. Transgenic potato plants through silencing the vacuolar invertase gene (Va-*INV*) and UDP Glouse Phosphatase (*UGPase*)

towards improving cold-chipping attributes of processing potato variety and dwarf potato transgenic by silencing the GA_{20} *Oxidase* gene has been developed (Fig. 4).

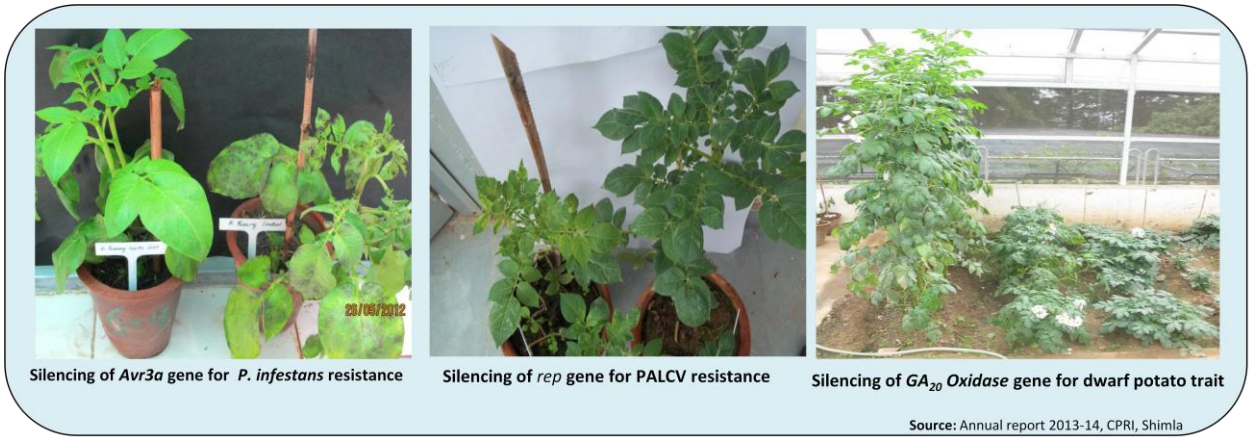

Fig.4 Transgenic potato lines developed at CPRI using RNAi technology

Transcriptomics

The complete set of RNA transcripts produced by an organism at any one time is called the transcriptome and is the link between the genome, the protein complement and the phenotype. Transcriptomics is the study of gene expression patterns and traditionally has used high throughput DNA microarray methodology. Over the past decade the cost of doing microarray experiments has decreased by an order of magnitude and the information obtained within a microarray has increased at least several fold. Plant transcriptomic resources were reviewed recently by Rensink & Buell (2005) who provided a comprehensive listing of the databases publicly available from genome-wide expression platforms, including: *Arabidopsis*, barley, Brassica, Citrus, grape, maize, Medicago, Populus, potato, rice, soybean, sugarcane, tomato and wheat. Rensink & Buell identify several strategic needs that must now be satisfied to accelerate functional discovery and begin the process of translational research:

- Generation of reference expression data sets (according to cell, tissue or developmental stage) for each plant species;
- Addition of gene expression data from mutants and treatments that will help to identify biological regulatory networks;
- Coupling expression and other molecular or phenotypic data to reveal the function of unknown genes;
- Consolidation and analysis of multiple datasets to make cross-species comparisons.

At CPRI, the transcriptome studies are being conducted using the modern genomics tools extensively. Detailed differential gene expression analysis has been executed using cDNA microarray for potato tuberization in response to heat stress in K. Chandramukhi (sensitive) and K Surya (tolerant) to decipher the genes and pathways involved in tuberization process at high temperature. In addition the gene expression studies were also carried in novel potato somatic hybrids for late blight resistance and reported two fold up-regulation of 320 genes at 72 hours post inoculation. Differential gene expression studies in *Phytopthora*

infestans were carried out using Nimblegen microarray to understand different developmental stages during asexual spore production to develop the combating strategies. Gene expression analysis was executed for ammonium transporter (AMT), asparagines synthetase (AS), nitrate reductase (NR) and nitrite reductase (NIR) genes for nitrogen use efficiency (NUE) among the indigenous potato cultivars using quantitative real time PCR and isolated these genes form efficient cultivars to be used to improve the NUE of other popular potato cultivars. Whole genomes of *Ralstonia solanacerarum* (4 strains) causing bacterial wilt and *Fusarium sambucinum* causing dry rot in potato were sequenced using pyrosequencing technology (Roche 454) to unravel the genetic constitution of these disease causing organisms towards developing different management and diagnostic technologies. NGS platforms (Ion-Torrent and Roche-454) are exclusively being used to study responsive genes and small RNAs in the plant-microbe interactions, stress responses and quality traits.

D] Plant metabolic engineering for crop biofortification

The health benefits of phenylpropanoids make them important targets for metabolic engineering. For most plant secondary metabolites, use of plants for engineering high level production remains an effective option compared to microbes. Plant metabolic engineering has traditionally adopted biochemical approaches based on modification of key rate-limiting steps in metabolic pathways or adding genes which encode enzymes of target biosynthetic pathways that are not expressed in tissues that are consumed as foods. This second strategy was adopted in the production of Golden Rice, enriched in b-carotene, a provitamin A with high bioactivity. The expression of four genes, encoding enzymes required for b-carotene biosynthesis, in endosperm resulted in rice that accumulated up to 1-2 mg b-carotene per gram (Ye et al, 2000). However, these levels were not adequate to provide the daily recommended allowance of provitamin A in a standard portion of rice. Consequently, considerable effort was invested in improving the efficiency of these enzymes to develop Golden Rice 2, which accumulates 37 mg provitamin A per gram of rice (31 mg per gram b-carotene), enough to provide the daily recommended allowance in a 100 g. Other reports of metabolic engineering include the accumulation of novel metabolites in food. A good example is the production of resveratrol in tomato, following the expression of the gene encoding *stilbene synthase* from grape (Giovinazzo et al, 2005). Once again, the final levels of resveratrol accumulated were low, meaning that tomatoes engineered for resveratrol production using this strategy, are unlikely to compete with natural food sources; red grapes, red wine and peanut products.

Fig. 5A Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors

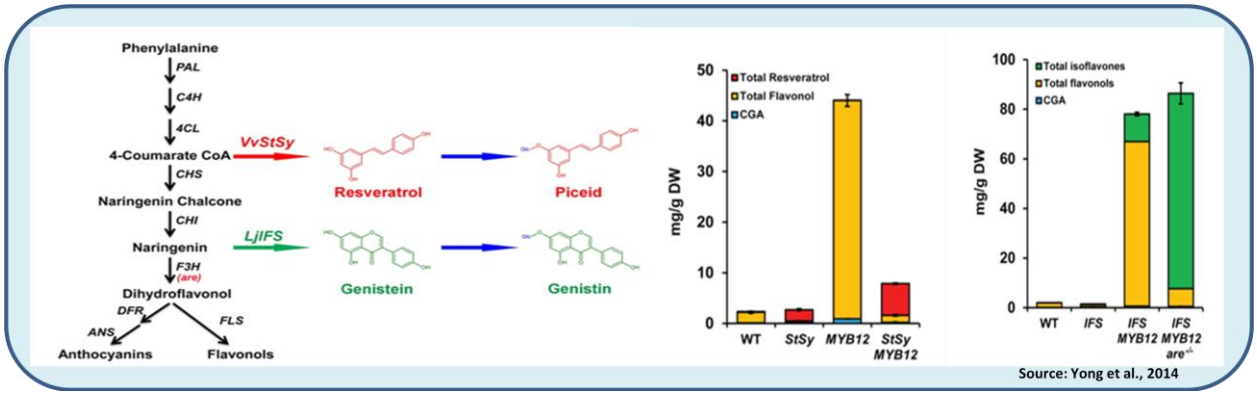

Fig. 5B Enrichment of tomato fruit with health-promoting resveratrols and isoflavonoids by co-expression AtMYB12 and structural genes (*VvStSy and LjIFS*)

These limitations on the levels of phytonutrients accumulated can be overcome by several strategies. One involves elimination of degradation or turnover pathways. And another to increase levels of phytonutrients effectively involves enhancing the levels of transcription factors that regulate specific biosynthetic pathways. It has been shown that AtMYB12 can be used as a general tool to engineer appreciable levels of phenylpropanoids in tomato. By direct activation of *ENO* and *DAHPS*, as well as genes of secondary metabolism, AtMYB12 can reprogram carbon flux towards the biosynthesis of phenylpropanoids. Co-expression of AtMYB12 with other TFs or structural genes resulted in the highest yields so far reported for anthocyanins, stilbenes and isoflavones in tomato fruit. Metabolic engineering that combines transcriptional regulation, flux control and pathway rerouting offers an excellent strategy for biofortification of foods and for the production of plant-derived phytochemicals and ingredients. It has been shown to be extremely effective in enhancing levels of phytonutrients that are secondary metabolites, such as anthocyanins (3 mg per g fresh weight; Butelli et al, 2008), flavonols (10 mg per g fresh weight; Luo et al, 2008) in tomato fruit. Recent study data confirmed that the original *35S:VvStSy* tomato produces about 0.5 mg/g DW trans-resveratrol in fruit. However, after co-expression with *AtMYB12*, the production of resveratrol and resveratrol derivatives reached between 5 and 6 mg/g DW, some 100-fold higher than in red grapes. In fruit expressing *LjIFS* only, the content of genistin, the only isoflavonoid detected, was about 0.3 mg/g DW. After the introduction of *AtMYB12*, the genistin content was significantly increased to about 11 mg/g DW. In lines homozygous for the *are*$^{-/-}$ mutation, the total amount of isoflavones increased to reach about 78 mg/g DW, some 100-fold higher than genistin levels in soya products such as tofu and natto (Yang et al, communicated fig 5 A & B).

E] Disease Diagnostics and quarantine:

Detection and diagnosis of plant viruses has included serological laboratory tests since the 1960s. With the advent of molecular biology and the ability to compare regions of genomic DNA representing conserved sequences, the development of laboratory tests increased at an amazing rate for all groups of plant pathogens. Comparison of ITS regions of bacteria,

fungi, and nematodes has proven useful for taxonomic purposes. Sequencing of conserved genes has been used to develop PCR-based detection with varying levels of specificity for viruses, fungi, and bacteria. Combinations of ELISA and PCR technologies are used to improve sensitivity of detection and to avoid problems with inhibitors or PCR often found in plants. The application of these modern technologies in plant pathology has greatly improved our ability to detect plant pathogens and is increasing our understanding of their ecology and epidemiology useful for their quarantine as well. CPRI being the Accredited Testing Laboratory (NCS-TCP) has developed the many modern, fast reliable detection tools for testing the pathogens. We have developed triplex lateral flow immune assay (LFIA) for simultaneous detection of PVX, PVA and PVM viruses in potato samples. A duplex RT-PCR assay for CMV and TMV and a multiplex RT-PCR assay for simultaneous detection of PAMV, PVS, PVM, PLRV and PVX are developed and are routinely being used in testing of the germplasm, mericlones and post entry quarantine materials (Fig. 6).

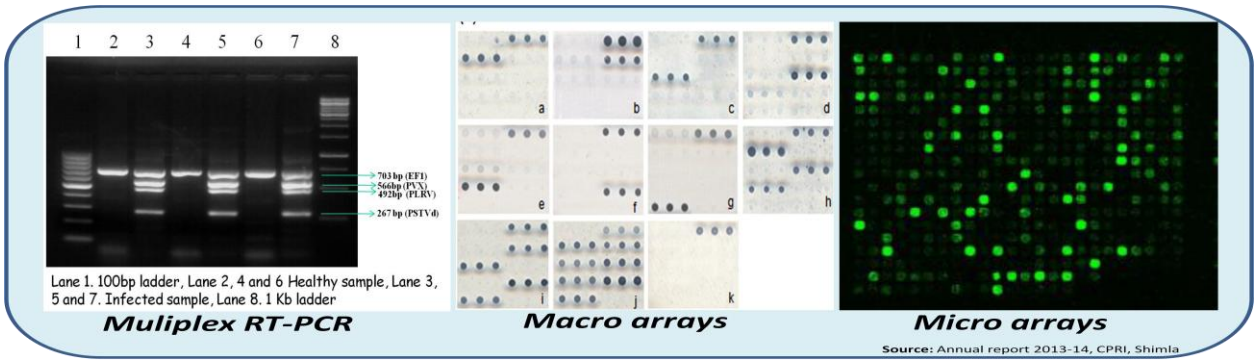

Fig. 6 Modern biotechnological tools used for virus detection at CPRI

F] Transgenic technology

Genetic modification of crops has enabled plant breeders to modify plants in novel ways and has the potential to overcome important problems of modern agriculture. Introduction of genes into plants has been made possible using *Agrobacterium* as a biological vector, and direct gene transfer techniques.

Indirect gene transfer using *Agrobacterium* as vector

The most widely used method for the introduction of new genes into plants is based on the natural DNA transfer capacity of *Agrobacterium tumefaciens*. In nature this soil bacterium causes tumor formation (called crown gall) on a large number of dicotyledonous plant species. During this infection a part of the Ti-plasmid of *Agrobacterium*, called T-DNA, is transferred and integrated into the plant genome. This natural capacity made us use this bacterium as a natural vector of foreign genes (inserted into the Ti-plasmid) into plant chromosomes. *Agrobacterium*-based and direct gene transfer techniques were developed in parallel, but the former is today the most widely-used method because of its simplicity and efficiency in many plants, although it still suffer limitations in terms of the range of species which are amenable to transformation. These limitations are due to the natural host range of *Agrobacterium*, which generally infects herbaceous dicotyledonous species most efficiently

and is less effective on monocotyledonous and woody species (De Cleene and De Ley, 1976).

The *Agrobacterium* transformation methods are using two different procedures. The first one is transformation that is dependent on a regeneration procedure from explants which is very widely used in many plant species. While the second is *in planta* methods for *Agrobacterium*-mediated transformation of that do not involve any tissue culture steps. In the first described procedure (Feldmann and Marks, 1987) imbibed seeds are infected with *Agrobacterium*, allowed to grow into mature plants and finally transformants were identified among the seeds harvested from these plants. Bechtold, *et al*. (1993) inoculated flowering *A. thaliana* plants by vacuum infiltration with an *Agrobacterium* suspension and managed to get transformants at even higher frequencies. Another technique which has been developed recently (Clough and Bent, 1998) is floral dip. It is a simple dipping of developing floral tissues into an *Agrobacterium* suspension. These *in planta* methods are now widely tried in regeneration recalcitrant leguminous crops for developing transgenic plants and are gaining importance in these days. The absence of any tissue culture step (so somaclonal variation does not occur), the simplicity and the relatively high efficiency of the transformation procedure would make such techniques attractive to adapt the technique to other plant species, recalcitrant to regeneration procedure.

Direct gene transfer

The development of novel direct gene transfer methodology, by-passing limitations imposed by *Agrobacterium*-host specificity and cell culture constraints, has allowed the engineering of almost all major crops, including formerly recalcitrant cereals, legumes and woody species. Direct gene transfer transformation methods are species and genotype-independent in terms of DNA delivery, but their efficiency is influenced by the type of target cell, and their utility for the production of transgenic plants in most cases depends on the ease of regeneration from the targeted cells, as most methods operate on cells cultured *in vitro*. As direct gene transfer referred methods such as particle bombardment, DNA uptake into protoplasts, treatment of protoplasts with DNA in the presence of polyvalent cations, fusion of protoplasts with bacterial spheroplasts, fusion of protoplasts with liposomes containing foreign DNA, electroporation-induced DNA uptake into intact cells and tissues, silicon carbide fiber-induced DNA uptake, ultrasound-induced DNA uptake, microinjection of tissues and cells, electrophoretic DNA transfer, exogenous DNA application and imbibition, macroinjection of DNA (Barcelo and Lazzeri, 1998· Walden and Schell, 1990). Worldwide, transgenic are being developed for number of traits, namely (i) resistance to herbicides (ii) pollination control mechanisms (iii) insect resistance (genes form bacteria and plants) (iv) virus resistance (v) resistance to fungi (antifungal proteins or R genes) (vi) nutritional improvement (vii) senescence retardation (viii) tolerance to abiotic stresses and (ix) production of valuable pharmaceuticals and secondary metabolites.

At CPRI transgenic technology is being used extensively for developing transgenic potato lines biotic, abiotic and quality traits. The replication-associated protein gene (AC1) of the virus was used successfully for engineering pathogen derived resistance against ToLCNDV virus in two popular potato cultivars K. Pukhraj and K. Badshah. The heat stress tolerance K. Bahar transgenic lines were developed using the 7.6 Kda *HSP* gene (476 bp) from heat tolerant variety K. Surya using the *Agrobacterium*-mediated transformation method. The late

blight resistance K. Jyoti transgenic lines were developed using 1.36 Kb F-Box gene isolated from late blight resistant *Solanum spegazinii* and being tested in containment facility. A review of most recent attempts of genetic transformation in other crop plants and transgenics for improved nutritional status are presented in Table 2 and 3 respectively.

Table 2: Review of most recent attempts of genetic transformation in other crop plants.

S.No.	Gene (source)	Methodology	Description of the transgenic	Reference
1.	*HVA1 (* Barley)	Biolistics	Drought and salt stress tolerant maize	Nguyen and Sticklen, 2013
2.	*SGTL1 (Withania somnifera)*	*Agrobacterium*-mediated (floral dip method)	Salt,heat & cold tolerant *Arabidopsis thaliana*	Mishra *et al,* 2013
3.	*NHX1 (Arabidopsis thaliana)*	*Agrobacterium*-mediated	Salt tolerance in *Brasica napus*	Dorani-Uliaie *et al,* 2012
4.	Synthetic promoters containing pathogen and/ or defence signalling inducible *cis*-acting regulatory elements (RE) fused to fluorescent protein (FP) reporter	*Agrobacterium*-mediated (floral dip method)	Pathogen sensing transgenic tobacco and *Arabidopsis* plants	Liu *et al,* 2013
5.	Acetyl-transferases (*Aspergillus nidulans)*	*Agrobacterium*-mediated (floral dip method)	Transgenic *Arabidopsis* and *Brachypodium* plants with decreased polysachharide acetylation and increased pathogen resistance	Pogorelko *et al,* 2013
6.	*Cry1Ab*	*Agrobacterium*-mediated	Insect resistant transgenic rice	Qi *et al,* 2013
7.	*ß-Glucuronidase*	Biolistics	Transgenic triticale	Karadag *et al* 2013
8.	Ribosome Inactivating Protein (Barley)	*Agrobacterium*-mediated	Enhanced resistance to *Rhizoctonia solani* in potato	M"hamdi *et al,* 2013
9.	ß-fructofuranosidase (FFase) of *Aspergillus niger* ATCC 20611 c	*Agrobacterium*-mediated	Fructooligosaccharide production in transgenic tobacco plants	Fukutomi *et al,* 2013
10.	*bar* and the *gus-intron* genes	*Agrobacterium*-mediated	Transgenic peach	Soliman, 2013

Table 3: An overview of reports on transgenic plants with improved nutritional status

S.No.	Transgenic plant	Description	Reference
Transgenic plants with improved amino acid/protein content			
1.	Transgenic canola and soybean seeds with increased Lysine	Feedback regulation system for lysine synthesis made insensitive	Falco *et al,*1995
2.	Transgenic lupins (*L. angustifolius* L.) expressing a sunflower seed albumin gene	Enhanced methionine levels and increased nutritive value of seeds	Molvig *et al,*1997
3.	Transgenic potato plants with increased protein content *ama I* from *A. hypochondriacus*	Non-allergenic seed albumin gene	Chakraborty *et al, 2000*
4.	Soybean seeds with enhanced methionine levels in seeds	Expresses feedback-insensitive cystathionine γ-synthase	Song *et al,* 2013
Transgenic plants with altered fatty acid composition			
5.	Transgenic canola having higher levels of 8:0 and 10:0 fatty acids	Overexpression of *FatB2 Cuphea hookeriana*	Dehesh *et al,*1996
6.	Transgenic rice plants with improved seed oil quality	Soybean microsomal omega-3 fatty acid desaturase gene expressing rice plants	Anai *et al,* 2003
Transgenic plants with altered starch content			
7.	Potatoes with freeze-thaw stable starch containing tubers	An amylose-free starch with short-chain amylopectin was produced by simultaneous antisense downregulation of three starch synthase genes	Jobling *et al,* 2002
8.	High-amylose potatoes	Antisense gene targeting of two branching enzymes coding genes *sbeI* and *sbeII*	Schwall *et al,* 2000; Hofvander *et al,* 2004; Andersson *et al,* 2006
9.	Sweet potato plants with increased amylose content in starch	RNA interference of the starch branching enzyme II gene (*IbSBEII*)	Shimada *et al,* 2006
Micronutrients and functional metabolites			
10.	Canola plants with increased Vitamin E content (α-Tocopherol)	Increased expression of expression of □-tocopherol methyltransferase	Shintani and DellaPenna, 1998
11.	Tomato fruits with increased β-carotene and lycopene	*β-Lcy* gene expression in tomato fruits modified	Rosati *et al,* 2000
12.	Rice with increased iron content with increased bioavailability	Rice plants contained ferritin gene from *Phaseolus vulgaris* for increased iron content in rice grains, a thermotolerant phytase	Lucca *et al,* 2001

		from *Aspergillus fumigatus* into the rice endosperm, for increased bioavailability and endogenous cysteine-rich metallothionein-like protein for increased absorption.	
13.	Tomato fruits with enhanced aroma and flavor on engineering of terpenoid metabolic pathway	*Overexpression of Clarkia breweri S*-linalool synthase (*LIS*) gene causes increased accumulation of S-Linalool	Lewinsohn *et al,* 2001
14.	Tomato fruits with increased flavonols	Overexpression of *Petunia chalcone* isomerase	Muir *et al,* 2001
15.	Transgenic maize plants with increased Vitamin C	Wheat dehydroascorbate reductase (DHAR) gene over-expressed in maize	Chen *et al,* 2003
16.	Enhanced zinc and iron accumulation in transgenic rice	Cloning and over-expression of soybean *ferritin* gene in rice	Vasconcelos *et al,* 2003
17.	Corn plants with increased Vitamin E	Overexpression of barley homogentisic acid geranylgeranyl transferase (HGGT) resulted in increased tocotrienol and tocopherol	Cahoon *et al,* 2003
18.	Higher vitamin E in Soybean seeds	*Arabidopsis* genes *At-VTE3* and *At-VTE4* (γ-tocopherol methyltransferase) expressed in soybean seeds	Van Eennemaan *et al,* 2003
19.	Transgenic multivitamin corn	Increased accumulation of ascorbate, folate and β-carotene in endosperm	Naqvi *et al,* 2009
20.	Transgenic tomato plants with increased carotenoid, tocopherol, phenylpropanoids, flavonoids, and anthocyanidins	Fruit-specific downregulation of the *DE-ETIOLATED1* (*DET1*) gene	Enfissi *et al,* 2010
Genetic manipulation of fruit ripening			
21.	Transgenic tobacco with altered ethylene production and perception	Silencing of ACS gene over expression of RTE1	Knoester *et al,*1997; Zhou *et al,* 2007
22.	Transgenic tomato fruits with altered cell wall softening	Silencing of *LeExp1* gene Silencing of PG gene	Brummell *et al,* 2002; Smith *et al,* 1988
23.	Transgenic fruits with altered sweetening	Over expression of □-fructosidase & Invertase gene	Klann *et al,*1993; Xie *et al,* 2007
24.	Transgenic fruits with altered volatile production	Over expression of Geraniol synthase gene	Davidovich-Rikanati *et al,* 2007
Designer traits			
25.	Parthenocarpic eggplants	*DefH9-iaaM* overexpression in eggplant	Acciarri *et al,*2002

As a result of consistent and substantial benefits during the initial years since 1996 of commercialization of transgenic or GM crops, farmers have continued to plant more biotech crops every single year. The rate of increase of acreage of GM crops is over 10% (James, 2010), and the most recent data suggests that GM crops being planted in more than 1.7 million ha land world over (Brookes and Barfoot, 2013). A database on field trials and commercialization of transgenic crops is maintained by Green Industry Biotechnology Platform (GIBP), an association of major European Plant Biotechnology Companies. A partial list of prominent transgenics released so far are is provided in Table 4. Thirty four countries, at present have allowed commercial cultivation of GM crops (Table 4), with nineteen of them being developing nations. China was the first country to grow a commercial transgenic crop. Interestingly, more number of developing countries is rapidly joining the list (Table 4). The benefits have been drawn more by farmers in developing countries than in the developed countries (Brookes and Barfoot, 2011). Commercialization of transgenic crops is often subject to independent approvals from several agencies, chiefly depending on the trait improved and ultimate use of the crop. In India, the jurisdiction of approval for commercialization rests on Genetic Engineering Action Committee (GEAC), jointly under Ministry of Science and Technology, and Ministry of Environment and Forests.

Indian Scenario: India, the largest cotton growing country in the world, where 60 million people are impacted by cotton, reported an average gain of 38% yield in cotton till 2011, translating to US$ 267 average income per hectare, by the use of GM cotton (Brookes and Barfoot, 2013). *Bt* cotton was first introduced for commercial cultivation in 2002, and has increased yield by up to 55%, reduced insecticide sprays by 63%, with environmental and health implications (Jagadish, 2012). The story of Bt cotton in India is remarkable. With political will and farmer support in place, adoption is projected to continue increasing with Bt cotton plantings escalating to upto 92% (James, 2010). As a result, India ranked fourth on the list of largest GM crop growing countries in the world in 2010 (James, 2010). *Bt* Brinjal became first GM vegetable crop in India to reach the approval stage for commercialization and consumption by humans (Kumar *et al,*2011). *Bt* Brinjal carries the gene *cry1Ac* from *Bacillus thuringiensis* and has been developed by the Maharashtra Hybrid Seed Company Ltd., (Mahyco), the Tamil Nadu Agricultural University, Coimbatore, the University of Agriculture in Dharwad (Karnataka) and Indian Councils of Agricultural Research (ICAR). The developers of *Bt* brinjal have carried out rigrous containment and confined field trials, as discussed by Kumar *et al,*(2011). However, following aggressive protests by non government players (As discussed later), release of *Bt* brinjal in to the market has been put on hold for "indefinite time" by Government of India. If released, *Bt* brinjal, based on its potential to reduce the consumption of pesticide by 77%, and increase in yield by 116%, is likely to increase average income of the farmer by US$300 per hectare (James, 2010). Other food crops like maize, sorghum, rice, cabbage, cauliflower, tomato, groundnut, etc. too are awaiting regulatory approvals for field trials and cultivation in India. However, their fate depends on the decision taken by the government based on the petitions filed both by supporters and protesters of GM crops in the country (Jagadish, 2012).

Table 4: Description of some commercialized transgenic crops

S.No.	Trade name and crop	Trait	Developer	Countries where commercialized
1	32138 SPT maintainer Maize	PCS	DuPont	USA
2	Amflora™ Potato	MPQ	BASF	EU
3	Atlantic NewLeaf™ Potato	IR	Monsanto	Australia, Canada, Mexico, New Zealand, USA
4	Bollgard™ Cotton	IR	Monsanto	Canada, Japan, New Zealand, USA
5	BT Shanyou 63 Rice	IR	HAU, China	China
6	Bt Xtra™ Maize	IR	Monsanto	Australia, Canada, Japan, New Zealand, Philippines, South Korea, Taiwan, USA,
7	BXN™ Cotton	HT	Monsanto	Australia, Canada, Japan, Mexico, New Zealand, USA
8	CDC Triffid Flax	HT	University of Saskatchewan	Canada, Colombia, USA
9	Cultivance Soybean	HT	BASF	Argentina, Brazil, Mexico, Philippines, Russian Federation, USA
10	Elizaveta plus Potato	IR	CB, Russian Acad.Sci.	Russian Fedretion
11	Enlist™ Maize	HT	Dow Agro Sciences LLC	Australia, Canada, Mexico, New Zealand, South Africa, Taiwan, USA
12	Enogen™ Maize	MPQ	Syngenta	Australia, Canada, Japan, Mexico, New Zeland, Philippines, Russian Federation, South Korea, Taiwan, USA
13	Fibermax™ Liberty Link™ Bollgard II™ Cotton	HT, IR	Bayer Crop Science	Australia, Japan, Mexico, New Zealand, South Korea
14	FLAVR SAVR™ Tomato	MPQ	Monsanto	Canada, Mexico, USA
15	Genuity® DroughtGard™ Maize	AST	Monsanto	Australia, Canada, Japan, Mexico, New Zealand, Taiwan, USA
16	Genuity® Roundup Ready™ 2 Xtend™ Soybean	HT	Monsanto	Australia, Canada, Japan, Mexico , New Zealand, USA
17	Glytol™ x Twinlink™ Cotton	HT, IR	Bayer Crop Science	Brazil
18	Herculex XTRA™ Maize	HT, IR	Dow Agro Sciences LLC and DuPont	Canada, EU, Japan, Mexico, Philippines, South Africa, South Korea, Taiwan, Turkey, USA
19	Hi-Lite NewLeaf™ Y Potato	DR, IR	Monsanto	USA
20	Huahui-1 Rice	IR	HAU, China	China
21	Huanong No. 1 Papaya	DR	SCAU, China	China
22	Intacta™ Roundup Ready™ 2 Pro Soybean	HT, IR	Monsanto	Argentina, Brazil, EU, Mexico, Paraguay, South Korea, Uruguay
23	InVigor™ Maize	PCS	Bayer Crop Science	Canada, USA
24	JK-1 Cotton	IR	JK Agri Genetics Ltd.	India
25	Laurical™ Canola	MPQ	Monsanto	Canada, USA
26	Liberty Link™ Independence™ Argentine	HT	Bayer Crop Science	Japan, USA

	Canola			
27	Liberty Link™ Sugarbeet	HT	Bayer Crop Science	Canada, Japan, USA
28	Lugovskoi plus Potato	IR	CB, R Acad. Sci.	Russian Fedretion
29	Mavera™ YieldGard™ Maize	MPQ	Renessen LLC and Monsanto	Japan, Mexico, USA
30	Moondust™ Carnation	MPQ	Florigene Pty Ltd.	Australia, EU, Japan, Norway
31	Moonlite™ Carnation	MPQ	Florigene Pty Ltd.	Australia, Japan
32	NaturGard KnockOut™, Maximizer™ Maize	HT, IR	Syngenta	Argentina
33	Navigator™ Canola	HT	Bayer Crop Science	Australia, Canada, China, Japan, New Zealand
34	New Leaf™ Plus Russet Burbank Potato	HT	Monsanto	Australia, Canada, Japan, Mexico, New Zealand, Philippines, South Korea, USA
35	Ngwe Chi 6 Bt Cotton	IR	Cotton and Sericulture Depart., Myanmar	Myanmar
36	Optimum® Gly Canola	HT	DuPont	Canada, Mexico, USA
37	Optimum™ GAT™ Maize	HT	DuPont	Argentina
38	Phytaseed Canola	MPQ	BASF	USA
39	Power Core™ Maize	HT, IR	Monsanto and Dow Agro Sciences LLC	Argentina
40	Rainbow, SunUp Papaya	DR	Cornell University & University of Hawaii	Canada, Japan, USA
41	Roundup Ready™ 2 Maize	HT	Monsanto	Argentina
42	Roundup Ready™ Bollgard™ II Cotton	HT, IR	Monsanto	Australia, Costa Rica, EU, Japan, Mexico, New Zealand, Philippines, South Korea
43	Roundup Ready™ Canola	HT	Monsanto	Australia, Canada, Chile, China, EU, Japan, Mexico, New Zealand, Philippines, South Korea, USA
44	Roundup Ready™ Wheat	HT	University of Florida	Australia, Colombia, New Zealand, USA
45	Seed Link™ Chicory	HT, PCS	Bejo Zaden BV	USA
46	Shepody NewLeaf™ Y Potato	DR, IR	Monsanto	Australia, Canada, Japan, Mexico, New Zealand, Philippines, South Korea, USA
47	Starlink™ Maize	IR	Bayer Crop Science	USA
48	Superior NewLeaf™ Potato	IR	Monsanto	USA
49	TruFlex™ Roundup Ready™ Canola	HT	Monsanto	Australia, Canada, Japan, New Zealand, USA
50	TwinLink™ Cotton	HT, IR	Bayer Crop Science	Brazil, Canada, South Korea, USA
51	VIPCOT™ Roundup Ready Flex™ Cotton	HT, IR	Syngenta and Monsanto Company	Costa Rica
52	Vistive Gold™ Soybean	HT. MPQ	Monsanto	Australia, Canada, Mexico, New Zealand, USA

53	Widestrike™ Roundup Ready Cotton	HT, IR	Monsanto and Dow Agro Sciecnes LLC	Costa Rica, Japan, Mexico, South Africa
54	YieldGard™ Plus Maize	IR	Monsanto	EU, Japan, Mexico, Philippines, South Africa, South Korea, Taiwan, USA,

AST- Abiotic stress tolerance, DR- Disease resistance; HT- Herbicide tolerance, IR- Insect resistance, MPQ- Modified product quality, PCS- Pollination Control System

In conclusion, the modern biotechnological tools will contribute in hasten the process of crop improvement. Tissue culture and transgenic technologies will provide modern crop plants for improved biotic and abiotic stress tolerance as well as improved quality traits. The post-genomics era, high-throughput approaches combined with automation, increasing amounts of sequence data in the public domain, and enhanced bioinformatics techniques will contribute to genomics research for crop improvement. However, the costs of applying genomics strategies and tools are often more than is available in commercial or public breeding programs, particularly for inbreeding crops or crops that are only of regional importance. Nevertheless, marker-assisted breeding or marker-assisted selection will gradually evolve into 'genomics-assisted breeding' for crop improvement. Newly developed genetic and genomics tools will enhance, but not replace, the conventional breeding and evaluation process. The ultimate test of the value of a genotype is its performance in the target environment and acceptance by farmers.

Structural Genomics and Metagenomics applying Next Genration Sequencing technology

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An Introduction to Genomics and Structural Genomics

Entire genetic composition of an organism is called the **genome**. There are several kinds of genomes in the nature like prokaryotic genome, eukaryotic genome, nuclear genome, mitochondrial genome and chloroplast genome. **Genomics** is the study of the molecular organization of these genomes, their information content, and the gene products they encode. It is a broad discipline, which may be divided into at least three general areas. **Structural genomics** is the study of the physical nature of genomes. Its primary goal is to determine and analyze the DNA sequence of the genome. **Functional genomics** is concerned with the way in which the genome functions. That is, it examines the transcripts produced by the genome and the array of proteins they encode. The third area of study is **comparative genomics,** in which genomes from different organisms are compared to look for significant differences and similarities. This helps identify important, conserved portions of the genome and discern patterns in function and regulation. The data also provide much information about microbial evolution, particularly with respect to phenomena such as horizontal gene transfer.

Structural genomics seeks to describe the 3-dimensional structure of every protein encoded by a given genome. This genome-based approach allows for a high-throughput method of structure determination by a combination of experimental and modeling approaches. The principal difference between structural genomics and traditional structural prediction is that structural genomics attempts to determine the structure of every protein encoded by the genome, rather than focusing on one particular protein. With full-genome sequences available, structure prediction can be done more quickly through a combination of experimental and modeling approaches, especially because the availability of large number of sequenced genomes and previously solved protein structures allows scientists to model protein structure on the structures of previously solved homologs.

Worldwide genome sequencing projects have produced an explosive growth of sequence information from many living species. Complete sequence information for more than 200 genomes is accessible through public domain sources, and about several times more genomes are currently being sequenced worldwide (http://maine.ebi.ac.uk:8000/services/cogent/stats.html;http://www.ncbi.nih.gov/Genomes/, http://www.tigr.org/tdb/). Although the currently known genome sizes range, in order of magnitude, from 10^6 to 10^{11} DNA base pairs, the number of genes is estimated to range only from 10^3 to 10^5 per organism. Taking the estimated 13.6 million species of living organisms on Earth into account, there are 10^{10} to 10^{12} different proteins encoded by organisms from three domains of life (eukaryotic, prokaryotic, and archaea). Thus, the protein universe is vast and diverse.

Next Generation sequencing technologies

Pyrosequencing

The pyrosequencing technology (http://www.454.com) was the first NGS, derived from technical combination of pyrosequencing chemistry and emulsion PCR. The basis of this technology was sequencing by synthesis, a different approach of DNA sequencing by pyrophosphate detection was also reported. A team lead by Nyren in 1993 came out with a sequencing approach based on chemi-luminiescent detection of pyrophosphate released during deoxynucleotide triphosphate (dNTP) incorporation. Later, up gradation of technique by Ronaghi and coworkers laid the foundation stone for the commercial development of pyrosequencing at Royal Institute of Technology, Stockholm in 1996. 454 Life sciences founded by Jonathan Rothberg in 2000, launched first commercially available NGS platform named GS 20 in 2005. The technology is incessantly being upgraded several times into a routine functioning method. In 2007, Roche introduced newer version as GS FLX with a unique flowcell referred 'picotiter plate' (PTP) comprising 3.4 $\times 10^6$ separate sequencing reaction wells allowing hundreds of thousands of sequencing reactions to be carried out in parallel and massive high-throughput way. The current pyrosequencer instrument, the GS FLX+ produces an average read length of approximately 1000 bp and throughput of approx 800Mb to 1Gb of high quality sequence data per 7-8 hr run (www.454.com/). Pyrosequencing is basically dual step approach. Firstly, single stranded DNA is fractionated into smaller fragments (300-1000bp), polished (made blunt end), and short oligo adapters having 5' biotin tag are ligated to the fragments. These adapters provide priming sequence for the attachment; amplification as well as sequencing the fragment. DNA fragments to be sequenced are then individually immobilized onto streptividin decorated beads which are amplified by the PCR in the water-oil emulsion droplets. These droplets act as individual amplification reactors producing manifold replicas ($\sim 10^7$) of the same DNA sequence on each bead. Template single stranded DNA is hybridized to sequencing primer and loaded onto the PTP plate along with DNA polymerase, ATP sulfurylase (a recombinant version from *Saccharomyces cerevisiae*), Luciferase (from firefly *Photinus pyralis*) nucleotide degrading enzyme Apyrase (from potato) along with the substrates adenosine 5' phosphosulfate (APS) and luciferin. One of the four dNTPs are added and if complementary DNA polymerase incorporates onto the template accompanied with the release of pyrophosphate (PPi) equal to molarity of incorporated nucleotide. The PPi released is quantitatively converted into adenosine tri phosphate (ATP) in presence of APS. The ATP acts as a fuel to lucifease mediated conversion of luciferin to oxyluciferin that generates light in comparative amount of ATP produced. Unincorporated nucleotides and ATPs are continuously washed away by apyrase and the next reaction start with another nucleotide addition cycle. One picomole of DNA in a pyrosequencing reaction yields $6x10^9$ photons at a wavelength of 560 nanometers, which is easily being detected by 16 mega pixel CCD camera maintained at -24 ^{0}C for its higher resolution and performance. The sequence of DNA is yielded in "pyrogram" corresponds to order of nucleotides that has been incorporated.

Pyrosequencing technology works best in cases where longer read length are in demand like *de novo* sequencing assembly and metagenomics. The run time required is also very short (few hours). The present strategy can generate more than 1,000,000 individual reads

with improved read length of 500-800 bases per 10 hour run. Additionally, the process requires short sample preparation time approximately two hours. A major limitation of pyrosequencing is difficulty in sequencing homopolymer or repetitive regions. Homopolymer regions cause dephasing due to asynchronous synthesis at the repetitive region. This will increase the sequence length and also effects error rate. Another disadvantage of pyrosequencing is its high cost ($45.0 per Mb data sequenced) as compared to other NGS technologies.

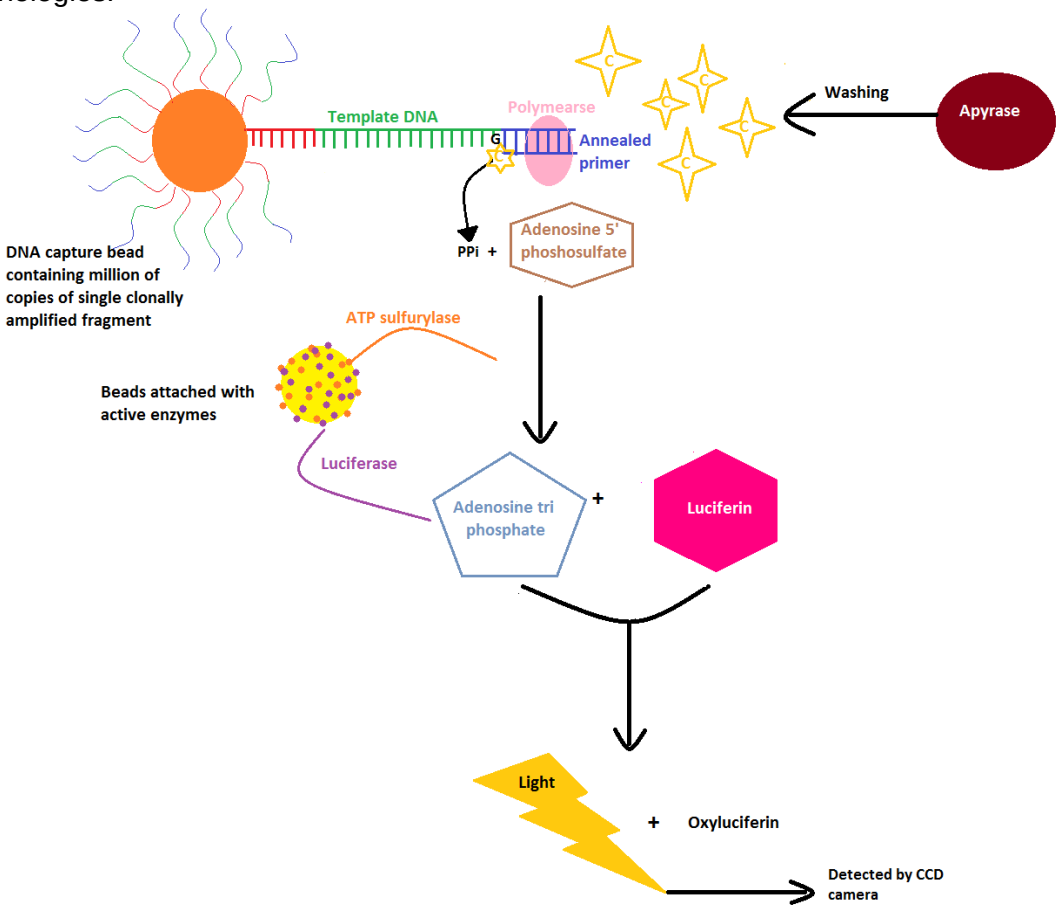

Figure 1. 454 workflow, Sequencing-by-synthesis approach inside PTP. The PPi released after successful incorporation of dNTP with substrate APS in presence of ATP sulfurylase produces ATP which emits light in proportional amount with luciferase coupled reaction.

Illumina genome analyzer

In 1997, British chemist Shankar Balasubramanian and David Klenerman conceptualized an approach for sequencing single DNA molecules attached to microspheres. They funded Solexa in 1998; however their goal of sequencing single DNA molecules was not fulfilled. The idea was then shifted towards sequencing clonally amplified templates. Year 2006, marks the commercial launch of the first 'short read' sequencing platform *Solexa Genome Analyzer*. Illumina is one of the high throughput sequencing technology among the NGS and it uses reversible terminator-based sequencing by synthesis approach. The templet DNA sample is fractionated to the average size ~800bp. The fragmented DNA ends are repaired;

5' end phosphorylated while at 3' poly A tail is added. Repairing of DNA is carried out using T_4 DNA Polymerase (digests 3' protruding ends), Klenow DNA polymerase (extension of 3'recessive ends) and T_4 PNK (phosphorylates 5'end and dephosphorylates 3'ends). Like pyrosequencing, illumina also requires template sequence to be converted to special sequencing library which insures the immobilization and amplification for sequencing. Therefore, two unique forked adaptors (adaptor oligonucleotides are complimentary to flow cell anchors) are added at the 5' and 3' end of the DNA fragment. The prepared samples are immobilized on 8 channeled flow cell surface allowing bridge amplification. Hybridization of library fragments and adaptor with that of flow cell occurs by active heating and cooling step. Subsequently, reactants and an isothermal polymerase are incubated to amplify the fragment in a discrete area 'cluster' on flow cell surface (for animation: http://www.illumina.com/) to form small clusters of single stranded fragments called 'bridge amplification'. Clusters are formed impulsively due to the fact that the newly produced copies of the fragment get attached in close proximity to the original fragment. After the bridge amplification is done, densely packed clusters of fragments formed, each cluster consisting of many copies of the same fragment, which begins the sequencing by synthesis step. For single strand sequencing of forward strands, clusters are denatured, chemically cleaved and washed. Sequencing of forward strand starts with the hybridization of sequencing primer complimentary to adaptor sequence followed by addition of DNA polymerase and mixture of four differently colored fluorescent dye terminator nucleotides. All four nucleotides are modified with distinct fluorochrome and reversible terminator group attached at its 3'hydroxyl group is chemically blocked, so that when one nucleotide is incorporated replication stops. This ensures the uniqueness of each event. DNA polymerase incorporates the appropriate nucleotide and unused nucleotides are washed away. After every incorporation cycle imaging step occurs for determining each incorporated nucleotide followed by chemical cleavage step which removes fluorescent nucleotide and unblocks the 3' end with the help of reducing agent tris (2-carboxymethyl phosphine) for next sequencing cycle. The process of adding nucleotides, imaging and removing the terminator is called a cycle. The illumina sequencing run offers very high throughput with 50×10^6 clusters per flow cell to generate of 2 to 15 GB in 2-8 days of run time and read length of 35 to 75 bases. The paired end module enables sequencing up to 2x100bp of fragments ranging from 200bp to 5kb with output reaching upto 45-50 GB. The latest technology 'Hi-seq 2500' produces around 600 Gb throughput per 11 day run with dual flow cell and another higher version of the same MiSeq® system with much more higher throughput and quality is at the doorstep for the release (http://www.illumina.com/). The run cost offered by this technology is also very low ($5.9 per Mb data sequenced). Currently, illumina is most widely used NGS platforms with 60% of global NGS installations. Though, it has very short read length with relatively higher error rates, but are compensated by coverage and throughput. The massively produced sequence data is difficult to manage and process as huge number of short reads complicate the assembly and alignment algorithm.

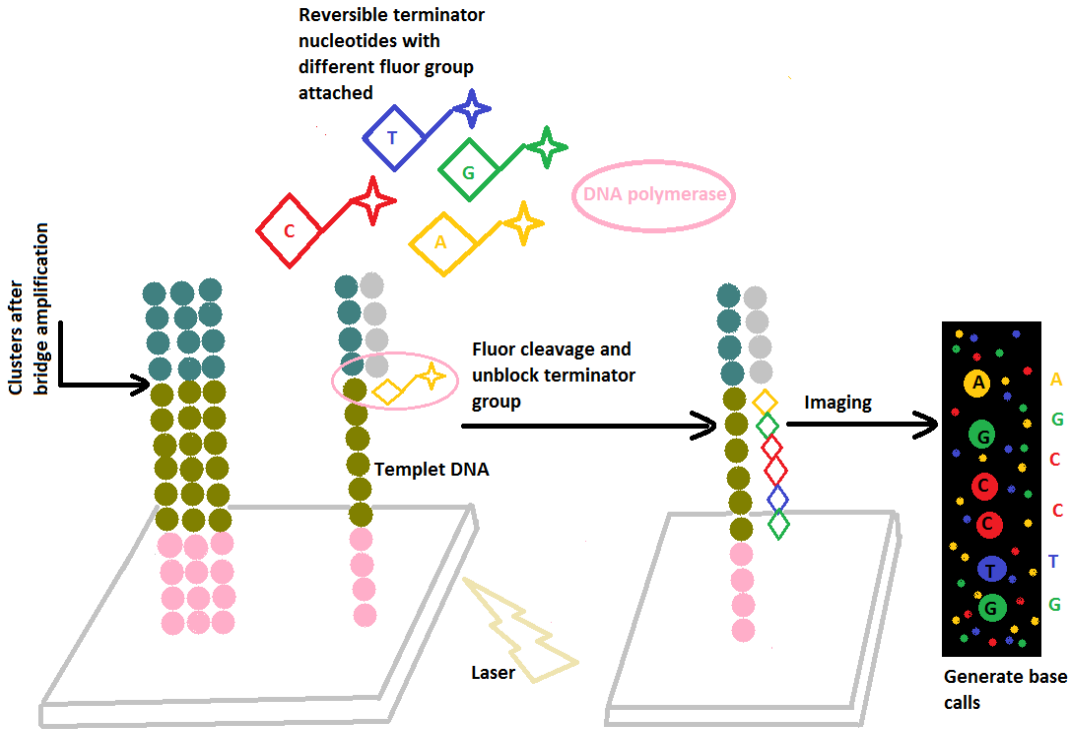

Figure 2. Illumina workflow, Reversible terminator nucleotides with different fluorescent group, primer and DNA polymerase are added to the flow cell after bridge amplification. Post incorporation fluorescence is recorded for generation of base calls subsequently fluor and block are removed before the next sequencing cycle begins.

Sequencing by Oligo Ligation and Detection (SOLiD)

The SOLiD strategy is the only NGS technology which is based on ligase mediated sequencing chemistry and di-based labeled probes, hence the name sequencing by oligo ligation and detection. The technology was developed at Harvard Medical School and was first time used successfully for the resequncing of *Escherichia coli* genome. The technology was further refined and commercially launched by Applied Biosystems in the year 2007. Sample preparation share similarities to that of pyrosequencing and Illumina. Oligo adapter is ligated to the DNA sequence to be determined, and then bound with the 1 µm magnetic beads decorated with complementary oilgos and DNA fragment is clonally amplified by emulsion PCR. After amplification the enriched beads are recovered for their immobilization onto derivitized-glass flow-cell surface by hybridizing adaptor sequence (P1 adaptor) with complimentary sequence on the flow cell. The ligation based sequencing initiate with annealing of universal sequencing primer complementary to the adapter sequence flanking the library fragment. Mixture of fluorescently labeled interrogated octamer probes hybridize to the fragment DNA. These octamers are dibase degenerate fluorescently labeled oligo nucleotides with specific dye at its 5'ends. In these octamers, first 2 nucleotides are

interrogated dinucleotide, 3rd, 4th and 5th bases are degenerate bases having cleavable site and 6th, 7th and 8th are universal bases with flouorophore attached to 5'end. The system uses sixteen possible dinucleotide combinations encoding four different fluorescent dyes. Ligase enzyme hybridizes complementary octamer oligonucleotide sequence to the target DNA; imaging signal identifies the attached oligonucleotide by the associated fluorescent dye. A chemical cleavage step cleaves off the 6th through 8th universal base thereby removing attached fluorescent group and enabling the next sequencing cycle to proceed. The cycle is repeated 7 times referred to as 'round' yielding 35bp read length. However, to increase read length additional cycles can also be performed. Second round of sequencing onsets with the stripping off synthesized strand and hybridizing another universal sequencing primer at the n-1 position followed by subsequent ligation and cleavage step. These steps are repeated with n-2, n-3, n-4 primers. Two flow cells in one run produces an overall output of 4 Gb data with two time enquiry of each base "two base encoding" which provides an extra quality check to discriminate measurement errors. High fidelity ligation chemistry coupled with interrogation of each nucleotides base twice yields sequence output with 99.9% accuracy. On an independent platform, the church laboratory at Harvard Medical School, Danaher Motion and Dover systems have collaborated to develop and introduce least expensive sequencing by ligation platform, the Polonator G.007 (http://www.polonator.org). The technology enables analyzing number of samples simultaneously at a smaller amount of reagents and low cost. The Polonator G.007 expected to produces 10-35 Gb of data per 2.5 day run. The only disadvantage with SOLiD technology is short read length which increases cost of data analysis and processing and significantly limits its applications in spite of low error rates than other NGS platforms.

Upcoming developments

True single molecule sequencing (tSMS™)

The first commercially available single molecule sequencing system, introduced by Braslavsky et al. 2003 and commercialized by Helicos Biosciences, USA (http:// www.helicosbio.com/). This is highly sensitive detection system used to interrogate single molecule of the DNA (or RNA without reverse transcription) in parallel without the need of amplification technique. Libraries are prepared by random fragmentation and poly dA tailing of the template allows it to hybridize with surface decorated poly dT oligonucleotide, anchored to glass cover slip. In every cycle called 'quad' DNA polymerase and single species of nucleotide labeled with the cyanine dye, cy5 (a non radioactive fluorescent dye) is sequentially added (ACGT, ACGT….), this causes extension of surface immobilized primer template duplexes. The cleavage of the fluorescent label enables the determination of particular nucleotide. The fluorescent 'virtual terminator' nucleotide (Cy5-12ss-NTPs) prevents the incorporation of any subsequent nucleotide until the nucleotide dye moiety is cleaved. The images from each quad are assembled to generate an overall set of sequence reads. On a standard run, 120 cycles of nucleotide addition and detection cab be carried out. Presence of 25 channel flow cells in a standard run enables 50 different samples sequencing simultaneously possible.

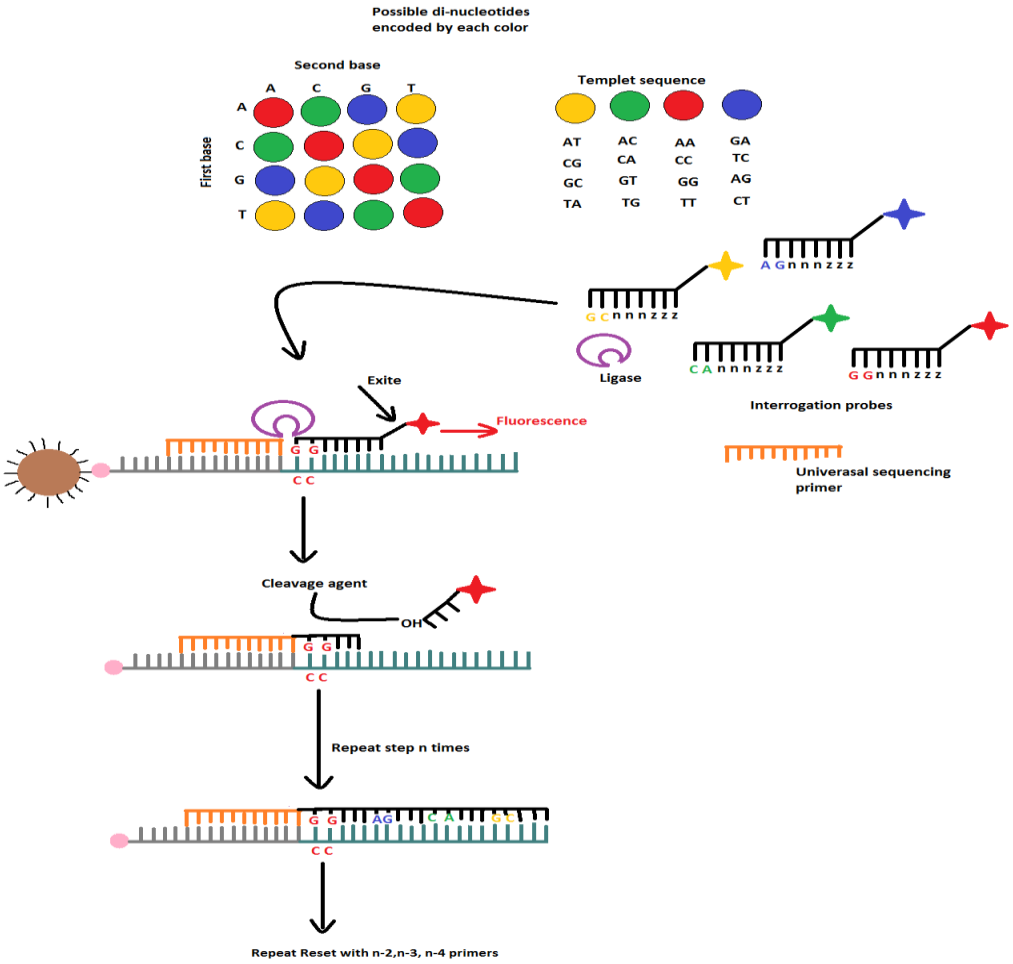

Figure 3. SoLiD work flow, DNA sequence to be determined bounded with magnetic beads immobilized on flow cell surface. Chemistry begins with the attaching universal sequencing primer complimentary to adaptor sequence flanking the library. Ligase along with fluorescently labeled octamer probes supplied to the sequencing reaction. Appropriate interrogation probes ligated to the library fragment and fluorescence group attached to it excites and fluorescence. The cycle is repeated 7 or more times for desired length. After first round the sequenced strand stripped off and the next cycle begins with n-1 position.

There are several unique features which makes tSMS an advanced sequencing strategy. Firstly, mis-incorporation arising from dephasing is not an issue. In tSMS, dephasing is bypassed as each template is monitored individually. Homopolymer is the second most error prone stage as several molecules need to be incorporated in the same cycle. In contrast, tSMS uses single molecule for interrogation therefore, the problem can be controlled by limiting the arêtes of incorporation events. Thirdly, error rate is reduced substantially by performing 'two pass' sequencing. The template strand is sequenced as usual (pass 1); the newly synthesized strand is surface tethered, and original template is denatured. Sequencing primed from distal adaptor yields second sequence for same template with opposite orientation (pass 2). Even the sub-nanogram amounts and very poor quality DNA,

including degraded or modified DNA, is sufficient for sequencing. This system has been used for variety of applications involving sequencing RNA sample providing unparalleled quantitative accuracy for RNA expression measurements. Precise expression measurement to be made with either RNA or cDNA has been possible due to very high read count per sample and also all classes of RNA molecules can be detected using this technology which was not possible previously. The technology has been utilized in sequencing ancient DNA, detection of BRCA1 mutation by single step target selection and Human gene therapy. Helicos claims the total output to be 25-35 Gb per run with a throughput of 1.1 Gb of data per hour and an average read length of 35 bp. (http://www.helicosbio.com). The only important weakness of tSMS approach is high raw error rate, which can be overcome by repetitive sequencing but increases cost per base for a given accuracy rate.

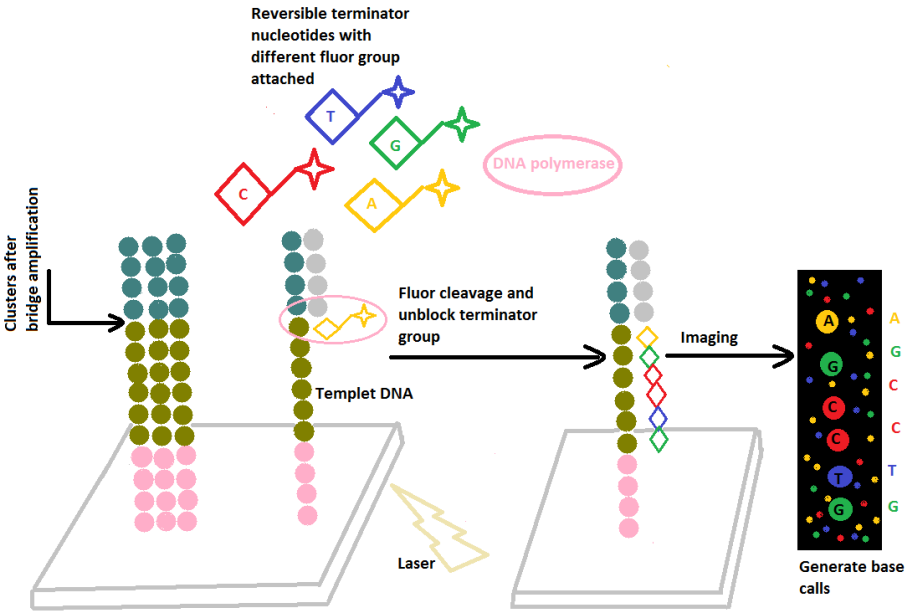

Figure 5. tSMS work flow, In every 'quad' same nucleotide moieties and DNA polymerase were added to surface hybridized by poly (dA) tailing on glass chip. Nucleotides Complimentary to suitable templet (templet 2 in above case) hybridizes and imaged followed by cleavage of dye

Single Molecule Real Time Sequencing (SMRT™)

SMRT is a paralleled single molecule DNA sequencing by synthesis developed by Pacific Biosciences (http://www.pacificbiosciences.com) based on the usage of Zero-Mode Waveguide (ZMW), developed in the laboratory of Harold Craighead at Cornell University. SMRT technology is unique in the field of DNA Sequencing and offers the ultimate combination of speed, long reads, and low costs. The axis of this technology involves specialized chip called SMRT chip, made of 100 nm thick metal film containing thousands of zero-mode waveguides (ZMW) which are basically wells of 10-50 nm diameters. Each well contains, a DNA polymerase molecule attached at the bottom, that elongates the primer

hybridized template by incorporating the ϒ-labeled dNTPs. All the four bases are fluorescently labeled at the phosphate group with distinguishable fluorophores. When a nucleotide is incorporated during DNA synthesis, the laser beam illumination of small detection volume (20 zeptoliters = 20×10^{-21}) causes attached fluorophore to light up allowing the identification of each nucleotide incorporated. During formation of the phosphodiester bond, a nucleotide is held up in the detection volume for a much longer time (milliseconds) than the time (microseconds) needed for a nucleotide to diffuse in and out of the detection volume. This increase in time facilitates proper detection[24]. The other unincorporated nucleotides float in the dark unilluminated volume of ZMW and do not light up. This strategy incorporates nucleotide at a speed of ten bases per second simultaneously in all thousands of ZMWs located on SMRT chips giving rise to a chain of thousands of nucleotides in length within minutes.

SMRT sequencing platform require minimal amount of sample and reagent for a complete run and also there are no laborious scanning and washing steps. Furthermore, this does not need any routine PCR amplification as needed in previous generation sequencing systems thereby avoiding any systematic amplification bias. The company claims the average read length of >3,000bp, which simplifies the assembly and mapping. However, premature termination caused by laser induced photo damage to polymerase and nucleotide causes short reads[33]. Turning off laser for short period of time during sequencing helps to produce longer reads named strobe reads. It takes less than a day for obtaining the result, starting from sample preparation. SMRT sequencing offers flexibility providing multiple protocols including standard, circular consensus and strobe sequencing. An additional benefit of this system is its ability to potentially detect modified bases. It is possible to detect 5-methylcytosine, although the role of sequence context and other factors affecting the accuracy of such assignments remains to be clarified[38]. Despite many potential advantages of SMRT sequencing, a number of challenges remain, like the raw read error rates can be in excess of 5%, with error rates dominated by insertions and deletions, particularly problematic errors when aligning sequences and assembling genomes. In addition, the throughput of SMRT sequencing will not initially match to that of data achieved by previous generations. Nevertheless, high raw error rates can be overcome by creating SMRT bell templates consisting double stranded region (insert of interest, 40-25,000 bp) flanked by single stranded loops on either side.

Nanopore sequencing

The launch of nanopore machine put an end to long decade wait as the method was under progress since 1995. The idea behind Nanopore sequencing was first conceived by David Deamer at the University of California. The technology is being commercialized by Oxford Nanopore technologies, which claims to sequence human genome within 15 minute. The technology involves the use of thin membrane that contains nanopore of ~1.5 to 2 nm diameter. The target DNA is placed on one side and current is applied across the membrane. The negatively charged DNA translocates through membrane and blocks the channel which generates alteration in electrical conductance leading to change in current in the range of pico amps (pA). This enables the discrimination of DNA molecules with different sequences[40]. Nanopores of great interest includes solid state nanopore like carbon nanotubes and thin films plastic materials biological protein nanopore like α-hemolysin and MspA have been investigated for sequencing. Of all these, protein nanopore proves to be

more advantageous as they can be genetically and chemically modified to optimize the detection of specific bases and translocation rate of DNA through the pore. The α-hemolysin is a toxic protein from *Staphylococcus aureus,* which is highly stable and remains functional even at boiling point. It was also demonstrated that inner diameter of the protein is as wide as single stranded nucleic acid; and helps locally to unknot the coiled nucleic acid enabling it to translate in strictly single file and sequential order.

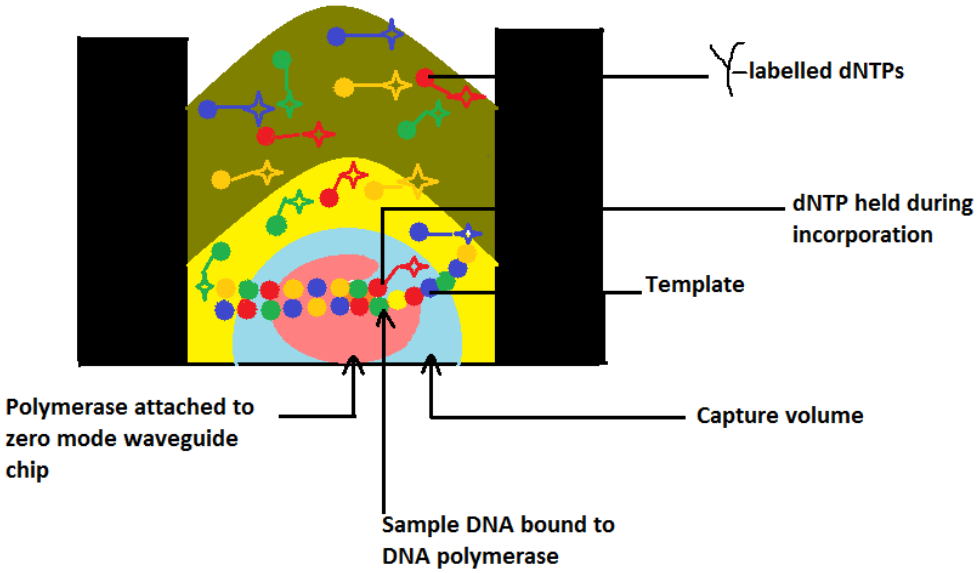

Figure 6. SMRT, Cross section of single ZMW. DNA polymerase immobilized on the bottom lengthens primed templet. Fluorescently labeled nucleotides when incorporated to growing nucleotide chain fluorophore group attached to the nucleotide bases lights up which aids in imaging and identifying characteristic nucleotide.

High translocation speeds (potentially millions of bases per second) creates barrier in the signal. In such cases detecting a signal over background noise can be a challenging task. Variety of methods have been used to control the pace of DNA through nanopores, including attachment of polystyrene beads[46] varied salt concentrations[47] viscosity magnetic fields introduction of regions of double-stranded DNA on a single-stranded target[41] and the attachment of polymerase to retach DNA through the α-hemolysin pore. Efforts to overcome this came out in the form of modification to basic nanopore technology described as *hybridization assisted nanopore sequencing* (HANS) developed by NABsys (http://nabsys.com/) which combines nanopore sequencing with sequencing by hybridization. The approach involves hybridizing molecule to be sequenced with known probe sequence and the resulting hybrid will pass through a nanopore. The changes in the current can be measured and subsequently hybridization sites can be determined. Another modified version of nanopore sequencing is '*design polymer'-assisted nanopore sequencing* developed by LingVitae (http://www.lingvitae.com/) which involves conversion of target DNA into magnified form called 'design polymer' and encoding the sequence by transformed nucleotide sequence, using binary code of molecular beacons.

The most important advantage of nanopore sequencing that makes it a unique and inexpensive technology over other methods is undemanding sample preparation, requiring minimal chemistries, eliminating the need of fluorescent nucleotides, enzymes, cloning and amplification steps. Oxford nanopore sequencing technologies expects to start selling its new machine in second half of this year in the high throughput electronic platform 'GridION' and MinION (a disposable sequencer of a USB memory stick size)[50]. Albeit of many advantages nanopore sequencing also possess some loopholes. Firstly, mechanical instability of lipid bilayer supporting nanopore requires continuous monitoring. Secondly, the sensitivity of biological nanopores to experimental conditions (such as pH, temperature and salt concentration) and finally, difficulty in integrating biological systems into large-scale arrays.

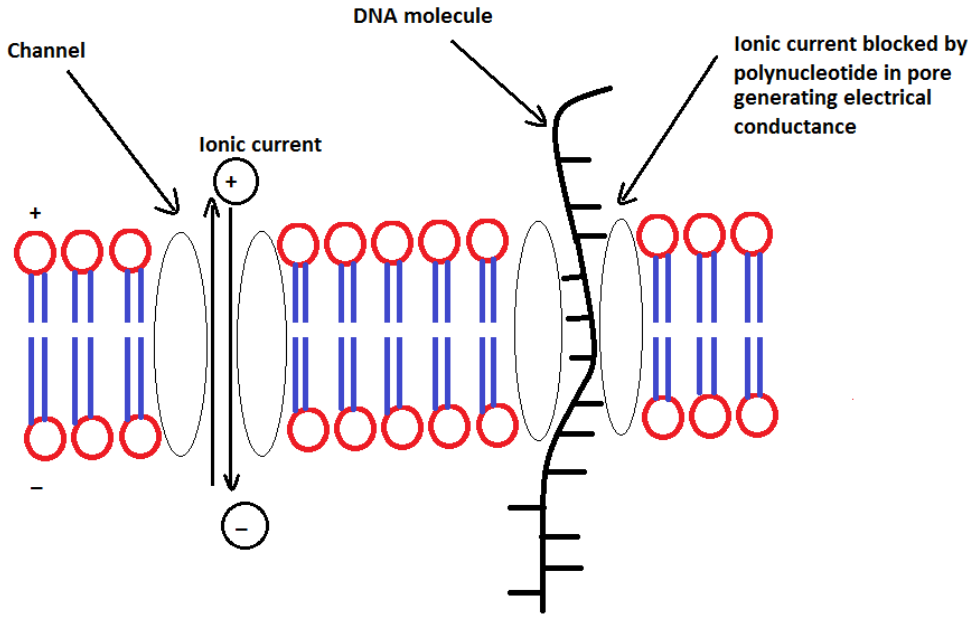

Figure 7. Nanopore sequencing, Target DNA when placed on one side of membrane translocates through sieve of nanopore. When current is applied negatively charged DNA translocation blocks the channel that alters electrical conductance and the change is translated into nucleotide sequence.

FRET based approach

Life Technologies, a major provider of both first and second generation sequencing systems, is developing fluorescence resonance energy transfer (FRET) based single molecule sequencing by synthesis approach technology, initially introduced by VisiGen. This type of approach could be considered an improvement over the Helicos technology. A theoretical throughput of 1 Mb per instrument second has been given, but right now it is difficult to measure progress. The basic idea behind this strategy was, monitoring of polymerase dependent incorporation of nucleotide bases into the DNA strand. The specialized DNA polymerase contains donar fluorophore, each nucleotide species carries one of the four differently colored acceptor fluorophor. When a nucleotide is incorporated, the proximity of donar-acceptor fluorophor results in a signal called 'FRET'. The FRET signal is specific for a

particular nucleotide incorporated at the particular position. After the nucleotide has been incorporated, the pyrophosphate containing fluorophor is released, thereby quenching signal and preparing for next step. Significant advances have been made with the commercial release of the technology in the form of 'starlight' system expected in near future. The current technology consists of a quantum-dot-labeled polymerase that synthesizes DNA using four distinctly labeled nucleotides in a real-time system. Quantum dots, which are fluorescent semiconducting nanoparticles, have an advantage over fluorescent dyes as they are much brighter, larger and less susceptible to bleaching, although they are much more susceptible to blinking. Interaction of fluorescently labeled nucleotides with the quantum dots (attached with DNA polymerase) causing an alteration in fluorescence of both nucleotide and quantum dots. The quantum dot signal drops, whereas a signal from the dye-labeled phosphate on each nucleotide rises at a characteristic wavelength. The real-time signal is captured and DNA sequence is determined. As each sequence is bound to the surface, it can be reprimed and sequenced again for improved accuracy.

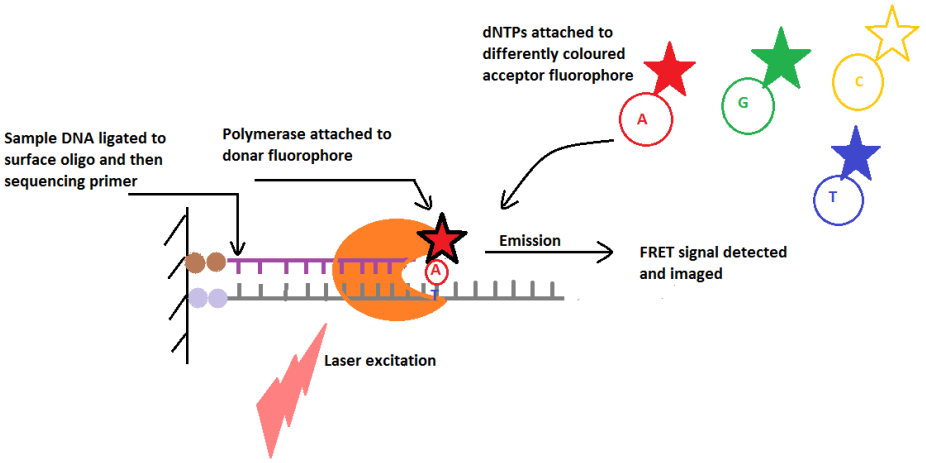

Figure 8. FRET, Specialized DNA polymerase conjugated with donor fluorophore employed for DNA synthesis. dNTPs coupled with acceptor fluorophore when comes in close proximity with acceptor fluorophore emits FRET signal which is detected and imaged.

Transmission electron microscopy (TEM) for DNA sequencing

Transmission electron microscopy for DNA sequencing is a new born third generation DNA sequencing system, the idea of which was introduced by ZSGenetics (http://zsgenetics.com/). The detail of the technology was first introduced at a sequencing conference in 2008 (http://www.healthtech.com/). The technology involves, denaturing templet DNA followed by heavy labeling. Natural DNA is transparent when viewed with TEM, as elements structuring it (C, O, N, H and P) have a low atomic number (Z=1-15). To circumvent this problem three of the four bases are labeled with heavy elements (bromine Z-35, iodine Z-53, and trichloromethane Z-63) and fourth remains unlabeled. Templet DNA molecules are linearised on a thin solid substrate by molecular cloning, a technique that utilizes the force of receding air-water interface to extend DNA molecules irreversibly bound to silane layer once dried[53]. Image can be viewed as dark and light bright spots on electron micrograph, corresponding to differentially labeled DNA bases. The technology helps in genetic information applications including enabling widespread adoption of genetics-based medicine and driving important innovation in disease research, drug discovery, forensics,

environmental impact studies, agriculture, anthropology and history. The ZSG claims for potential of sequencer to be 10-20 Kb with rate of 1.7 Gb per day, at comparatively lower cost than second generation sequencers and has already released images of 23kb DNA. The TEM approach is a technically challenging task. Selective heavy atom labeling and attaching and straightening the labeled DNA to a substrate are a serious technical challenge. Further, the DNA sample should be stable to the high vacuum of electron microscope and irradiation by a focused beam of high-energy electrons.

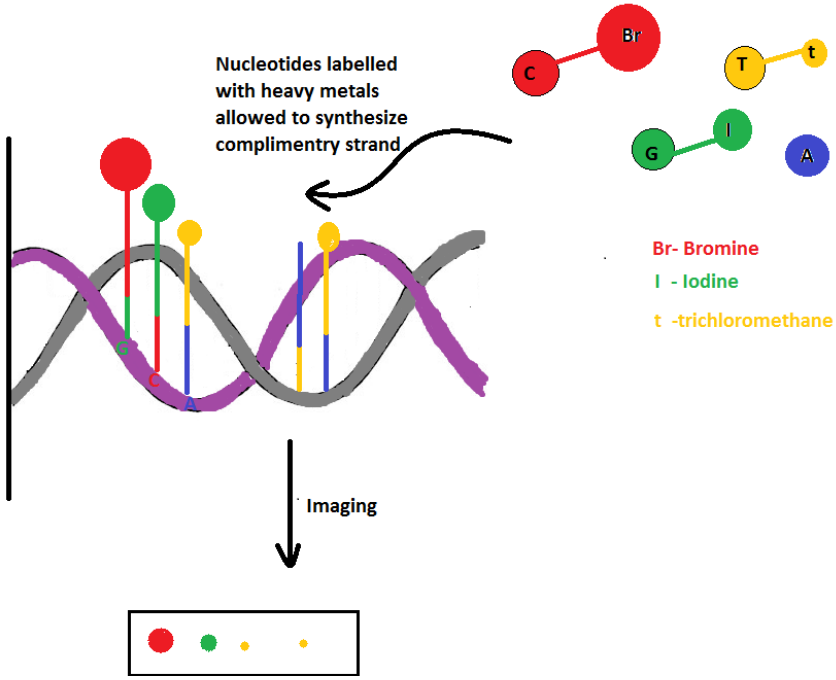

Figure 9. **Transmission electron microscopy for DNA sequencing**, Nucleotide bases labeled with heavy metals (bromine Z-35; iodine Z-53; trichloromethane Z-63) are allowed to synthesize complimentary strand of templet DNA. These nucleotides make newer heavier strand and attached nucleotide could be imaged by TEM as dark and light bands.

Ion torrent

A scalable, low cost DNA sequencing technology has always been the choice of scientists for genome sequencing. The next in the list for above attributes is PostLight^{TM} sequencing technology (Ion torrent) introduced by Life technologies (http://www.iontorrent.com/). It is the first commercial sequencing technology which does not depend upon light for determining the sequence. It produces sequencing data quickly and in an unsophisticated manner. The sequencing chemistry is incredibly simple based on a well characterized biochemical process. When a nucleotide is incorporated into the growing DNA strand by polymerase, H^+ ion is release as byproduct causing a detectable local change in the pH. The whole process occurs inside micro-wells on ion torrent sequencing chip, with each well holding a different DNA template. Change in solution pH of the well is detected by the ion sensor placed beneath the well, essentially going directly from chemical information to digital information. Change in pH is proportional to number of nucleotides added. If voltage is double there must be two identical bases on the DNA strand, and the chip records two identical bases. If the

nucleotide that floods the chip is not a match, no voltage change is recorded and no base is called. Technology does not involve the use of fluorescent nucleotides or imaging, base call is relatively fast and cost effective compared to other sequencing technologies. Currently, the company offers one time use Ion 314^{TM} chip (consisting 1.0 million wells and produces 10 Mb of good quality sequence with read length of 200bp), Ion 316^{TM} (6 million wells and 100Mb data) and Ion 318^{TM} (11 million wells and 1 Gb data). In the near future, company claims to release Ion Proton I^{TM} (165 million wells and ~7 GB throughput) and Ion Proton II^{TM} (660 million wells and ~4 Gb throughput) which further scale up the current throughput 1000 times higher (www.appliedbiosystems.com). The technology utilizes natural biochemistry using inexpensive reagents. The major advantage of this technology is its fast run time (2 hours). A small, light weight machine which brings sequencing to the doorstep of every lab. Short read length is the major problem with this sequencing tool which would create a significant burden on assembly process. Also, error accumulation is remarkably high if reaction wells are not properly cleansed between reaction steps. In this technology, homopolymer region is the most error prone area. The sequencing accuracy of 5-mer homopolymer region of *E. coli* DH10B sequenced through Ion torrent reported accuracy of around 97.5%.

Notably, both NGS and NNGS technologies differ in features like template preparation involving clonal amplification of immobilized template by NGS technology, while NNGS technology involves single molecule detection, thereby bypassing the biasness introduced by PCR amplification. The read length offered by NGS technologies are very low ranging from 70-400bp. In contrast, NNGS provides read length greater than 1000 bp and even more. Moreover, few of the NNGS involves real time detection of fluorescent dye in polymerase active site during incorporation. Even two of the technologies (Nanopore and Ion torrent) do not need modified bases, for this reason optics need is eliminated in these NNGS technologies. On the other hand NGS technology needs optics as they are dependent on fluorescently labeled nucleotide for detection. In addition, NNGS technologies offer very high throughput of the rate 100 Gb per hour while NGS provides only 0.4 to 20 Gb per run. Despite moderate read accuracy offered by NNGS technologies they are cost effective when assessed with NGS technologies. Overall, whatever the firms dealing with NNGS technologies claim regarding their performance, it's too early to tell whether these third generation sequencers will truly provide a breakthrough 'if the technology works' and could completely turn the things around for genome sequencing, but there are a lot of ifs and buts.

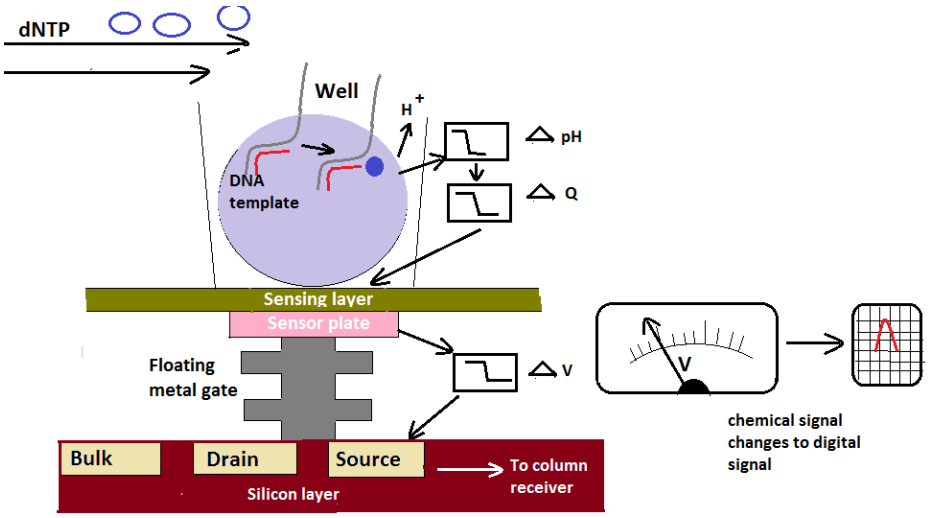

Figure 10. **Ion torrent sequencing,** Cross section of a single well showing template DNA is attached to bead. Incorporation of nucleotide causes release of protons (H^+), which subsequently changes the pH of the surrounding. Sensing layer beneath the bead detects the change in pH and translates this chemical signal to digital signal

Metagenomics

In recent years NGS technologies have been used to study whole population rather than just individuals. The study known as population genetics and Handelsman in 1998 coined the term *Metagenomics* for the same. In today's world metagenomics studies are expanding due to the decreasing cost of sequencing. It has the power of exploring the varying microbial population, community structure and composition with respect to diverse environmental condition like soil deep sea and deep mines. There are certain obstacles in studying microbial composition of an environmental sample. One of the first reason is that sheer diversity of microbes that are present in most extreme environments as well as only small portion of the total microbes are culturable. Genomic studies allow molecular phylogenetic analysis of non culturable organisms which can be employed to study taxonomic diversity of organisms present also, analysis of gene content enlightens metabolic potential of an environment. Large scale shotgun sequencing approaches allow the discovery of many novel genes found in the environments independent of cultivation efforts. Whole genome shotgun sequencing technology has been applied to study microbial population from Sargasso sea near Bermuda and identified 1.2 million of previously unknown genes including 782 new rhodopsin like receptors.

It has been estimated that 10 grams of soil would consist of 10^7 distinct microbial populations. Large sequencing centre would have to dedicate its entire resource for years to sequence all the genomes of the species present. In soil new highly parallel sequencing technologies offer a cost effective production of genomic data as they can generate much more sequence than classical method. NGS for the first time applied to study genome sequences from two sites in Soudanmine, Minnesota USA. Comparison of microbes and subsystems identified in two samples highlighted important differences in metabolic potential in each environment with respect to carbon utilization, iron acquisition mechanism, nitrogen

assimilation and respiratory pathways by assessing gene function and homology searches of sequence reads against the metabolic database. The 16S reads were used to identify species present and it was proved that oxygenated environment possess more diverse species as compared to oxygen poor environment. Approximately ~118,000 amplicons were sequenced that spanned the V6 hypervariable region of ribosomal RNAs from the DNA sampled at different depth and location of Atlantic and Pacific Ocean. The resulting sequences were trimmed and compared with reference database (V6RefDB) to identify the closest match for accessing closest taxanomic diversity. Although this study was inadequate to cover the whole diversity however efficiency of estimates of natural diversity was greater than other methods. 'Zebra chip 'disease of solanaceous crops, caused by '*Candidatus liberibacter solanacearum'* (CLs) and transmitted by the potato psyllid, *Bactericera cockerelli*. Recently 16s DNA tag encoded amplicon pyrosequencing was performed to determine total bacterial microbiota in CLs-uninfected and CLs infected potato psyllid strains and potato leaf tissues and identified five bacterial species in *B. cockerelli* including the P-endosymbiont, *C. carsonella ruddii*, the facultative endosymbiont, *C. carsonella wolbachia* plant pathogen. Very recently seasonal pattern of microbial species diversity and richness was shown using samples collected from deep waters of English Channel over 6 years. Deep sequencing of V6 hypervariable region of 16S rDNA using Illumina platform, providing ~1000X coverage indicated the changes in relative abundances of taxas that are always present. NGS technology has accelerated the study of population level of plant diversity. Sequencing of 1001 accession of model plant is accomplished by using Solexa, Roche/454 and ABI SOLiD technologies expected to provide genome wide LD structure and haplotype data that have broad application for evolutionary biology and plant breeding (http://1001genomes.org/index.html).

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Functional Genomics in crop improvement

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The complete nucleotide sequence of the Arabidopsis thaliana, Rice, Potato, Tomato, Chickpea, Pigeon pea, Egg Plant viz., genome has made it really possible to proceed to the solution of the major problem in the molecular genetics of higher plants: the identification of gene functions. To determine the functions of genes methods of direct and reverse genetics are used. The approach of direct genetics makes it possible to obtain a collection of mutants among which candidate genes are selected by phenotype. The approach of reverse genetics is based on data concerning the state of the genome and the availability and localization of mutations. In this case an extrapolation of data obtained for different plants is possible.

In the case of microorganisms and animals, the functional significance of genes is determined by various methods like including expression arrays, microRNA arrays, array CGH, ChIP-on-chip, methylation arrays, mutation analysis, genome-wide association studies, proteomic analysis, integrated functional genomic analysis and related bioinformatic and biostatistical analyses.

Microarray technology

With the completion of genome sequencing of more and more organisms, research focus has now been shifted from sequencing to delineating the biological functions of all genes coded within the genome of a particular organism. Methodologies of biological research are evolving from "one gene in one experiment" to "multiple genes in one experiment" paradigm.

Introduction to Microarray

Molecular Biology research evolves through the development of the technologies used for carrying them out. It is not possible to research on a large number of genes using traditional methods. DNA Microarray is one such technology which enables the researchers to investigate and address issues which were once thought to be non traceable. One can analyze the expression of many genes in a single reaction quickly and in an efficient manner. DNA Microarray technology has empowered the scientific community to understand the fundamental aspects underlining the growth and development of life as well as to explore the genetic causes of anomalies occurring in the functioning of the human body.

A typical microarray experiment involves the hybridization of an mRNA molecule to the the DNA template from which it is originated. Many DNA samples are used to construct an array. The amount of mRNA bound to each site on the array indicates the expression level of the various genes. This number may run in thousands. All the data is collected and a profile is generated for gene expression in the cell.

Microarray Technique

An array is an orderly arrangement of samples where matching of known and unknown DNA samples is done based on base pairing rules. An array experiment makes use of common assay systems such as micro plates or standard blotting membranes. The sample spot sizes are typically less than 200 microns in diameter usually contain thousands of spots.
Thousands of spotted samples known as probes (with known identity) are immobilized on a solid support (a microscope glass slides or silicon chips or nylon membrane). The spots can be DNA, cDNA, or oligonucleotides. These are used to determine complementary binding of the unknown sequences thus allowing parallel analysis for gene expression and gene discovery. An experiment with a single DNA chip can provide information on thousands of genes simultaneously. An orderly arrangement of the probes on the support is important as the location of each spot on the array is used for the identification of a gene.

Types of Microarrays

Depending upon the kind of immobilized sample used construct arrays and the information fetched, the Microarray experiments can be categorized in three ways:

1. **Microarray expression analysis:** In this experimental setup, the cDNA derived from the mRNA of known genes is immobilized. The sample has genes from both the normal as well as the diseased tissues. Spots with more intensity are obtained for diseased tissue gene if the gene is over expressed in the diseased condition. This expression pattern is then compared to the expression pattern of a gene responsible for a disease.

2. **Microarray for mutation analysis:** For this analysis, the researchers use gDNA. The genes might differ from each other by as less as a single nucleotide base.

A single base difference between two sequences is known as Single Nucleotide Polymorphism (SNP) and detecting them is known as SNP detection.

3. **Comparative Genomic Hybridization:** It is used for the identification in the increase or decrease of the important chromosomal fragments harboring genes involved in a disease.

Applications of Microarrays

Gene discovery: DNA Microarray technology helps in the identification of new genes, know about their functioning and expression levels under different conditions.

Disease diagnosis: DNA Microarray technology helps researchers learn more about different diseases such as plant (Viral, Bacterial, Fungal and phytoplasm diseases) and animal diseases (heart diseases, mental illness, infectious disease and especially the study of cancer). Now, with the evolution of microarray technology, it will be possible for the researchers to further classify the types of diseases on the basis of the patterns of gene activity in the cells. This will tremendously help the scientific community to develop more

effective drugs as the treatment strategies will be targeted directly to the specific type of diseases.

Drug discovery: Microarray technology has extensive application in *Pharmacogenomics.* Pharmacogenomics is the study of correlations between therapeutic responses to drugs and the genetic profiles of the patients. Comparative analysis of the genes from a diseased and a normal cell will help the identification of the biochemical constitution of the proteins synthesized by the diseased genes. The researchers can use this information to synthesize drugs which combat with these proteins and reduce their effect.

Toxicological research: Microarray technology provides a robust platform for the research of the impact of toxins on the cells and their passing on to the progeny. Toxicogenomics establishes correlation between responses to toxicants and the changes in the genetic profiles of the cells exposed to such toxicants. Eg. Mycotoxin resistant of *Phytophthora infestans* strain.

GEO

In the recent past, microarray technology has been extensively used by the scientific community. Consequently, over the years, there has been a lot of generation of data related to gene expression. This data is scattered and is not easily available for public use. For easing the accessibility to this data, the **National Center for Biotechnology Information** (**NCBI**) has formulated the **Gene Expression Omnibus** or **GEO**. It is a data repository facility which includes data on gene expression from varied sources.

Microarray probe design parameters

For 25-35 mers

Parameter	Minimum Value	Maximum Value	Default Value	Unit
Probe Length	10	99	30	bases
Probe Length tolerance	0	15	3	
Probe Target Tm	40	99	63	°C
Probe Tm Tolerance (+)	0.1	99	5	
Hairpin Max ÄG	0.1	99.9	4	Kcal/mol
Self Dimer ÄG	0.1	99.9	7	Kcal/mol
Run/Repeat	2	99	4	bases

For 35-45 mers

Parameter	Minimum Value	Maximum Value	Default Value	Unit
Probe Length	10	99	40	bases
Probe Length tolerance	0	15	3	
Probe Target Tm	40	99	70	°C
Probe Tm	0.1	99	5	

Tolerance (+)				
Hairpin Max ÄG	0.1	99.9	6	Kcal/mol
Self Dimer ÄG	0.1	99.9	8	Kcal/mol
Run/Repeat	2	99	5	bases

For 65-75 mers

Parameter	**Minimum Value**	**Maximum Value**	**Default Value**	**Unit**
Parameter	Minimum Value	Maximum Value	Default Value	Unit
Probe Length	10	99	70	bases
Probe Length tolerance	0	15	3	
Probe Target Tm	40	99	75	°C
Probe Tm Tolerance (+/- above)	0.1	99	5	
Hairpin Max ÄG	0.1	99.9	6	Kcal/mol
Self Dimer ÄG	0.1	99.9	8	Kcal/mol
Run/Repeat	2	99	6	bases

The introduction of real-time PCR technology into the field of molecular diagnostics has simplified the quantification of nucleic acids. Enormous amounts of data can be generated within a short time. Although most real-time PCR assays in themselves are characterized by high precision and reproducibility, the accuracy of the obtained data is largely depended on several other factors such as sample preparation, quality of the standard and choice of housekeeping gene. Therefore, the accuracy of the obtained data has to be checked during the establishment of the assay by comparison with other established assays.

Transcriptomic sequencing: A tool for crop improvement

An organism genes are expressed that is transcribed from the genomes DNA code into messenger RNA (mRNA) and subsequently, may be translated into proteins that function within the organisms cells. Some genes are expressed more than others, some at different life stages, and some at different times according to environmental conditions or the state of the organism or the individual cell. This complex regulation of gene expression means that the organisms cells can respond to their environment, and to the development of the organism.

Transcriptome: The set of genes which are transcribed in any one condition is known as the transcriptome and the process of determining the genetic codes contained in the transcriptome and their relative proportions is known as transcriptome sequencing.

Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells, tissues and also for understanding development and disease. The key aims of transcriptomics are to catalogue all species of transcript, including mRNAs, non-coding RNAs and small RNAs to determine the transcriptional structure of genes in terms of their start sites 5′and 3′ ends, splicing

patterns, other post-transcriptional modifications and to quantify the changing expression levels of each transcript during development and under different conditions.

In many cases, the introduction of new technologies to a field can overcome previously existing limitations and obstacles and can lead to significant leaps in our understanding of a biological process. For example, the introduction of microarrays two decades ago enabled the study of changes in the expression levels of many genes simultaneously. In principle, this technology also allows one to comprehensively monitor gene expression in both the pathogen and the host during their interaction. However, the technical difficulties associated with simultaneously determining two often very different transcriptomes, including issues such as probe selection, cross-hybridization and the required design and cost of custom chips, make microarray-based studies challenging and expensive when they are applied to determining both the host and pathogen transcriptomes.

Recently, the development of novel high-throughput DNA sequencing methods has provided a new method for both mapping and quantifying transcriptomes. This method, termed RNA-Seq (RNA sequencing), has clear advantages over existing approaches and is expected to revolutionize the manner in which eukaryotic transcriptomes are analysed. It has already been applied to *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, mous, human cells, Rice, Potatoand tomato.

In general, a population of RNA (total or fractionated, such as poly(A)+) is converted to a library of cDNA fragments with adaptors attached to one or both ends (FIG. 1). Each molecule, with or without amplification, is then sequenced in a high-throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing).The reads are typically 30–400 bp, depending on the DNAsequencing technology used. In principle, any high-throughput sequencing technology can be used for RNA-Seq, and the Illumina IG, Applied Biosystems SOLiD, Ion-Proton and Roche 454 Life Science.

Benefits of RNA seq

- RNA-Seq particularly attractive for non-model organisms with genomic sequences that are yet to be determined.

- RNA-Seq can reveal the precise location of transcription boundaries, to a single base resolution. Furthermore, 30-bp short reads from RNA-Seq give information about how two exons are connected, whereas longer reads or pair-end short reads should reveal connectivity between multiple exons. These factors make RNASeq useful for studying complex transcriptomes.In addition, RNA-Seq can also reveal sequence variations (for example, SNPs) in the transcribed regions.

- A second advantage of RNA-Seq relative to DNA microarrays is that RNA-Seq has very low, if any, background signal because DNA sequences can been unambiguously mapped to unique regions of the genome. RNA-Seq does not have an upper limit for quantification, which correlates with the number of sequences obtained. Consequently, it has a large dynamic range of expression levels over which transcripts can be detected.

- By contrast, DNA microarrays lack sensitivity for genes expressed either at low or very high levels and therefore have a much smaller dynamic range (one-hundredfold to a few-hundredfold).

- RNA-Seq has also been shown to be highly accurate for quantifying expression levels, as determined using quantitative PCR (qPCR) and spike-in RNA controls of known concentration.

- The results of RNA-Seq also show high levels of reproducibility, for both technical and biological replicates18,22. Finally, because there are no cloning steps.

- RNA-Seq requires less RNA sample.

Taking all of these advantages into account, RNA-Seq is the first sequencing based method that allows the entire transcriptome to be surveyed in a very high-throughput and quantitative manner.

This method offers both single-base resolution for annotation and digital gene expression levels at the genome scale often at a much lower cost than either tiling arrays or large-scale Sanger EST sequencing.

Somatic cell genetics through protoplast fusion in potato improvement

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Introduction

Gene transfer is the basis for almost all crop improvement including potato. Conventionally, this is achieved through sexual hybridization; this rather limits the range of species from which gene flow can occur into a crop species. Wild species have contributed remarkably to the success of latter, they allowed the crops to retain their commercial status. As a result plant breeders have sought to utilize an increasing number of wild species as a source of valuable genes ranging from disease resistance to grain yield, and produce quality. But many sources of useful genes cannot be included in crop improvement programme primarily because of sexual incompatibilities. Genetic transformation, a focussed and direct gene transfer approach, require identification, isolation and cloning of the concerned genes. Further it is expensive and technically most exacting, although it may represent the ultimate strategy. However, some characters of interest may be govern by two or more and yet unknown genes; transfer of such characters through genetic transformation may pose many difficulties. Finally transfer of cytoplasmic organells, *viz.*, chloroplast and mitochondria may often be desired objectives; this, however is not possible through genetic transformation, while it can readily achieved by somatic hybridization.

Wild and cultivated species of potato have been effectively used in potato breeding but represent only a tiny fraction of the available potato biodiversity. Utilization of the wild tuber-bearing diploid species has been remained untapped potential source for transferring resistance trait into common potato (Bradshaw et al. 2006). Wild tuber-bearing *Solanum* species are widely distributed from southwestern USA to central Argentina and Chile. This extensive geographical range has resulted in types adapted to a broad range of climatic and soil conditions. In the course of evolution, these plants have also developed resistance/tolerance to different pathogens and pests. Much of this effort has involved the examination of wild species for various resistance traits related to potato. This trait is particularly attractive to breeders to widen the potato genetic base, but the barrier between the cultivated potato and the many wild species has proved a difficult task, even when unconventional crossing methods are used (Orczyk et al. 2003).

Many useful genes derives from wild sources cannot be transferred through conventional technique because of sexual incompatibilities are primarily due to difference in ploidy and endosperm balance number (EBN) (Spooner and Salas, 2006). It is extremely difficult to cross 1 EBN wild species directly with common cultivated 4 EBN potato. Limited success has been obtained by utilizing bridging species but the incompatibility of 1 EBN wild species has generally prevented the use of this particularly valuable trait. However, modern research and new techniques have made it possible to expand considerably the genetic resources available for use in breeding programs. A few methods have now become available to overcome this problem. These methods include: manipulation of ploidy and endosperm balance number (EBN), bridge crosses, mentor pollination and embryo rescue, hormone treatment, and reciprocal crosses (Jansky 2006). Somatic hybridization, which removes

prezygotic and some postzygotic barriers, can likewise surmount the barrier between cultivated and wild species. Somatic hybridization can provide a means of bypassing sexual incompatibility between *Solanum* species, leading to fertile plants that can be used directly in breeding programs.

Somatic hybridization generates functional combinations of large sets of genetic material, which makes it similar to sexual hybridization. This method can also be used to overcome limitations of genetic transformation. Many of the important traits are predominantly polygenic such as late blight resistance and thus unavailable as isolated and characterized sequences that are ready for genetic transformation. Therefore, efficient methods of transformation are yet to be available for multiple genes that are expressed in a coordinated manner (Orczyk et al. 2003). On the other hand, somatic hybrids obtained directly after fusion contain all organelles from the cytoplasms of both parents. Somatic hybridization via protoplast isolation, electrofusion and regeneration is a useful tool to transfer polygenic traits such as late blight resistance in a single step. It enables a development of tetraploid somatic hybrid between diploid wild species and dihaploid of common potato. As a result, tetraploid somatic hybrids may be utilized in conventional breeding for late blight resistance and improvement of other traits. Thus, production of somatic hybrids between tetraploid 4EBN *S. tuberosum* and diploid 1EBN wild species has been envisaged for imparting durable resistance to late blight. In consequence, aim of this somatic fusion technology is creditable to enrich the cultivated potato gene pool by incorporating genes from a new exotic wild species, in order to enhance resistance to late blight disease.

Hence, somatic hybridization is the technique enables to transfer agronomically important traits by bypassing such sexual barriers, besides the conventional and recombinant-DNA technologies approaches. Despite these crossing-barriers, many researchers have used this technique and subsequently produced somatic hybrids with cultivated potato. Production of hybrid plants through the fusion of protoplasts of two different plant species/varieties is called somatic hybridization, and such hybrids are called somatic hybrids. Therefore, somatic hybridization can be resorted to only when the following two criteria are satisfied: i) isolation of protoplast in large quantity and ii) totipotency of the isolated protoplasts.

Procedures

The protoplast fusion by electric field requires sufficient amount of suitable plant material for protoplast isolation and their culture after electrofusion for obtaining plant regeneration. The procedure involves the following stages.

- *In vitro* culture of donor plants
- Protoplast isolation from leaf meshophyll tissues
- Verification of protoplast viability and protoplast fusion
- Protoplast fusion by electric field
- Regeneration and culture fusion products
- Characterization of putative somatic hybrids

1) Prepare materials:
   - Three-week-old *in vitro*- grown microplants, raised from single nodal cutting

2) Microplant incubation condition:
- 16 h photoperiod/40 µ mol m^{-2} s^{-1}/20ºC.

3) Pre-isolation (protoplast) incubation:
- 48 h/dark/20ºC

4) Protoplast isolation:
- Mince young leaf tissues (1-2 g) in a ϕ 90 mm Petri dish containing digestion solution: 10 ml digestion solution for 1 g tissue.

5) Incubation (for protoplast isolation):
- 16 h/ dark/ 25ºC/ optional: gyratory shaking at 40-50 rpm; not exceeding 50 rpm.

6) Post-isolation handling:
- Add 0.3 M KCl (sterile) to the digestion medium/ solution containing released protoplasts (protoplast suspension) in a 1:1 ratio (KCl: solution). For example, add 15 ml M KCl to 15 ml digestion medium/solution.
- Filter the suspension through 40 µ nylon mesh, and collect in centrifuge tubes; 60 µ can be used but debris and/ or undigested tissues will be much.

7) Protoplast purification:
- Centrifuge the filtrate at 50 x g (50 RCF) for 5 min.
- Discard supernatent and then resuspend the pellets in 10 (or 9) ml of 0.6 M sucrose (sterile).
- Overlay 1 ml of 0.3 M KCl onto this protoplast suspension.
- Centrifuge at 50 x g (50 RCF) for 5 min.
- Recover the protoplast (green upper portion/live protoplast) from sucrose: KCl interface
- Dilute the recovered protoplasts with 10 ml of 0.3 M KCl.
- Centrifuge at 50 x g (50 RCF) for 5 min to form pellet of the protoplast.
- Resuspend the pellets in 1-2 ml 0.5M mannitol (sterile), centrifuge at 50 RCF) for 5 min., then retain pellets and discard supernatants
- Finally resuspend the pellets in 400-500 µl 0.5M mannitol (sterile) to a final density of $1x1^{06}$ protoplast/ml for electrofusion.

8) Electrofusion medium:
- 0.5 M mannitol (sterile)/ sterile-filtered (0.2 µ)/ pH 7-7.3/ adjust pH with 0.1 N NaOH

9) Symmetric fusion:
- 1:1 of each species

10) Electrofusion settings:

Chamber	BTX Microslide Model 453/3.2 mm gap
Alignment amplitude	16 V
Alignment time	25-30 s
Alignment field strength	50 V cm^{-1}
Electrofusion amplitude	260 V

DC pulse width	60 µs
Number of pulses	2
Electrofusion field strength	812 V cm^{-1}

11) Post-fusion culture:

- Dispense 50 µl Na-Alginate in each box of castor rack and mix well the 50 µl fusion products into it
- Add 2-3 ml Solution No 3 in each box and incubate at RT inside laminar for 30 min followed by add 2-3 ml Solution No 2 and incubate same for 1.30-2 h
- Remove Sol 2 & 3 by Pasteur pipette and add 5 ml VKMG (VKM Glucose) and tightly wrapped with parafilm.
- Incubate the castor racks under dark at 25ºC for the regeneration of calli.

Applications

Symmetric protoplast fusion approaches involving diploid *Solanum* species in combination with dihaploid *S. tuberosum* have been essentially used to develop tetraploid somatic hybrids potato having desirable introgression from wild relatives (Table 1). In the current years, application of this technology has been observed widely for the production of multiple resistant somatic hybrids. Interspecific potato somatic hybrid between commercial cultivars of potato *S. tuberosum* Agave and Delikat and wild diploid species *S. cardiophyllum* (1 EBN) has been produced for resistances to Colorado potato beetle, foliage blight and PVY (Thieme et al. 2010). In addition, somatic hybrids between a diploid potato clone DG 81-68 susceptible to *P. infestans* and a resistant diploid tuber-bearing species *Solanum* x *michoacanum* were generated (Szczerbakowa et al. 2010). Polzerová et al (2011) have developed interspecific somatic hybrids between wild diploid species *S. pinnatisectum* (1 EBN) and *S. tuberosum* for the late blight resistance in potato. Following the successful production, somatic hybrids have been applied in the potato breeding for the development of advance progenies for transferring the resistance trait. For example, Thieme et al. (2008) have developed novel somatic hybrids and their fertile BC_1 progenies having resistances to late blight, Colorado potato beetle and PVY from a diploid wild species *S. tarnii* into common potato.

Interspecific potato somatic hybrids at CPRI

Interspecific potato somatic hybrids between 1 EBN wild *Solanum* species *S. pinnatisectum* (+) dihaploid *S. tuberosum*, and ii) S*. etuberosum* (+) dihaploid *S. tuberosum* have been produced following optimized protocol of the protoplast isolation, electrofusion and regeneration of plantlets (Sarkar et al. 2011; Tiwari et al. 2010, 2011). The somatic hybrids *S. pinnatisectum* (+) *S. tuberosum* have resistance for late blight resistance, whereas *S. etuberosum* (+) *S. tuberosum* have resistance for potato virus Y. These potato somatic hybrids have been confirmed for the hybridity through molecular (RAPD and SSR markers), phenotypic assessments. Hybrids were also evaluated for the disease resistance. Ploidy level (tetraploid) of somatic hybrids has been examined through flow cytometry and guard cell count. At present, work is under progress on the development of more interspecific potato hybrids involving diverse 1 EBN wild species obtained from foreign gene banks. Molecular analyses of the potato somatic hybrids produced at CPRI, Shimla are shown in Figs. 1 and 2.

Table 1. Symmetric protoplast fusion in potato for incorporation of desirable traits from wild diploid species into *S. tuberosum*

Diploid wild species	Trait(s) transferred	References
S. acaule	PVX resistance	Yamada et al. 1997
	Bacterial ring rot resistance	Rokka et al. 2005
	Glycolkaloid composition	Kozukue et al. 1999
S. berthaultii	Salinity tolerance	Bidani et al. 2007
S. bulbocastanum	Late blight resistance	Bołtowicz et al. 2005
	Meloidogyne chitwoodi resistance	Mojtahedi et al. 1995
	Bacterial stem rot resistance	Rokka et al. 1994
S. brevidense	Tuber characteristics and insect resistance	Serraf et al. 1991
	Tuber soft rot resistance	Polgar et al. 1999
	Early blight resistance	Tek et al. 2004
S. cardiophyllum	Late blight, PVY, Colorado potato beetle resistances	Thieme et al. 2010
S. chcoense	Colorado Potato Beetle resistance	Cheng et al. 1995
S. circaefolium	Late blight and nematode resistances	Oberwalder et al. 2000
S. commersonii	Frost resistance	Nyman and Waara 1997
	Bacterial wilt resistance	Kim-Lee et al. 2005
	Tuber traits	Caruso et al. 2008
S. etuberosum	Tuber characteristics	Novy and Helgeson, 1994
	PVY resistance	Novy et al. 2007
	PLRV resistance	Novy et al. 2007
S. x michoacanum	Late blight resistance	Szczerbakowa et al. 2010
S. nigrum	Late blight resistance	Szczerbakowa et al. 2003
S. phureja	Bacterial wilt resistance	Fock et al. 2000
S. pinnatisectum	Late blight resistance	Polzerová et al 2011
S. stenotomum	Bacterial wilt resistance	Fock et al. 2001
S. tarnii	Late blight, Colorado Potato Beetle and PVY resistance	Thieme et al. 2008
S. torvum	Resistance to *Verticillium dahliae*	Jadari et al. 1992
S. tuberosum	Late blight resistance	Rasmussen et al. 1998

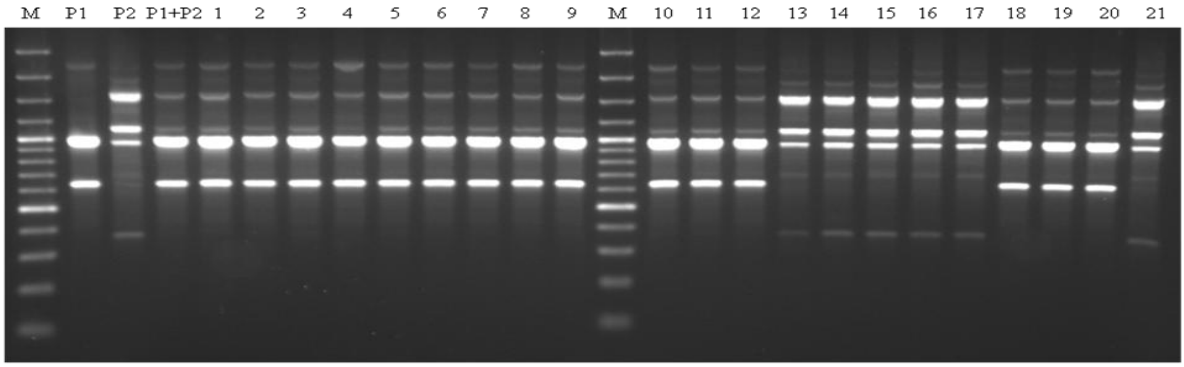

Fig. 1 RAPD profiles generated by primer OPAC-13 on 1.6% agarose gel. M = 100 bp ladder, P_1 (Parent 1) = C-13, P_2 (Parent 2) = *S. etuberosum* P_1+P_2 = Pooled parental DNA, 15 clones (No. 1 to 12 and 18-20) were confirmed as somatic hybrid. Whereas, 6 clones (No. 13-17 and 21) were not somatic hybrids (continued on Fig. 2)

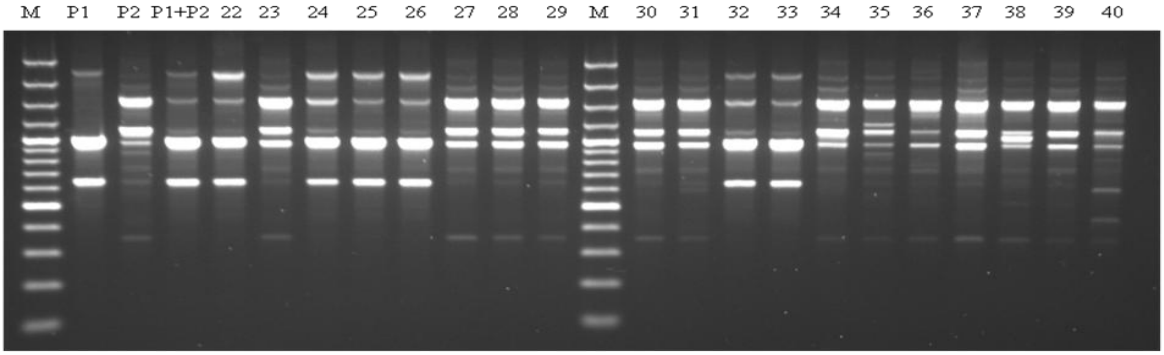

Fig. 2 RAPD profiles generated by primer OPAC-13 on 1.6% agarose gel. M = 100 bp ladder, P_1 (Parent 1) = C-13, P_2 (Parent 2) = *S. etuberosum* P_1+P_2 = Pooled parental DNA, 6 clones (No. 22, 24, to 26, 32, 33) were confirmed as somatic hybrid. Whereas, 13 clones (No. 23, 27-31, 34-40 were not somatic hybrids

Phenotypes of the interspecific potato somatic hybrids *S. tuberosum* spp. *tuberosum* (+) *S. pinnatisectum*, and *S. tuberosum* spp. *tuberosum* (+) *S. etuberosum* produced at CPRI, Shimla are shown below (Figs. 1 and 2).

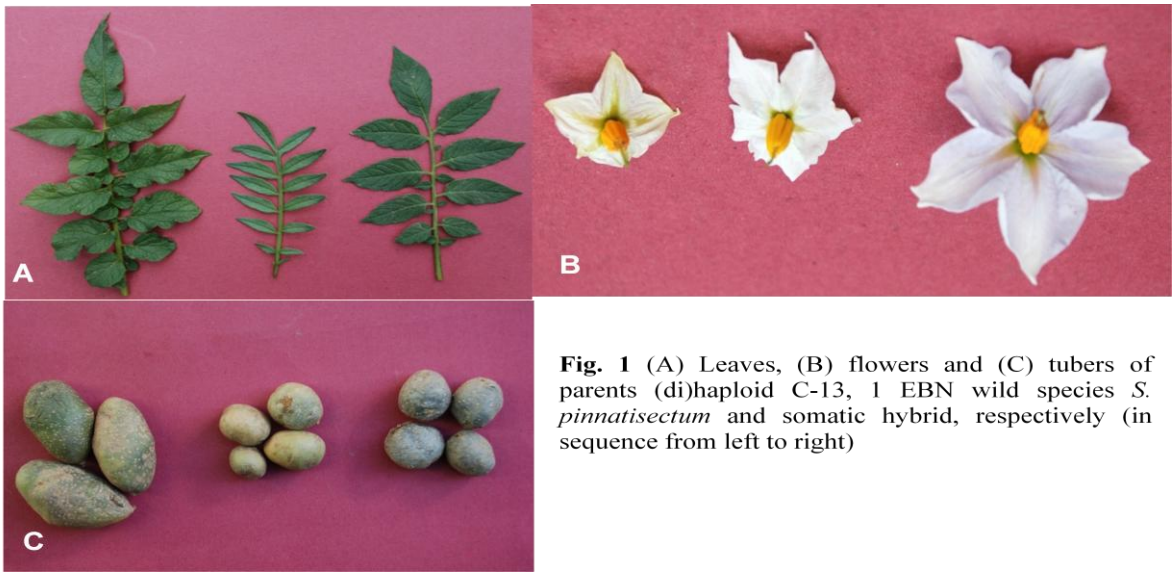

Fig. 1 (A) Leaves, (B) flowers and (C) tubers of parents (di)haploid C-13, 1 EBN wild species *S. pinnatisectum* and somatic hybrid, respectively (in sequence from left to right)

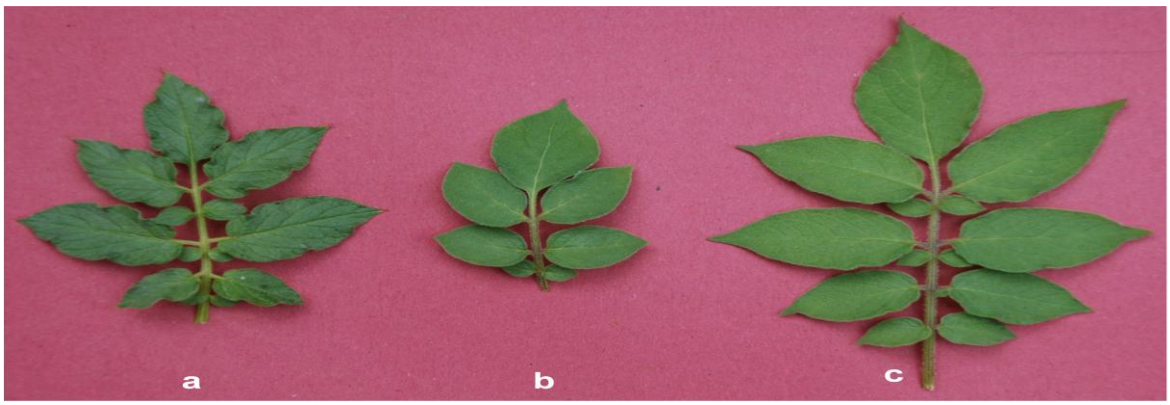

Fig. 2 Leaf of (a) parent S. tuberosum dihaploid C-13, (b) parent *S. etuberosum* and, (c) somatic hybrid clone C-13 (+) *S. etuberosum*

Advantages

- Somatic hybrids can be produced between species, which cannot be hybridized sexually.
- Somatic hybrids can be readily used in breeding programme for transfer of resistance genes
- Hybrids can be produced even between such clones, which are completely sterile.
- Cytoplasm transfer can be done in one year, while back crossing may take 5-6 years. Even where backcrossing is not applicable, cytoplasm transfer can be made using this approach.
- Mitochondria of one species can be combined with chloroplast of another species. This may be very important in some cases, and is not achievable by sexual means even between easily crossable species.
- Recombinant organelle genomes, especially of mitochondria, are generated in somatic hybrids. Some of these recombinant genomes may possess useful features.

Limitations

- Techniques for protoplast isolation, culture and fusion are very complicated.
- In many cases, chromosome elimination occurs from somatic hybrids leading to asymmetric hybrids. Such hybrids may be useful, but there is no control on chromosome elimination.
- Many somatic hybrids show genetic instability, which may be an inherent feature of some species combinations.
- Many somatic hybrids either do not regenerate or give rise to sterile regenerants. Such hybrids are useful for crop improvement. All interfamily somatic hybrids are genetically unstable and/or morphologically abnormal, while intergeneric and intertribal hybrids are genetically stable, but produce abnormal and/or sterile plants.

Conclusion

Somatic hybridization allows transfer of cytoplasmic organelle in a single generation and offer unique opportunities for combining mitochondria of one species and chloroplast of another species in a single hybrid. This capability may permit improvement of characteristics certain cytoplasmic male sterile line, which may lead to their commercial exploitation. In addition, even non-flowering and non-tuber bearing species can be utilized in breeding programme. The transfer of gene governing resistance to biotic and abiotic stresses is an important objective. In potato, this technique is already being used in commercial breeding programme of the Netherlands and Germany. In general, somatic hybrids have low pollen fertility, but they can often be used as female parents in backcrossing with one of fusion parents. It is likely that the approach of somatic hybridization will find grater applications in potato improvement for enabling transfer of useful genes from sexually incompatible species. In this context, it is important that the DNA segment carrying the desired gene from wild species is introgressed into the genome of cultivated potato and stable inherited. The possible mechanissm for such introgression are homeologous pairing leading to crossing over, and intergenomic translocation. An understanding of the gene introgression mechanism may enable their enhancement using suitable treatment; this, in turn will enhance the opportunities for utilization of somatic hybrids in potato improvement.

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Application of proteomics and metabolomics in crops

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The availability of genome sequences at large scale has helped to gain better insights into the genome structure and organization to a great extent. However, real challenge still remains in deciphering the function of the encoded proteins, their interaction with other proteins or biomolecules through which these contribute to the functioning of the organism as an entity and at systems level. A systems-level understanding is essentially required if we intend to fully capitalize on plants to address global challenges such as food, and fuel shortages, changing global climate etc. The first step in a systems approach is to identify all of the system components (e.g., genome, transcripts, proteins, metabolites). Because information regarding the function of the system as a whole is desired, all of the measurements and experimental observations are most informative when conducted on a global scale at high spatial and temporal resolution. This approach has given rise to new field known as Systems biology. Systems biology is the study of the interactions and interplay between all properties of biological systems. It involves integrating the existing knowledge about biological components, building a model of the system as a whole, and extracting unifying organizational principles that explain the form and function of living organisms. This lecture will briefly describe the two important components of systems biology. These are: proteomics and metabolomics.

Proteomics

The proteome refers to the protein complement of a particular biological system at any given point of time. The term "proteome" was first used by Wilkins et al. (1995) to describe the protein complement to the genome. Though proteomics is theoretically the analysis of the full protein population of complex biological systems, the available technologies are not capable of fulfilling this description experimentally. Conceptual progress in proteomics has made possible the analysis of entire proteomes at previously unprecedented density and accuracy. Various steps involved in proteomics studies are described below.

Methodologies for plant proteomics

1. Extraction of proteins

Sample preparation procedures for proteomic analysis require the reliable and consistent extraction of proteins from tissue samples. Plant tissues usually contain high levels of proteases and secondary metabolites which cause problems with protein extraction and proteomic procedures. The existence of secondary metabolites displays species/tissue-specificity and varies with age or developmental stage. Secondary metabolites can severely affect the performance of protein extraction and separation. For example, phenolics can build irreversible complexes with proteins, and the oxidation of phenolics by phenoloxidases and peroxidases can cause streaking on 2-DE gels. Pigments, polysaccharides, and lipids can also cause severe disturbances in 2-DE gels. For total protein extraction, an ideal protocol should reproducibly capture all the protein species in a proteome with low

contamination of other molecules. In many cases, the extraction procedure must be optimized for plant species, tissue, or cell compartment. The three main extraction protocols commonly employed for plant proteomics are (1) TCA/acetone precipitation, (II) phenol extraction and (III) TCA/acetone/phenol extraction.

TCA/acetone extraction: The method is based on protein denaturizing under acidic and/or hydrophobic conditions that help to concentrate proteins and remove contaminants. TCA/acetone extraction involves the cleanup of tissue powder with 10% TCA/acetone, which is usually more effective than either TCA or acetone alone. Plant tissues are rich in proteases, which cause the proteolytic degradation of proteins and the loss of high molecular weight proteins. However, homogenizing plant tissue in TCA/acetone almost immediately inactivates proteases and precipitates proteins. Also, TCA/acetone extraction is valuable for removal of interfering compounds and the enrichment of very alkaline proteins such as ribosomal proteins from total cell lysates. **Limitation:** The resulting pellets from TCA/actone precipitation may be difficult to dissolve.

Phenol-based extraction: In this method, plant tissue powder is extracted in the extraction buffer/buffered phenol (pH 8.0) and followed by methanol (or acetone) precipitation of phenol phase. Phenol dissolves proteins (including membrane proteins) and lipids leaving water-soluble substances (polysaccharides, nucleic acids, etc.) in the aqueous phase, thus proteins in phenol phase are purified and concentrated simultaneously by subsequent methanol (or acetone) precipitation. In the case of tissues containing high levels of phenolic compounds pulverizing plant tissues with PVPP can help to remove phenolic compounds. The important points for successful use of phenol extraction are (i) keeping samples at low temperature during the first extraction step and (ii) careful recovery of the phenolic phase after centrifugation. Phenol can minimize protein degradation resulting from endogenous proteolytic activity. Many studies showed that phenol extraction gave satisfactory results in recalcitrant plant tissues rich in components which inhibit electrophoresis. Overall, phenol protocol is more efficient than TCA/acetone precipitation. **Limitation**: toxicity nature of phenol and more time-taking.

TCA/acetone precipitation/phenol extraction: This is integrated phenol-based extraction with TCA/acetone cleanup steps prior to protein extraction. The protocol holds the merits of both TCA/acetone precipitation (for removal of phenolics, lipids, and pigments) and phenol extraction (for removal of phenolics, lipids and pigments, polysaccharides, nucleic acids, and salts). In the TCA/acetone/phenol extraction protocol, tissue powder purged by TCA/acetone is used as the starting material in phenol extraction of proteins. The protocol has been proved to be very effective to handle with recalcitrant tissues and provides enhanced 2-DE-based proteomic analysis of plant tissues. Although the TCA/acetone/phenol extraction is time consuming and laborious compared to both the TCA/acetone precipitation and the phenol-based extraction, it has the potential to generate high-quality protein samples for some kinds of recalcitrant plant tissues. **Limitations:** Possible protein loss due to multiple cleanup steps.

2. Separation and detection of proteins

There are two preferred methods for separation of complex protein or peptide samples: (1) two-dimensional (2-D) gel electrophoresis; and (2) liquid chromatography (LC).

Two dimensional gel electrophoresis

Majority of proteome studies undertaken on plant samples have used two dimensional gel electrophoresis (2D-GE) for separation of proteins. This is mainly because of advances in isoelectric focusing (IEF) which now routinely utilize immobilized pH gradient (IPG) strips in the first dimension. This has significantly enhanced the ease and reproducibility of 2D-GE for proteomic analysis. The basic procedures and techniques for undertaking 2D-GE are available in many biochemistry books/ manuals. After the IEF has completed, strips are equilibrated in an SDS loading buffer and placed on top of a precast acrylamide gel. Once the sample has successfully been arrayed in the second dimension acrylamide gel, sample analysis requires a successful digestion, and peptide extraction to be undertaken. The basic procedure has been widely reported and used with many minor modifications. One of the underlying objectives of 2D-PAGE is the reproducibility and consistency of protein extractions. Many attempts have been made to improve extraction reliability and performance utilizing a variety of detergents, chaotropes and mechanical disruption techniques. Such optimization is often required due to the nature of different samples and the lack of completer solublization in non-denaturing homogenization buffers.

After 2D-GE the gels need to be stained for detecting proteins. Although several methods for protein detection have been reported, silver and Coomassie Brilliant Blue (CBB) staining methods are still the preferred methods for in-gel protein detection. Silver staining is ~ 10-fold more sensitive than CBB, with a detection range of 0.1–1 ng. However, silver staining has problems such as inferior reproducibility, poor linear dynamic range, and non-quantitative negative-staining of some modified proteins, all of which complicate downstream quantitation and spot matching. Broad dynamic range fluorescent protein stains including SyproRuby™, Deep Purple™, and ruthenium II, which have detection sensitivities around 10–20 ng are promising, but expensive, alternatives to Coomassie and silver staining as general protein stains.

3. Identification of proteins:

Protein identification using mass spectrometry

Mass spectrometry is the most preferred technique employed for identification of proteins. The development and commercialization of two different "soft" ionization approaches, electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) enabled large macromolecules such as proteins to be analyzed either in flowing, liquid solution or in a dry, crystalline state, respectively. The importance of these developments was appreciated by the scientific community and in 2002 led to John Fenn and Koichi Tanaka sharing the Nobel prize in Chemistry. The utilization of MS for the identification of proteins has become the primary technique for large scale protein analysis in recent years. The choice of sample delivery methods and subsequent analysis are dictated by availability of hardware, costs and experimental objectives. As stated above the two most popular sample delivery devices currently being used in proteomics are MALDI and ESI. These sources are connected to various types of mass spectrometers, which further influence the type of data and quality produced. Both delivery methods share similarities as they rely on the processing of an isolated sample with cleavage agent such as trypsin and the resulting peptides are introduced into the mass spectrometer as peptide ions in the gas phase.

Matrix assisted laser desorption ionization

The most widely used method of sample delivery and analysis uses the MALDI source attached to time of-flight mass spectrometer (MALDI-TOF). This combination has been successfully employed in the proteomic identification of proteins. This method of analysis is best suited for gel-separated proteins producing samples of relatively low complexity. This approach uses the absolute mass of each peptide to produce a peptide mass fingerprint (PMF) of the protein, which is then used to search a database of theoretical peptide masses. The analysis of samples by MALDI-TOF to produce PMF data is relatively high-throughput technique but produces data of poorer quality when compared to MS/MS –type analysis. This has a direct impact on pattern-matching confidence scores and creates more ambiguity when interrogating a large database for a match. The delivery process relies on aromatic acid matrices (e.g. dihydroxybenzoic acid or hydroxycinnamic acid) mixed with a digested sample. The matrix transforms the energy from LAZER to the sample leading to a release of peptides from the matrix. The LASER desorbs the peptides from a solid dried sample into the gas phase for analysis by mass spectrometer. No amino acid sequence information is obtained by basic MALDI-TOF analysis. Matching of data is based on the similarity of the masses to the predicted patterns from an *in silico* digestion of the sequence database. In most MALDI-TOF instruments there are now techniques that provide some sequence information by a post-source decay (PSD) process. However, PSD will generally only provide sequence tags for three to four amino acids from 10-15 amino acids peptide. While these can be useful and provide an increase in matching confidences, they do not offer the same matching capabilities as authentic MS/MS spectra from a tandem mass spectrometer.

More recently, MS/MS capabilities have been made available with the coupling of the MALDI source to tandem mass spectrometers allowing full-peptide fragmentation. The MALDI source has commonly been attached to a tandem mass spectrometer with a TOF component due to synergistic reasons involving timed points of ionization and the pulsing nature of the TOF (e.g. TOF-TOF and Quadrupole-TOF (Q-TOF), but more recently has also been coupled to Ion trap (IT) mass spectrometers. This arrangement of MS components provides the advantages of sample throughput and high-confidence matching of MS/MS spectra into a single system. Moreover, these systems now have capacity to deal with complex lysates with the development of LC-MALDI applications allowing chromatographic separations to be directly spotted onto MALDI plates. One of the considerable advantages of this technique is the ability of lockdown samples providing the capacity to return to a plate for a subsequent detailed analysis of a particular spot of interest. The LC-MADLI setup appears to be providing an excellent means of obtaining proteomic depth and protein coverage when compared to other methods. These systems thus provide significant advances in the large-scale analysis of complex systems with unambiguous matching capabilities, high-throughput applications and the capability to analyse complex samples.

Electrospray ionization

The ESI source is commonly used to analyse complex mixture and is capable of interfacing with a wide range of mass spectrometers that usually provide MS/MS capabilities. ESI is an excellent method for the ionization of a wide range of polar molecules and relies on the sample of interest being dissolved in a solvent and delivered to the source in a thin capillary. A high voltage is to the tip of the capillary, producing a strong electric current which causes

the emerging sample to be dispersed into an aerosol of highly charged droplets. These droplets are vaporised by an inert gas such as nitrogen-releasing charged species for analysis by the mass spectrometer. Whatever the arrangement of MS components that is utilized, this approach relies on the fragmentation of each peptide in some sort of collision cell within the mass spectrometer yielding a fragmentation spectra or MS/MS spectra. Each MS/MS spectrum can then be interpreted individually to suggest a probable amino acid sequence (de novo sequencing) or can be used to search a database containing theoretical MS/MS spectra to suggest both an amino acid sequence and probable identity of the original protein.

The analysis of sample using ESI can readily be achieved manually using a syringe apparatus often provided with the system, but by far the most convenient and sensitive procedures involve a high-performance liquid chromatography (HPLC) connected to the ionization source. This provides sample automation and higher throughput but also has an added level of complexity. Samples are usually injected onto a reverse phase HPLC column such as C18 allowing the concentration and separation of peptides prior to analysis by the mass spectrometer. This analysis produces MS/MS data from the fragmentation of a parent ion (peptide) in the process termed collision-induced dissociation (CID). These CID spectra are high –quality data for pattern matching and interpretation of peptides.

Data analysis:

Peptide mass fingerprints: The ability to rapidly screen many hundreds of gel-separated samples at relatively low costs still makes PMF-based analyses a practical route. Since no sequence data are obtained (unless using PSD), data matching is heavily reliant upon mass accuracy and sequence availability. Most modern TOF mass spectrometers provide a high mass accuracy (±10-30 ppm) which is a crucial aspect to the analysis of PMF data. Thus the real limitation to using PMF in proteomic studies is sequence availability.

Peptide fragmentation data (MS/MS): The use of MS/MS data produced by a mass spectrometer capable of targeted peptide fragmentation provides an unsurpassed method of data matching and analysis in species with both a well characterized genome and those lacking genomic data. The data format provides the mass of the analysed peptide as well as some fragmentation data which can either be used directly for interrogation with a database using available software (e.g. MASCOT of SEQUEST) or can be deconvoluted to provide some sequence information (*de novo sequencing*), which can then be used to match to the sequence databases using the BLAST. The use of either method provides a far greater confidence in the resulting match, but just as with PMF matching, the final interpretation still requires some level of assessment.

Application of plant proteomics

Proteomics is a vital component of functional genomics and systems biology. Proteins being the actual functional entities of the biological systems their in-depth analysis using proteomics approach will be helpful in all the research areas aiming to have deeper molecular insights into the complex metabolic processes influencing plant growth, development, biomass potential and their interactions with environment.

Metabolomics

Metabolites represent a diverse group of low-molecular-weight structures including lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols and carbohydrates, which make global analysis a difficult challenge. It is estimated that more than 100,000 secondary metabolites are produced by plants, while the total number is estimated to exceed over 500,000. To assess this diversity of structurally complex chemical compounds, various approaches have been initiated largely due to the tremendous advances in the instrumentation and data handling capabilities. The comprehensive assessment of endogenous metabolites and attempts to systematically identify and quantify metabolites from a biological sample is known as metabolomics.

Methodologies for metabolomics

Sample preparation: With the exception of nondestructive nuclear magnetic resonance (NMR)-based, nevertheless still have certain limitations to application with plants, essentially all metabolomics approaches give us what is basically a 'metabolic snapshot' which is specific to the time of sampling. This so-called 'point-in-time-chemistry', provides us with a hugely valuable metabolic insight but it is also important to bear in mind that failing to take full and proper care of the cultivation and sampling of the plants will result in potentially misleading and incorrect conclusions. Full consideration must be given to these temporal and spatial dynamics of plant metabolism when wishing to perform comparative analyses on contrasting samples. Producing and comparing metabolic profiles of any biological materials is always possible but determining the significance and biological relevance of the differences observed is a challenging task.

Extraction, separation and detection: Chemical complexity, metabolic heterogeneity, dynamic range and ease of extraction remain the main challenges in developing an effective metabolomics technology platform. Multiparallel technologies are required to gain the desired broad metabolic picture. Careful selection of suitable combinations of extraction, separation and detection protocols can however, lead to a rapid build-up of complementary biochemical data on the composition of biological samples (LC-MS, GC-MS, high-pressure liquid chromatography (HPLC)-PDA); (GC-MS, LC-MS); (HPLC-PDA, FT-ion cyclotron resonance (ICR)- MS); (CE-MS, CE-PDA)). Consequently, both polar/semipolar (e.g. methanol (MeOH)/water) and lipophilic (e.g. chloroform) extractions are usually analysed and, especially for plants, an additional analysis of the volatile components via solvent extraction (e.g. pentane) or through headspace extraction (e.g. solid phase (micro)extraction, SP(M)E) is often desirable. The main detection technologies are described as follows:

Mass spectrometry (MS): is the primary detection method of choice for plant metabolomics due to its sensitivity, speed and broad application. Depending upon the type of extract made, GC (gas chromatography) or LC (Liquid Chromatography) are most routinely used for metabolite separation before the samples pass into the mass spectrometer. Capillary electrophoresis separation is, however, gaining interest.

*Gas chromatography–mass spectrometry (GC-MS) i*s currently proving to be the most popular global analysis method. Popularity stems primarily from the robustness of both the separation and the electron impact spectrometry technique and the availability of some excellent deconvolution and metabolite identification software. Together, these can be used for complete metabolite identification and quantification. Running standards and comparing with commercially available compound databases can be used to confirm metabolite identity. Gas chromatography-MS is the principal technique for separation and detection of metabolites that are naturally volatile at higher temperatures. However, the technology is more broadly applicable to groups of nonvolatile, polar (mainly primary) metabolites, such as amino acids, sugars and organic acids, by converting these into volatile and thermostable compounds through chemical derivatization. These derivatized samples can then be analysed by GC-MS.

Liquid chromatography-MS is a particularly important additional, versatile technology for plants as it also provides a means to analyse many large groups of 'secondary' metabolites often present in plant tissues. Advances in chromatographic technologies (e.g. the ultraperformance liquid chromatography (UPLC) system from Waters Corporation, Milford, MA, USA) together with advances in column chemistry (e.g. hydrophilic interaction chromatography (HILIC) and long monolithic columns) are yielding significantly improved separation potentials. The technology is inherently restricted to molecules which can be ionized, either as positively or negatively charged ions, before moving through the MS. A range of possible techniques is available in order to ensure the ionization of a broad range of (LC-separated) metabolites (e.g. electrospray ionization (ESI); atmospheric pressure chemical ionization (APCI); photoionization (PI)). The high analytical precision of modern LC techniques combined with the high sensitivity and mass accuracy and resolution of MS systems, in particular timeof- flight (TOF) and Fourier transform ion cyclotron resonance (FT-ICR)) instruments, is proving very useful in the analysis of complex metabolite mixtures typified by plant extracts. Working in positive and negative ion modes can provide broader coverage of molecules which more readily either gain or lose a proton. Rather than performing separate analyses, some machines now have the capacity to switch continuously between positive and negative modes during each run. Unlike GC-MS, few mass spectral libraries are available for LC-MS and this is a key topic being given considerable attention at present.

Capillary electrophoresis is an alternative separation technology growing in popularity when combined with MS (CE-MS) for extra selectivity and sensitivity. The value of application with the less complex microbial extracts has already been clearly demonstrated and wider use with plants can be expected. High-resolution chromatographic separation and sensitive detection of water soluble extracts make a strong combination suitable for the analysis of a diverse range of primary and secondary metabolites.

Fourier transform-ICR-MS (abbreviated as FTMS) is a much discussed but, as yet, rarely used technology for plant metabolomic analysis. More recent applications are beginning to emerge. This technique has the advantage of providing more accurate molecular mass estimations through a high resolving power. The extremely high mass resolution means that high chromatographic resolution becomes less important and is often omitted (though isomers, which can be very abundant in plant extracts, consequently cannot be

distinguished). Up to 2400 compounds analysed simultaneously, of which 781 were putatively identified, has been reported. Recently introduced orbitrap FTMS instruments offers not only faster and more sensitive analyses but also are significantly less expensive than the cyclotron FTMS machines are also worthy of attention. The technology is predicted to have a great future in the field of metabolomics, although for many the inability to separate structural isomers which have identical mono-isomeric masses is still seen as a significant limitation to its application. Linking up to some sort of prior isomeric separation strategy will be required to overcome this.

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) seems currently to be the method of choice for medical metabolomics (metabolomics). For plants, its application has so far been limited because of its currently much poorer sensitivity compared to MS-based methods. Nuclear magnetic resonance analysis is a favored choice for the major metabolites but many other important compounds will typically be missed as they occur in plant extracts at levels below the usual NMR detection threshold. It does, however, have the advantage that it is a more uniform detection system and can directly be used to identify and quantify metabolites, even *in vivo*.

Application of metabolomics in plants:

Plant metabolomics has a potentially broad field of application in plant science in terms of monitoring crop quality characteristics or identifying potential biochemical markers to detect product contamination and adulteration. Metabolomics is expanding the general knowledge of plant materials and is illustrating how organisms function as integrated biological systems. Studies in plant biology involving the four different types of metabolite analysis (target analysis, metabolite profiling, metabolomics, metabolite fingerprinting) may be classified into following five broad research areas viz: (i) Bioengineering of metabolism, (ii) metabolic pathway analysis, (iii) deciphering the complex physiological processes, (iv) development of plant metabolomics methods, and (v) food science.

In bioengineering, metabolite analysis is typically restricted to only a few target compounds or select pathways are profiled to conclude the effectiveness of a certain treatment. If metabolite profiling or target analysis is used, hundreds of lines involving thousands of analyses can easily be scrutinized to guide the bioengineering efforts because both sample purification and instrumental methods can easily be optimized for this task. The result of metabolomic analyses is a series of measurements of metabolite levels, that is, snapshots of metabolism. However, this is just one way to study the control of metabolism. Metabolic snapshot data are usually not sufficient to directly derive enzyme activities and hierarchical structures of pathways and, even more importantly, the dynamics of carbon partitioning between the different organs or the different metabolic cycles. A range of possible scenarios may explain a finding of altered metabolite levels: anabolic reactions might be faster, the catabolic fate might be different or transport activities may have been changed. For vascular plants, a highly suitable way to analyze fluxes is to use NMR-based techniques. Physiological adaptation to environmental stress has been the focus of several studies. Investigating metabolic relationships has been the focus of a physiological study on sink–source transitions in plant. Novel insights into the control of metabolism go hand in hand

with improved methods. For this reason, various research groups validated the usefulness of novel methods or improved protocols by using model plant experiments. Metabolomics has also found application in control of metabolism is food science. Food quality is easily and rapidly deteriorated by a number of different pests, and therefore it must be tightly monitored to prevent major losses. Metabolomics based methods enable a rapid survey during food storage by observing metabolic effects for disease diagnostics rather than trying to understand the biochemical or physiological control mechanisms.

Systems biology approach seems to be very valuable in answering the complex issues related to plant growth, development and their interaction with environment. A natural shift is taking place in the approaches being adopted by plant scientists in response to the accessibility of systems-based technology platforms. Metabolomics is one such field, which involves a comprehensive non-biased analysis of metabolites in a given cell at a specific time. Metabolomics now plays a significant role in fundamental plant biology. Plants collectively produce a huge array of chemicals, far more than are produced by most other organisms; hence, metabolomics is of great importance in plant biology. Metabolomics has gained importance in biotechnology applications, as exemplified by quantitative loci analysis, prediction of food quality, and evaluation of genetically modified crops. Systems biology driven by metabolome data will aid in deciphering the secrets of plant cell systems and their application to biotechnology.

Pathogenomics and its role in fungal disease management - *Phytophthora infestans* as an example

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Introduction

Late blight caused by oomycete *Phytophthora infestans* (Mont.) de Bary has historically been an important disease of potatoes and tomatoes worldwide. In the mid 1800, late blight caused widespread crop failures throughout Northern Europe including Ireland where it was responsible for the Irish famine (Elansky *et al*., 2001). Since then, it has spread far and wide and now occurs wherever potatoes are grown. Crop losses and costs of late blight control constitute significant financial burden on the potato industry. Losses due to *P. infestans* have been estimated to € 12 billion per annum of which the losses in developing countries have been estimated around € 10 billion per annum (Haverkort *et al*., 2009). Predicting the sustainability of disease management strategies is clearly dependent on an understanding of the pathogen and its population dynamics. This is especially true of potato late blight, as *P. infestans* has been classified as 'high risk' based on evolutionary potential. *Phytophthora infestans* is thus a moving target and the bodies responsible for practical long and short term advice to the potato industry need data on contemporary pathogen populations. The resurgence of late blight in the past two decades and the presence of new highly virulent genotypes of the pathogen emphasize the need to intensify research on the biology of *P. infestans*. With the emergence of the new population of late blight pathogen, monitoring the population structure of *P. infestans* has been on the agenda of scientific community. In light of the threats from changing *P. infestans* populations, particular emphasis should be placed on the utility of existing phenotypic and genotypic markers and the potential of new methodologies for examining *P. infestans* populations.

Characterization of variability in *Phytophthora infestans*

With the introduction of A2 mating type, there has been a quantum change both in disease behaviour and *P. infestans* population structure. Late blight is appearing with regularity even in sub-tropical plains, resulting in heavy crop losses. Monitoring population structure of *P. infestans* has been on the agenda of scientific community and new techniques have become available. Earlier biological (phenotypic) markers including mating types, race pattern and metalaxyl sensitivity were used for monitoring the population structure of *P. infestans.* But now with the introduction of molecular techniques, other markers have gained importance. However, no single marker system is adequate for all aspects of *P. infestans* research.

Phenotypic markers

There are relatively few reliable morphological characters by which *P. infestans* isolates can be discriminated. The most studied and informative traits are mating type, virulence and fungicide resistance.

Virulence (Physiological races): *Phytophthora infestans* is highly variable. Genetic analysis of the resistance introgressed into *Solanum tuberosum* from wild *Solanum* species demonstrated a gene-for-gene interaction with single R genes in the host and corresponding virulence genes in the pathogen. An isolate's race or virulence phenotype is determined by inoculating a series of 11 genetically defined 'differential' potato genotypes, each carrying a specific R gene. The most effective method of control of late blight lies in the breeding and cultivation of blight resistant varieties. However, resistance to late blight is not stable. In due course of time, new virulences develop in the pathogens which overcome the host resistance. Therefore, for the success of resistance breeding, information on prevalence and development of new virulences (physiological races) is essential. The rate of development of race complexity in the pathogen appears to be governed by the duration of the conditions favourable for disease development and multiplication of the pathogen. The longer duration of favourable conditions will produce more generations of the pathogen thereby increasing the chances of mutation, somatic recombination and adaptation. Once the race spectrum becomes complex as it has happened now, the utility of monitoring races becomes irrelevant. However, prevalence and frequency of different virulence genes need monitoring to understand their associations and disassociation, and to select broad spectrum host resistance. The frequency of different virulence genes varies from place to place and year to year.

Mating type: *P. infestans* is heterothallic and requires two different mating types designated as A1 and A2 for sexual reproduction. Prior to 1984 the A_2 mating type was restricted to Mexico and Andean mountains which is the centre of origin of cultivated potatoes. Studying the spatial and temporal distribution of the A1 and A2 strains of *P. infestans* is fundamental to understanding the genetic diversity and to disease aetiology. So monitoring of mating types is essential to know whether sexual reproduction can occur in the given region or the country.

Metalaxyl insensitivity: The development of resistance to fungicides is a major problem in the management of plant diseases. Phenylamide fungicides such as metalaxyl, when they were first introduced, displayed excellent performance as systemic fungicides with protective, curative, and long-lasting activity against many important plant pathogenic fungi within the Peronosporales. However, strains insensitive to phenylamides have since appeared in many important crop systems. For example, not long after the introduction of metalaxyl to the European market in the 1970s, insensitive strains of the late blight pathogen *P. infestans* were detected and, by the 1990s, insensitivity was reported in North America and other regions. Monitoring of *P. infestans* population for metalaxyl sensitivity revealed that the pathogen has acquired resistance to metalaxyl within a period of 3-4 years of its commercial use. The metalaxyl sensitivity analysis of the Indian isolates showed that tolerance levels to phenylamide fungicides increased significantly in most of the locations, probably due to increased fungicide usage in the last decade. Almost all the isolates prior to 2000 were metalaxyl-sensitive, while most isolates post-2002 showed a sharp rise in resistance levels at most of the locations in India.

Genotypic markers

Ploidy status: While polyploidy is known to play an important role in the evolution of higher plants and animals, its role in the evolution of fungi has been emphasized by some and

dismissed by others. Polyploidy can provide an important mechanism for the maintenance of genetic variation, and thus has been considered an important evolutionary factor. In *P. infestans*, polyploidy, as measured using Feulgen-DNA cytophotometry, appears to be quite common in populations outside Mexico. The presence of polyploidy may be advantageous to *P. infestans* in two ways. One that tetraploid may be better adapted to the cooler conditions found in temperate zones compared with their related diploids. Secondly, polyploids can harbour a wider array of virulence allele combinations compared with diploids, polyploidy could contribute to the development of the many virulence phenotypes. Data on ploidy status reveals that *P. infestans* population in India consists of diploids, triploids and tetraploids. Temperate population consisted of diploids, triploids and tetraploids. A1 mating type isolates were predominantly diploids followed by triploids. Frequency of different polyploids in A2 type strains was equal. Sub-tropical population (A1 mating type) was predominantly diploid and tetraploid. Studies on sexual compatibility amongst different polyploids revealed that isolates of the same ploidy status mated freely i.e. diploid x diploid or tetraploid x tetraploid whereas those with varying ploidy status did not mate that frequently indicating that sexual reproduction in nature would be conditioned by the ploidy status of the *P. infestans* genotypes. Polyploids also differ in their aggressiveness.

Isozymes: Isozymes are ideal tools for population genetics as they are easily assayed, stable, co-dominant which is important in a diploid organism like *P. infestans*, and generally under simple genetic control. Glucosephosphate isomerase (*Gpi*) and peptidase (*Pep*) are two systems for which simple genetic control has been demonstrated. Allozyme genotypes are described in terms of relative mobilities of their bands of activity during electrophoresis. The most common allele is assigned a mobility of 100 and other alleles are assigned a number based on their relative mobility. Thus 90/100 refers to a heterozygous genotype with two alleles, one allele being the most common type, and the other producing an enzyme that migrates 90% as far as the common type. The 90 allele for *Gpi* and the 83 allele for *Pep* were detected only in Europe for the first time after detection of the A_2 mating type: the changes in allozyme alleles occurred concomitant with the change in mating type structure. The allozyme genotypes *Gpi* 90/100 *Pep* 83/100; *Gpi* 90/100 *Pep* 100/100; *Gpi* 100/100 *Pep* 83/100 and *Gpi* 100/100 *Pep* 100/100 are characteristic of the new population. US-1 probably has two copies of the 100 allele and one of the 86 allele. US-8 probably has three different alleles at the *Gpi* locus: 100, 111, and 122. This would give a six-banded phenotype on a gel, with three homodimer (100/100, 111/111, and 122/122) and three heterodimer (100/111, 111/122, 100/122) bands. US-6 genotype has a *Gpi* banding pattern 100/100, US-7 has 100/111, US-11 has 100/100/111, US-10 has 111/122, and US-17 has 100/122. As for Pep, US-1 and US-6 have bands 92/100; US-7, US-8, US-11, and US-17 have genotype 100/100. In India, *P. infestans* population has been analysed for *Gpi* since 1998. In India, all the isolates were monomorphic as single band was resolved for *Gpi*. This makes the *P. infestans* Indian population distinct from European and American populations.

RFLPs: The moderately repetitive RFLP probe RG57 yields a genetic fingerprint of 25-29 bands and has proved a valuable tool in monitoring *P. infestans* genetic diversity. Many thousands of isolates worldwide have been fingerprinted and an international database of the results constructed. The dataset has been important in defining and monitoring lineages of *P. infestans* and tracking inoculum sources.

Mitochondrial DNA haplotypes: Mitochondrial DNA variation may be more useful than nuclear DNA variation when studying the migration events in *P. infestans,* since it evolves rapidly, uniparentally inherited and no segregation, elimination, or recombination of haplotypes has been observed. This has undoubtedly played an important role in the recent evolution of this species, and this molecule may also be useful for inferring phylogenetic relationships among *Phytophthora* species. The most straightforward approach to analyzing polymorphisms in mitochondrial and nuclear DNA is by comparison of digestion profiles produced by type II restriction endonucleases. These enzymes recognize specific sequences of nucleotides and cleave double stranded DNA wherever such sequences occur, producing a range of lengths of linear DNA known as restriction fragments. Two mitochondrial types, type I and type II, by digestion of total DNA with the frequently cutting restriction enzymes *Msp*I or *Cfo*I have been defined. Type I is further differentiated into haplotype Ia and Ib; similarly, type II is subdivided into haplotypes IIa and IIb. The principal method for characterizing *P. infestans* mtDNA type is PCR-RFLP method.

AFLPs: Amplified fragment length polymorphisms have proved very powerful marker, since they yield many loci per primer combination. They have been central to the genetic mapping of *P. infestans* and resolve at a level appropriate for examining intrapopulation diversity. The method is sensitive to changes in DNA quality, time consuming and requires very pure DNA, which means it cannot be applied to infected plant material.

SSRs: Simple sequence repeat markers, or microsatellites, have revolutionized the fields of molecular ecology and phylogeography as well as proving to be powerful tools for genetic analysis. Microsatellites are short fragments of DNA in which motifs of 1-6 bases occur in tandem repeats. Slippage during DNA replication results in periodic alteration of the repeat length, which is scored by accurate sizing of the PCR amplified repeat and its immediate flanking sequence. They offer a taxonomic resolution suitable for the analysis of individual isolates within a population and phylogenetic relationships between closely related taxa. Both alleles at a locus are amplified and discriminated simultaneously, yielding codominant data appropriate for detailed population genetic analysis.

SNPs: Single base pair differences in DNA which occur as a result of point mutations are termed as single nucleotide polymorphisms. They represent the main source of genetic variation in the genome. SNP based markers share many of the advantages of SSRs and they are thus powerful tools for genetic analysis, as well as for the estimation of population parameters. SNP markers do, however, have specific advantages for particular applications. Since these markers are based on the specific SNP responsible for an amino acid replacement in a functional protein, they are vital in the direct monitoring of the frequencies of functional alleles in natural and experimental populations.

Detection of *Phytophthora infestans*

Most *Phytophthora* species cannot be isolated from diseased tissues as readily as many other fungal pathogens, much research has been devoted to the development of supplemental strategies to increase the frequency of selective isolation of *Phytophthora*. The following methods are employed for the detection of *Phytophthora* species.

Serological methods: There is a need to supplement conventional methods of diagnosis that utilize symptoms and direct isolation with newer technologies that might be less time consuming and possible more accurate. Both serology and DNA technologies are rapidly becoming available for research and commercial purposes. Serology is based on the ability of antibodies from the blood of a mammal to recognise specifically a foreign protein or carbohydrate. There is a range of specificities of different antibody systems. Monoclonal antibodies are much more specific. The most practical use of serology for detection is the well-known enzyme linked immunosorbant assay (ELISA), which is now prepared at commercial levels to detect *Phytophthora* species in soil and plant samples. The ELISA method is also being used to quantitate the amount of *Phytophthora* in plant tissue.

DNA probes: The use of DNA probes to identify pathogens provides a highly specific tool. This exciting new technology is rapidly becoming a new frontier for research on detection and identification of *Phytophthora* species and biotypes and may help not only to differentiate species with similar morphology but also to detect species in roots and soil with more precision. In recent years PCR has emerged as a powerful tool in plant disease diagnostics as it is more sensitive, accurate, robust and rapid, less labour intensive, and more economical than conventional diagnostics. Rapid detection of *P. infestans* from potato seedlot has been established. Amplified products were detected with DNA from tissue with obvious symptoms as well as those without any symptoms. Primers (INF 2 & ITS 3) based on ITS2 region of rDNA could detect pathogen in potato leaf and tuber and yielded a PCR product of 456 bp with a sensitivity limit of 1 to 10 pg of DNA. These specific primers and the rapid NaOH extraction method could detect late blight in artificially and naturally infected tubers. PCR could detect *P. infestans* from artificially infected tubers at 4 days post-inoculation, before any visible symptoms were present. In addition, sensitivity of the PCR assay was limited to 1pg of DNA. Similarly, detection of inoculum in soil was validated and up to 10 oospores per 0.5 g soil were detectable.

Real-time PCR combines the sensitivity of conventional PCR with the generation of a specific real-time fluorescent signal throughout the reaction. Real-time PCR enables automation of the technique and is suitable for large scale sample processing and has the potential for an accurate quantification of target DNA. Quantitative analyses are of basic importance for development of predictive diagnostic test to identify high risk fields where pathogens inoculum is above threshold values. Among the PCR-based real-time techniques, four main methods are currently used for the application of this technique in plant pathology, which can be grouped into amplicon sequence non-specific (SYBR Green I) and sequence specific (TaqMan, Molecular beacons & Scorpion-PCR) methods. The detection and sensitivity limit is 10^4-10^5 times more than conventional PCR and allowed the processing of many samples in a short time.

Suggested Readings

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Molecular tools for bacterial disease management

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Bacteria are single-celled microorganisms, generally ranging from 1-2 µm in size that cannot be seen with the unaided eye.Of the over 15,000 identified species of bacteria, around 200 species are phytopathogenicwhich cause many serious diseases of plants throughout the world. The absolute control of these bacterial diseases, at present, is difficult to achieve as chemical control options are very limited. Chemical sprays have only rarely proved effective in controlling bacterial diseases. In some cases, copper-based sprays are effective at minimizing inoculum build-up. But copper-resistant bacteria have also arisen. Another example is the use of the antibiotic streptomycin in America and New Zealand to control fire-blight of pome fruit caused by *Erwiniaamylovora*. However, streptomycin resistant strains of the pathogen have appeared and are no longer effectively controlled. In many countries, the use of antibiotics of human medical value to control plant diseases is discouraged. Disease resistance is considered an important method for control of bacterial diseases but for many phytopathogenic bacteria, the resistance sources are either limited or not available. Also, sometimes resistance breaks down when temperatures are high. The development of new virulent strains that can attack previously resistant cultivars may also occur. Biological control has been investigated but is still in its infancy. Other methods of control include sanitation and control by cultural methods such as crop rotation. The most appropriate method of control depends on the nature of the disease and on the crop that is affected. Therefore, while considering management measures for bacterial diseases, it is essential to know how the pathogen is spread and how it survives from one crop to the next. For example, if the pathogen is seed-borne, it will probably be easier to ensure a supply of healthy seed than to control the disease in the field.

Consequently, prevention is essential to avoid the dissemination of bacterial pathogens through different vehicles such as contaminated propagative plant material, vectors, irrigation water, soil, etc. The prevention measures demand pathogen detection methods of high sensitivity, specificity and reliability because many phytopathogenic bacteria can remain latent in propagative plant material and in other reservoirs. Accuratedetection of phytopathogenic organisms is crucial for virtually all aspects of plant pathology, from basic research on the biology of pathogens to the control of the diseases they cause. Moreover, the need for rapid techniques of high accuracy is especially necessary forquarantine pathogens, because of the risk of the disease and the spread of the inoculum must be reduced to nearly zero.

Traditionally, the available detection and diagnostic techniques for plant pathogenic bacteria have been microscopic observation, isolation, biochemical characterization, serology (mainly through immunofluorescence and Enzyme-Linked Immunosorbent Assay (ELISA) using polyclonal and/or monoclonal antibodies), bioassays and pathogenicity tests. Biological indexing, electron microscopy and some biochemical and staining tests have been used for testing pathogens of the genus *Spiroplasma* and phytoplasmas.

Standard protocols for detection of plant bacteria based on isolation and further identification are time consuming and not always sensitive and specific enough. Consequently, they are obviously not suited for routine analysis of a large number of samples. Other handicaps are the low reproducibility of identification by phenotypic traits, frequent lack of phylogenetic significance and false negatives due to stressed or injured bacteria, or those in the viable but non culturable state (VBNC), which escape from isolation.

Nucleic acid-based techniques are becoming popular and are being used increasingly for detection of plant pathogenic microbes. These methods are sensitive, specific and allow genetic relationships to be determined. In plant pathology, the most frequently used molecular techniques have been, first, molecular hybridization and, afterwards, the polymerase chain reaction (PCR). Compared to traditional methods, PCR and its variants offer several advantages, because, organisms do not need to be cultured before their detection, it affords high sensitivity at least theoretically, enabling a single target molecule to be detected in a complex mixture, and it is also rapid and versatile. However, nucleic acids extraction protocols are usually necessary to obtain a successful result when processing plant or environmental samples by molecular methods. This specific aspect, as well as primer design for PCR, is considered below.

Plant sample preparation: Accuracy of molecular analysis for pathogen detection in plant material requires efficient and reproducible methods to access nucleic acids. The preparation of samples is critical and target DNA or RNA should be made as available as possible for applying the different molecular techniques. There are a great many published methods for preparing the plant tissues, soil, water or other type of samples before molecular detection of plant pathogenic bacteria. Depending on the material to be analysed the extraction methods can be quite simple or more complex. The use of commercial kits, either general or specifically designed for one type of plant material, has gained acceptance for extraction, given the ease of use and avoidance of toxic reagents during the purification process. Among those: DNeasy and RNeasy Plant System, Qiagen; Ultra Clean Plant RNA and DNA isolation kits, MoBio; Easy-DNA-Extraction kit, Invitrogen; Nucleon plant tissue, Amersham; EaZy Nucleic Acid Isolation Plant DNA kit, Omega Bio-tek; Wizard Genomic DNA and SV Total RNA Isolation System, Promega; Extract-N-Amp Plant PCR kit, Sigma; Powersoil DNA kit, MoBio; RNA/DNA/Protein Purification Kit, Norgen; Quickpick Plant DNA, Bio-Nobile; and others, are applied in different models with success. Nevertheless, simple laboratory protocols have also been developed with few steps and minimal handling, reducing the risk of cross contamination, cost and time, with similar results to those of longer and more expensive. In addition, several commercial automated systems allow the extraction and analysis of nucleic acids from plant and microorganisms and even equipment performing automatic separation has also been developed (QIAcube, QIAgen, CA, USA; DNA extractor, Applied Biosystems, USA; X-Tractor Gene, Corbett, USA).

Primers and probes: The molecular methods for detection of plant pathogens are based on the use of specific sequences (oligonucleotides/probes), and their accuracy is basic for designing a good protocol. Partial or complete nucleotide sequences of each DNA or RNA target can be found in the Nucleotide Sequence Search program located in the Entrez Browser program provided by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Genbank/). Conserved regions for each target can be studied using the similarity search Advanced BLAST 2.2.18, with the blastn program designed for

analysis of nucleotides (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). Specific nucleotide regions should be selected and, by using this methodology, appropriate PCR primers to different DNA or RNA targets can be easily and properly designed for bacteria and other micro-organisms.

The DNA sequences from which the primers are designed for bacteria come from three main origins: pathogenicity/virulence genes, ribosomal genes, and plasmid genes. The sequences of published primers for phytobacteria have been compiled by various authors. A recent compilation (Palacio et al, 2009) reports more than two hundred PCR protocols for detection and identification of more than 50 bacterial species, 9 sub species and more than 40 pathovars. The pathogenicity genes used as targets can be involved in any of the several steps leading to symptoms development and can be related to virulence factors, virulence or avirulence genes, toxin products, other factors.

Several variants have been developed to improve sensitivity of conventional PCR. Among the first described, nested-PCR, with both internal and external primers to the target sequence, was reported to increase sensitivity and reduce the effect of inhibitors. However, in nested-PCR the risk of cross-contamination in routine analysis of large numbers of samples is increased by the introduction of a second round of amplification and the simultaneous manipulation of the previously amplified products. To avoid these problems, nested-PCR in a single closed tube has been developed.

A new method named co-operational polymerase chain reaction (Co-PCR) has been described for highly sensitive detection of plant viruses and bacteria. Co-PCR is based on the simultaneous action of three or more primers that produce three or more amplicons by the combination of the primers and the co-operational action of amplicons for the production of the largest fragment amplified by the external primers. As it is performed in a single reaction, it minimizes contamination risks and has a level of sensitivity similar to nested-PCR and real-time PCR.

Multiplex PCR protocols using specific primers have also been set up for simultaneous detection of two genes of the same bacterial pathogen, thus limiting false positives, or allowing amplification of several pathogenic bacteria in seed or plant material or even detection of one bacterium and four viruses in olive plants.

Further advances have also been made through the use of real-time PCR, which offers advantages over conventional PCR because data are available in real-time, do not require time consuming post-PCR processing and can be quantitative. Moreover, it is a high-throughput technique for many plant pathogens from different sample types. The ability to quantify pathogen populations using quantitative real-time PCR holds great potential for epidemiological studies, for determining seed-borne or plant-borne inoculum and for establishing and monitoring transmission thresholds as bases for improved disease management.

Real-time PCR and melting curve analysis (MCA) are state-of-the-art techniques for quantifying nucleic acids, mutation detection, genotyping analysis as well as for detection and diagnosis purposes. Many different systems have been developed, including probe-based methods, such as TaqMan Probes, molecular beacons, Scorpion etc. In general, the

protocols developed are based on hybridisation of the probe to the target amplicon, thus achieving maximum sensitivity and confirming the identity of the amplified product. Real-time PCR, which can provide accurate and rapid detection of bacterial pathogens, is becoming the gold standard for diagnosis of plant-pathogenic bacteria, as well as of other organisms.

Accurate diagnosis or detection of plant pathogenic bacteria often requires multiple complementary tests to achieve definitive identification. Besides, PCR-based approaches require thorough studies of target pathogens to both characterize their diversity and identify common stable markers for designing specific primers. It is necessary to indicate that, although most protocols are claimed to be specific, they must be validated against a large collection of strains of the target bacterium and other pathogens of the same host, as well as against organisms of its environment, before they can be used as standards. The reliability of the protocols will eventually be demonstrated after years of use. A standard protocol must subsequently be established and optimized based on results.

The development of protocols withhigher and well-balanced sensitivity, and specificity fordetection of plant pathogens will have a positive effecton the sanitary status of thecultivated plants, reducingthe long distance spreading of new or emergentpathogens in a globalized world. This should drasticallyreduce the need for pesticide treatments, increase theprotection of ecosystems and enhance the quality offood and the environment. The accuracy of new detection protocolsbased on molecular methods will lie behind theavailability of plants free of a wide range of pathogens ina near future.

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Molecular approaches in disease diagnostics

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Early and reliable diagnosis methods allow the monitoring of the pathogen and enable farmers to administer suitable management strategies in a timely fashion and would greatly increase the effectiveness of the treatment. In general, the disease can be diagnosed by the presence of symptoms like blight, spots, wilting or by the presence of mycelia growth or other structures and bacterial ooze or can be diagnosed by mosaic patterns on leaves, stunting of the plant, leaf malformations, and tuber malformations in case of viral diseases. However, recent advances in biotechnology and molecular biology have played a significant role in the development of rapid, specific and sensitive tests. They are either serology based (ISEM, Dot blot assay, ELISA & LFIA) or nucleic acid based (NASH, PCR, real time PCR, RCA, LAMP, Micro or macro array and small RNA deep sequencing) techniques. Sensitivity of serology based techniques is poor compared to nucleic acid based techniques. Moreover, reliability of those techniques is dependent on requirement of polyclonal or monoclonal antibody sera specific for each pathogen that does not cross-react with plant proteins.

Microscopic techniques

Almost all diagnostic techniques like biological, serological and molecular gives an indirect evidence of the causal agent (etiology) whereas, Electron Microscopy gives a direct access to see the causal agent. TEM is one of the most powerful scientific tools for carrying out detail structural studies of biological materials. In TEM studies, negative staining made it easy for detection of viruses from liquid samples. This led to the widespread application of TEM in the field of basics of virology and rapid diagnosis of viruses. Due to the advancement of the science, electron-opaque gold nano particles labelled immunoglobulin (IgG) complexes were used successfully for electron microscopic detection of plant viruses at the ultra-structural level. Immune-gold electron microscopic technique (also known as GLAD *i.e.,* gold-labelled antibody decoration) with colloidal gold-labelled IgG was used for localization of *Barley stripe mosaic virus* (BSMV) in ultrathin sections of wheat cells, *Tobacco mosaic virus* (TMV) in tobacco, *Wheat streak mosaic virus* (WSMV) in wheat, *Cowpea mosaic virus* (CPMV) in cowpea, Brome mosaic virus (BMV) in barley, *Potato leafroll virus* (PLRV) in potato and *Barley yellow dwarf virus* (BYDV) in oats by simple leaf dip method. Here visualization of the antigen-antibody reaction is achieved by using colloidal gold (CG) labelled antibodies. Therefore, this technique is more sensitive in comparison with classical direct electron microscopy and immune electron microscopy. At the same time it has the advantage of being highly specific and can detect the viruses under low concentration in infected tissues. FTIR microscopy can be used for identification of fungal pathogens directly from the surface of infected potatoes, as a representative model for *in vivo* rapid detection of plant fungal pathogens (Erukhimovitch *et al*., 2010).

Enzyme Linked Immuno sorbent assay (ELISA)

The technique utilizes the ability of antibodies raised in animals to recognize proteins, usually the coat protein, of the virus of interest. Antibodies are fixed to the surface of a well within a microtitre plate, and a sap extract from the plant is added to the well. If the virus of interest is present in the plant, it will bind to the antibodies fixed on the surface. Any unbound extract is washed-off before a secondary antibody that recognizes the first antibody is added. The secondary antibody allows for indirect detection of the virus because it has a reporter molecule attached to it, usually an enzyme that acts on a substrate that changes colour, which is detected visually by a calibrated microtitre plate spectrophotometer.

Lateral flow immuno assay (LFIA)

Rapid diagnosis of viral infections for virus-free plant culture industry or individual use requires inexpensive, sensitive, and simple technologies that make possible analysis without special skill and equipment even under field conditions. One of the promising solutions for overcoming this challenge is lateral flow immuno assay (LFIA), based on the interaction between the target virus and immunoreagents (antibodies and their conjugates with colored colloidal particles) applied on the membrane carriers (lateral flow test strips). When the test strip is dipped into the sample being analyzed, the sample liquid flows through membranes and triggers immunochemical interactions resulting in visible coloration in test and reference lines (Fig. 1). Test strips were developed for detection of five plant viruses varying in shape and size of virions: spherical carnation mottle virus, bean mild mosaic virus, rod shaped tobacco mosaic virus, and filamentous potato viruses X and Y by Byzova *et al*., (2009). CPRI has developed LFIA kits for the detection of five viruses *viz*., PVX, PVA, PVS, PVM and PVY either individually or in combination of two viruses viz., PVA & PVX, PVA & PVS, and PVY & PVM or three (PVX, PVA & PVM) using a single strip.

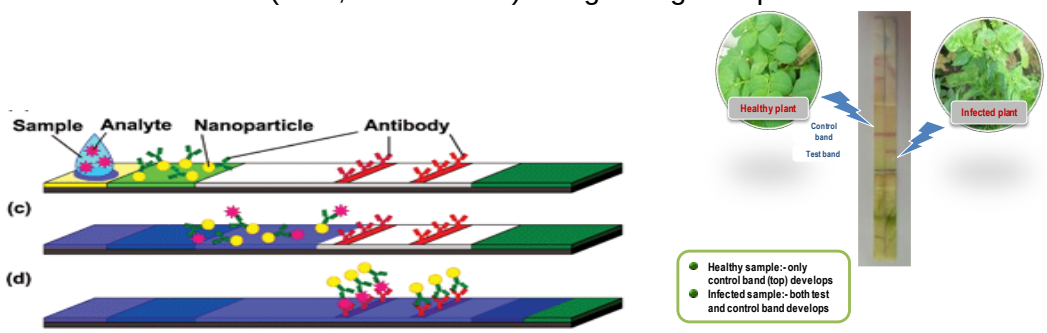

Fig. 1. Working principle of LFIA and interpretation results

Polymerase chain reaction (PCR)

RT–PCR and PCR are popular techniques for detection and identification of RNA and DNA plant viruses, respectively. The procedures are extremely sensitive, fairly inexpensive and require minimal skill to perform. In the case of RNA viruses, a cDNA strand complementary to the virus is made with reverse transcriptase (RT). Oligonucleotide primers, flanking part of the genome of the virus, are extended by a thermostable DNA polymerase in a series of denaturation and extension steps that exponentially increase the target DNA. PCR-based methods can be adapted to high-throughput applications. In addition to detection of the virus,

an additional advantage of the method is that the amplicon can be sequenced to provide further data about strain types. Possible drawbacks of the method are the need for a thermocycler, which can be expensive, and sequence information for design of primers. With databases containing ever-growing numbers of virus sequences, access to sequence information for many viruses is possible. Careful primer design is crucial, whether to detect only a single strain, or all the members of a genus. The sensitivity of the method is its major advantage. High sensitivity, however, can easily lead to false positive results from contamination; so adequate controls are essential. RT-PCR protocols for detection of major potato viruses have been standardized that can detect very low level of virus inoculums (Singh and Nie, 2003, Lorenzen *et al.*, 2006). Several variations of RT-PCR like Immuno Capture PCR (IC-PCR), nested PCR and multiplex RT-PCR have been standardized for detection of potato viruses or to differentiate strain variation of a particular virus. Multiplex RT-PCR is a time- and reagent-saving amplification technique in which multiple primer sets are used to amplify multiple specific targets simultaneously from the same sample. Phylotype specific multiplex PCR is used to identify and differentiate different phylotypes of *Ralstonia solanacearum*, bacterium infecting potato, tomato, brinjal, Ginger etc. ITS and 16s rRNA based PCR assays are used to detect fungal and bacterial pathogens. The enrichment of the bacteria in semi selective liquid medium prior to PCR (BIO-PCR) is an important initial step for the success in PCR detection extremely low number of the target bacteria from tubers. *Erwinia carotovora* subsp. *atroseptica* (Eca), *Erwinia carotovora* subsp. *carotovora* (Ecc) and *Erwinia chrysanthemi* (Ech) are the different sub species of *Erwinia* that cause the diseases commonly known as blackleg, aerial stem rot and soft rot on potato. The different strains appear to differ in their ability to compete with other saprophytes and reach the target detection limit of bacterial population during the enrichment culture of the potato peel extract.

Real time PCR or quantitative PCR

Real time PCR allows quantification of the target DNA as it is amplified. Real time RT-PCR protocols are now being standardized for potato viruses in several laboratories (Agindotan *et al*., (2007); Mortimer-Jones *et al*., (2009). For potato viruses, TaqMan® duplex RT-PCR have been used for the detection of *Tobacco rattle virus* (TRV) and *Potato mottle top virus* and for PLRV and PVY. Agindotan *et al*., (2007) reported an assay where four common potato-infecting viruses, *Potato leafroll virus*, *Potato virus A*, *Potato virus X* and *Potato virus Y*, were detected simultaneously from total RNA and saps of dormant potato tubers in a quadruplex real-time RT-PCR. A single tube real time RT-PCR was reported by Mortimer-Jones *et al*., (2009) to detect single infections of PLRV, PVX, PVS and TSWV simultaneously in a single assay from bulked samples equivalent to 300 dormant tubers. Real time PCR protocols for detection of very low level of virus inoculums in nucleus seed stocks and mericlones have been standardized for all common potato viruses at CPRI (Fig. 2).

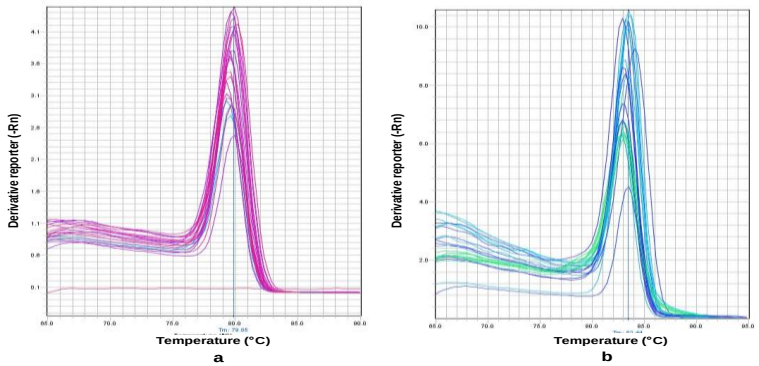

Fig. 2. Realtime RT-PCR detection of ToLCNDV-potato and PVX from field samples- Meltcurve analysis showing specific peaks (a). ToLCNDV-potato and b. PVX

Molecular beacons

Molecular beacon consists of a single-stranded DNA with a stem-loop structure (Fig. 3a). The loop portion consists of a probe sequence that is complementary to a target sequence. The stem portion is formed by the annealing of the 5' and 3' arm sequences, which are unrelated to the target sequence. A fluorescent moiety is attached to the 5' arm terminus, while a quenching moiety is attached to the 3' arm at the opposite end. The molecular beacon is added to a solution to be tested for the presence of the target nucleic acid. The mixture is heated to 80°C and then allowed to cool to 20°C. The fluorescence intensity is monitored continuously during the entire period. In the absence of a target, the arms associate to form a stem-loop conformation. The fluorescence emitted from the fluorophore is quenched by fluorescence resonance energy transfer (FRET) *via* the quencher due to their close proximity to each other (Fig. 3a). In the presence of a target, the probe forms a hybrid with the target via its complementary sequence within the loop region, resulting in the displacement of the fluorescent moiety from the quenching moiety, thus resulting in fluorescence emission (Fig. 3b). The molecular beacons emit fluorescent signals only upon hybridization with their complementary target nucleic acids.

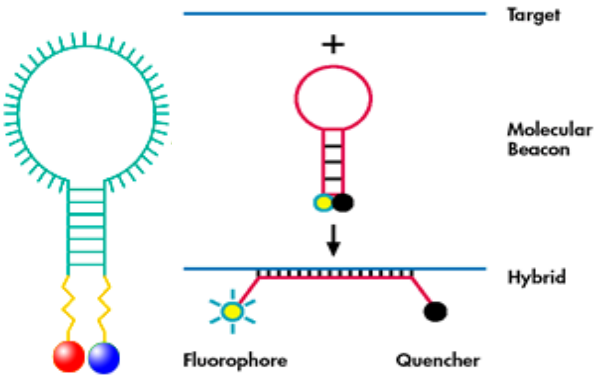

Fig. 3a **Fig. 3b**

Fig. 3. Structure of molecular beacons (3a) and its function (3b)

Multiple molecular beacons can be employed in a single reaction tube. This is feasible, since each molecular beacon could be tagged with fluorescent moieties that possess different

emission wavelengths. Eun and Wong, (2000) designed four molecular beacons specific to the RNA-dependent RNA polymerase and coat protein genes of two orchid viruses, namely *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) and successfully applied to detect as little as 0.5 ng of viral RNA of both orchid viruses simultaneously in 100 mg of coinfected *Oncidium* orchid leaves.

Rolling circle amplification (RCA)

RCA is an isothermal method which is reliable for diagnosis of geminiviruses and presumably all viruses with small single-stranded circular DNA genomes. The results showed the efficiency of this technique in characterizing viral DNA components of several geminiviruses from experimental and natural host plant sources. RCA amplified viral DNA can be characterized by restriction fragment length polymorphism (RFLP) analysis and directly sequenced up to 900 bases in a single run, circumventing cloning and plasmid purification. RCA is better, easier and cheaper than polymerase chain reaction (PCR) or antibody-based detection of geminiviruses. RCA in combination with PCR assay increased the sensitivity and specificity of the assay (Rocha *et al*., 2010). RCA-PCR has been used to detect ToLCNDV-potato, causing apical leaf curl disease which is an emerging disease of potato (Fig. 4).

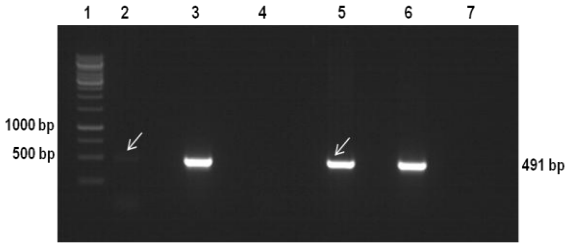

Fig. 4. RCA-PCR detection of ToLCNDV-potato from suspicious leaf samples
Lane 1. Marker; PCR products (Lane 2- suspected sample, Lane 3-infected sample, Lane 4- Negative control); RCA-PCR products (Lane 5- suspected sample, Lane 6-infected sample, Lane 7- Negative control).

Nucleic acid spot hybridization (NASH)

NASH technique has also been standardized for detection of many potato viruses. Nucleic acid hybridization of DNA or RNA probes has the advantage of being able to detect the nucleic acid of the virus in both forms, single-stranded and double-stranded. cRNA probes can be labelled with isotopes or nonradioactive probes. cRNA probes are preferable to cDNA probes when used to detect RNA viruses, because RNA/RNA hybrids are more stable than DNA/RNA hybrids. An RNA extraction from infected tissue is blotted onto a membrane and the probe hybridized to it and detected. Polyprobe for the simultaneous detection of several viruses can be designed utilizing the sequence information of all the target viruses. Nucleic acid spot hybridization is routinely used for detection of PSTVd.

Micro and Macroarrays

The principle of microarrays is the hybridization of fluorescently labeled sequences (targets) to their complementary sequences spotted on a solid surface, acting as probes. The main advantage of this method is the opportunity to detect many pathogens simultaneously. Microarrays are high-density arrays with spot sizes smaller than 150 microns. A typical microarray slide can contain up to 30,000 spots. Arrays printed with probes corresponding to a large number of virus species (or indeed, any type of pathogen) can be utilized to simultaneously detect all those viruses within the tissue of an infected host. Bystricka et al., (2005) described a microchip using short synthetic single-stranded oligomers (40 nt) instead of PCR products as capture probes for detection of PVA, PVS, PVM, PVX, PVY and PLRV, in both single and mixed infections. Sip *et al*., (2010) reported oligonucleotide microarray for the detection of mixed infections of PVA, PVS^{O}, PVM, PVX, PVY^{O}, PVY^{N}, PVY^{NTN} and PLRV.

Macroarrays are generally membrane-based with spot sizes of greater than 300 microns. Agindotan and Perry, (2007) reported a macroarray sytem using 70-mer oligonucleotide probes immobilized on nylon membrane for the detection of CMV, PVY and PLRV. Sugiyama *et al*., (2008) used convenient, cost-effective macroarray and microtube hybridization (MTH) system in which cDNA probes immobilized on nylon membrane, target viruses were amplified and labelled with biotin and then hybridization was carried in hybridization oven and colorimetrically detected using nitro blue tetrazolium (NBT)/bromo-4-chloro-3-indolyl phosphate (BCIP) reagent.

Loop mediated isothermal amplification (LAMP) assay

The technique uses four to six primers that recognise six to eight regions of the target DNA, respectively, in conjunction with the enzyme *Bst* polymerase, which has strand displacement activity. The simultaneous initiation of DNA synthesis by multiple primers makes the technique highly specific. The LAMP test is carried out under isothermal conditions (60–65°C) and produces large amount of DNA (amplified 10^{9}–10^{10} times) in 15–60 min. Alternatively, gene amplification can be visualised by the naked eye either as turbidity or in the form of a colour change when SYBR Green, a fluorescent dsDNA intercalating dye, is employed. LAMP does not require a thermal cycler and can be performed simply with a heating block and/or water bath. At CPRI, this technique is being standardized for the detection of potato viruses.

Deep sequencing of small RNAs

Small RNAs (sRNA), including microRNAs (miRNA) and small interfering RNAs (siRNA), are produced abundantly in plants and animals and function in regulating gene expression or in defense against virus or viroid infection. Analysis of siRNA profiles upon virus infection in plant may allow for virus identification, strain differentiation, and *de novo* assembly of virus genomes. Next Generation Sequencing **(**NGS) technology that is able to sequence viruses from samples without the need for laborious and costly purification, cloning and screening techniques. NGS technologies can be used as a diagnostic tool to identify a virus in an unbiased fashion when no prior knowledge of the aetiology of the virus is available. Li *et al*., (2012) used small RNA deep sequencing for virus and viroid identification and strain

differentiation in tomato. Gutierrez *et al*. (2013) exploited this technique for identification and determination of complete genome of a novel strain of PVS using GS FLX 454 Life Sciences (Roche). Song *et al*., (2013) used small RNA deep sequencing technique to identify different potato viruses and found to have rich genetic diversity. Small RNA sequencing is being used to detect and determine the whole genome of known and unknown viruses of potato using ion PGM at CPRI.

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Developing nutrient rich potatoes

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Introduction

Potato is consumed by one and all due to its versatility in way of cooking viz. boiling, baking, deep frying etc. Potato is popularly known as the "Vegetable King". Potatoes account for only 2% of the world's dietary energy supply (FAO, 2009) compared to other staple food crops like rice (20%), wheat (18%) and maize (5%). Potatoes are composed of approx. 75% water, 21% carbohydrates, 2.5% protein and less than 1% fat. Potato is a good option for food and is capable of producing nutritious food more quickly on lesser land compared to any other major food crop. Potato is considered as the most productive vegetable and provides a major source of nutrition and income to many population and communities. Its content of dry matter, edible energy and edible protein makes it a good choice for nutrients availability. Potato is known to everyone as a supplier of energy but its ability to supply vital nutrients is vastly underestimated. Potato is an excellent source of complex carbohydrates, dietary fibres and vitamin C. Potatoes also contain a variety of health-promoting compounds, such as, phytonutrients that have antioxidant activity (Ezekiel *et al.,* 1999). Among these, important health-promoting compounds are carotenoids, flavonoids, and caffeic acid, as well as unique tuber storage proteins, such as patatin, which exhibit activity against free radicals. Potato is a substantial source of ascorbic acid, thiamine, niacin, pantothenic acid and riboflavin. Potato is highly desirable in human diet as many of the compounds present in potato are important because of their beneficial effects on human health. The nutritive value of a potato containing food depends on other components served with it and on the method of preparation. By itself, potato is not fattening, however, preparing and serving potatoes with high-fat ingredients raises the caloric value of the dish. Since the starch in raw potato cannot be digested by humans, they are prepared for consumption by boiling (with or without the skin), baking or frying.

Potato (*Solanum tuberosum* L.) belongs to the genus Solanum and section Petota that contains approx. 2000 species that are distributed from the south western United States (38 ◦ N) to central Argentina and adjacent Chile (41◦ S) between 2000 and 4000m altitude. Presently, there are four cultivated and approximately 110 wild tuber-bearing *Solanum* species. Potato originated in the highlands of southern Peru over 10,000 years ago from *S. brevicaule*. Today, potatoes are grown in 149 countries from latitudes 65◦N to 50◦S and at altitudes from sea level to 4000m. It is the fourth most important food crop after wheat, maize and rice. Wild potatoes have ploidy ranging from diploid, 2n=24 to hexaploid, 2n=72 that are intercrossable within and between ploidy with ploidy manipulations. Cultivated potatoes have ploidy ranging from diploid to pentaploid. Several new resistances genes from wild relatives of potatoes have been incorporated for pest and pathogen resistance and quality improvement. The populations generated from interspecific and interploidy crosses between wild and cultivated potato have been important for both crop improvement and genetic studies. Though considered low in phytonutrient content, potatoes have received

considerable attention in the present time owing to wide variations available in wild germplasm and landraces.

Health benefits

Potatoes contain a complete range of nutrients, including those necessary for growth and development of human beings. They are rich in vitamin C and thus prevent scurvy. More than half of dietary fiber in potato is in the form of pectic substances, which helps in lowering cholesterol levels in human. Moreover, the dietary fiber dilutes highly caloric components in food, stimulates peristaltic movement and improves digestion. Because of its low sodium content, potatoes can be used in diets given to patients with high blood pressure. Potato is a good source of vitamin B6. Many of the building blocks of protein, amino acids require B6 for their synthesis, as do the nucleic acids used in the creation of our DNA. Amino acids and nucleic acids are critical parts of new cell formation and vitamin B6 is essential for the formation of virtually all new cells in the body.

Potato is a good source of resistant starch. Natural resistant starch helps maintain a healthy colon and a healthy digestive system via several mechanisms and prevents colorectal cancer and type 2 diabetes. Resistant starch is a valuable tool for formulators of reduced-calorie foods. Resistant starch may also help to burn fat and may lead to lower fat accumulation. Resistant starch contributes to oral rehydration solutions for the treatment of diarrhea. It is predicted to help maintain "regularity" with a mild laxative effect due to increased microbial activity in the large intestine. Consumption of natural resistant starch by humans has been shown to result in decreased glycemic response in healthy individuals, decreased glycemic response in diabetics, increased insulin sensitivity in healthy individuals, individuals with Type II diabetes as well as insulin resistant individuals (Nugent, 2005). When resistant starches are included in a meal, it slows down the absorption of sugars from other foods, which means there is more gradual rise and fall in blood sugar levels after eating. That's particularly helpful for diabetics, who need to keep their blood sugar levels steady.

Potato carotenoids viz., lutein and zeaxanthin have no provitamin A activity, but prevents age related macular degenerations. Carotenoids have antioxidant properties (Palozza and Krinsky, 1992). Antioxidant properties of potato carotenoids provide protection against degenerative ailments such as diabetes, cardiovascular diseases and cancer. Anthocyanin has also higher antioxidant capacity (Brown *et al*., 2005). Hidden Hunger due to micronutrient deficiency holds importance both for developed and developing nations in post Green Revolution era. At present the figure lies at an estimate of over 60% of the world's population with iron deficiency and over 30% with zinc deficiency. Iron deficiency is the most common and widespread nutritional disorder worldwide and affects immunity, cognitive development, temperature regulation, energy metabolism, and work performance. Zinc is vital for normal growth and development, DNA synthesis, immunity, neurosensory function and plays a functional role in many zinc-containing proteins and a large number of zinc-dependent enzymes.

Genetics of potato phytonutrients

Genetic diversity is important to choose the best parents for breeding, to design proper crossing schemes and selection strategies. Outside the centre of origin, very less variation is available in potatoes with respect to tuber colour. Generally, the potato colour (skin and flesh) is governed by two groups of compounds. Anthocyanin imparts red, blue, and purple pigmentation, while carotenoids, predominantly xanthophylls, produce yellow flesh colour. Violaxanthin, antheraxanthin, lutein and zeaxanthin are the major forms of carotenoids predominantly present in cultivated potato in different ratios, whereas neoxanthin, β-cryptoxanthin, and β-carotene are available in minor constituents.

Yellow pigmentation is governed by a single gene (Y located on chromosome 3) with the yellow flesh dominant over the white flesh (y) (Fruwirth, 1912) and role of modifying genes in controlling the degree of pigmentation. An another allele *Or* at the *Y* locus control synthesis of increased content of zeaxanthin leading to orange flesh and is dominant to *y* and *Y,* which control white and yellow pigmentation, respectively, at this locus (Brown *et al*.,1993) . In contradiction to this Wolters *et al*. (2010) has reported that the dominant Chy2 allele 3 in combination with the recessive zeaxanthin epoxidase allele (Zep allele 1) when present in the homozygous condition leads to orange-flesh colour and higher levels of zeaxanthin. The beta-carotene hydroxylase gene (*bch* or *Chy2*) have been located on same Y locus and had been mapped (Thorup *et al*., 2000). Expression and SNP analysis had depicted the importance of gene *Chy2* in controlling flesh colour (Kloosterman *et al*., 2010; Wolters *et al*., 2010). There are eleven different alleles of the *Chy2* gene, out of which only one leads to difference between yellow or orange flesh colour (Wolters *et al*., 2010). Specific expression of an allele to get the desired phenotype is vital factor in any breeding process with respect to marker development for the trait and thus facilitates marker assisted breeding.

Anthocyanin accumulation in the phelum and epidermis layer of the tuber is responsible for the skin colouration. Tuber skin colour is expressed due to the complementary action of locus involved in anthocyanin biosynthesis and locus involved in tissue-specific regulation of anthocyanin expression. Three loci affects skin colour in tetraploid potato viz., D (developer, known as I locus in diploid potato), required for tissue-specific accumulation of anthocyanin in tuber skin, R (red) required for the production of red anthocyanins and P (purple) for the production of purple pigments and have been localized on the potato chromosomes number 10, 2 and 11, respectively. The P locus encodes for flavonoid 3', 5'-hydroxylase (*f3'5'h*) (Jung *et al*., 2005) and the D locus harbours a R2R3 MYB transcription factor that regulates the expression of anthocyanin biosynthetic genes (Jung *et al*., 2009). However, microarray experiment conducted on pigmented and non-pigmented tuber samples suggests 27 genes were differentially expressed, including a novel single domain MYB transcription factor (Stushnoff *et al*., 2010). Another locus *Psc* on chromosome 10 controls purple skin colouration (Gebhardt *et al*., 1989). Red pigments are derivatives of pelargonidin and peonidin while purple anthocynanins are derived from delphinidin, petunidin and malvidin.

Breeding for micronutrients and phytonutrients

Biofortification to enhance the micronutrient density through breeding approaches hold a promise especially for a crop like potato with wider consumption, bioavailability (relatively high concentrations of vitamin C, protein cysteine and various organic and amino acids that stimulate the absorption of mineral micronutrients by humans in the mean time low concentrations of compounds that limit their absorption such as phytic acids and oxalates) as well as considering the available variability. With rapid increase in potato production in developing countries particularly India and a projection of rise in production to 122 million tonnes by 2050 and per capita consumption to 48 kg (Singh *et al*., 2014) potato holds importance for nutrient and phytonutrient biofortification.

Several factors viz., genotypic, agronomic, post-harvest handling and storage conditions, method of cooking and processing and environmental factors (intensity of light, diurnal temperature differences) affects presence and availability of phytochemicals of tuber (Ezekiel *et al*., 2011). Among these the varietal or genotypic component is most important (Toledo & Burlingame, 2006). Presence of significant variations and proportion of that variation under genetic control is important for breeding any trait.

Genotypic variation as a prerequisite for genetic advance

The presence of genetic variation and its heritability is important for achieving desirable increments through breeding. Presence of heritable variations facilitates exploitation in form of additive gene effects, transgressive segregants and heterosis. Wide variation in iron content of potato genotypes have been reported (Burgos *et al*., 2007; Brown *et al*., 2010) while zinc represents lesser variations (Burgos *et al*., 2007; Brown *et al*., 2011). The carotenoids content range from 50 µg/ 100 g fresh weight in white fleshed potatoes to above 1,000 µg/ 100 g fresh weight in intense yellow coloured potatoes (Brown *et al*., 2008). Genetic variation in diploid cultivated potato is easily incorporated into tetraploid varieties e.g 'Papa Amarilla', composed of the diploid groups *S. phureja, S. stenotomum* and *S. goniocalyx*, have highest levels of carotenoids (2,600 µg/ 100 g fresh weight) and have been successfully introgressed in tetraploid germplasm. Similarly very high zeaxanthin concentration (2000 µg/ 100 g fresh weight) had been observed in diploid potatoes viz., *S. stenotomum* and *S. phureja*. Anthocyanins are only present in red or purple genotypes and range upto 48 mg/ 100 g fresh weight have been observed in a solidly pigmented purple skinned, purple-fleshed breeding line (Brown *et al*., 2008).

Heritability of micronutrients and phytonutrients

Broad-sense heritabilities estimate the ratio of genetic variation to the proportion of the total variation. Higher broad sense heritabilities for total carotenoids (0.96) and its constituents viz., zeaxanthin (0.89), antheraxanthin (0.93), violaxanthin (0.68), neoxanthin (0.51), lutein (0.85) have been observed in tetraploid germplasm (Haynes *et al*., 2010) depicting heritability of the traits. Similarly in tetraploid × diploid potato crosses also high estimates of broad-sense heritability have been obtained for total carotenoids (0.81) (Haynes *et al*., 2011). With wider heritable genetic variation for total and individual carotenoid content in potatoes, improving the levels of carotenoids in tetraploid potatoes is feasible. Similarly large genetic variation along with wider broad-sense heritability for potato iron (0.49) and zinc

(0.82) content (Haynes *et al*., 2012) micronutrient content of potatoes can also be improved through breeding approaches.

Consumers are becoming increasingly aware of potential benefits from choosing a healthy diet and they are willing to pay more for food they believe will benefit their health. One of the global health goals is to increase the availability of nutrients to a large population of the world. A sensible approach to achieve this goal would be to increase the nutritional content of highly consumed crops. Potatoes are grown throughout the country and are consumed in large quantities. Furthermore, potatoes have higher phytonutrient content. Due to relatively high consumption of potatoes compared to most of fruits and vegetables, they can significantly contribute to overall population nutrition. Therefore, potato cultivars with high phytonutrient content are desirable from health point of view.

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Application of Bioinformatics

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A recent google search for "definition of bioinformatics" returned over 6,00,000 results. The terms bioinformatics and computational biology have become completely interchangeable terms. **Computational biology and bioinformatics are multidisciplinary fields.**

• **Bioinformatics:** Typically refers to the field concerned with the collectionand storage of biological information. All matters concerned with biologicaldatabases are considered bioinformatics.

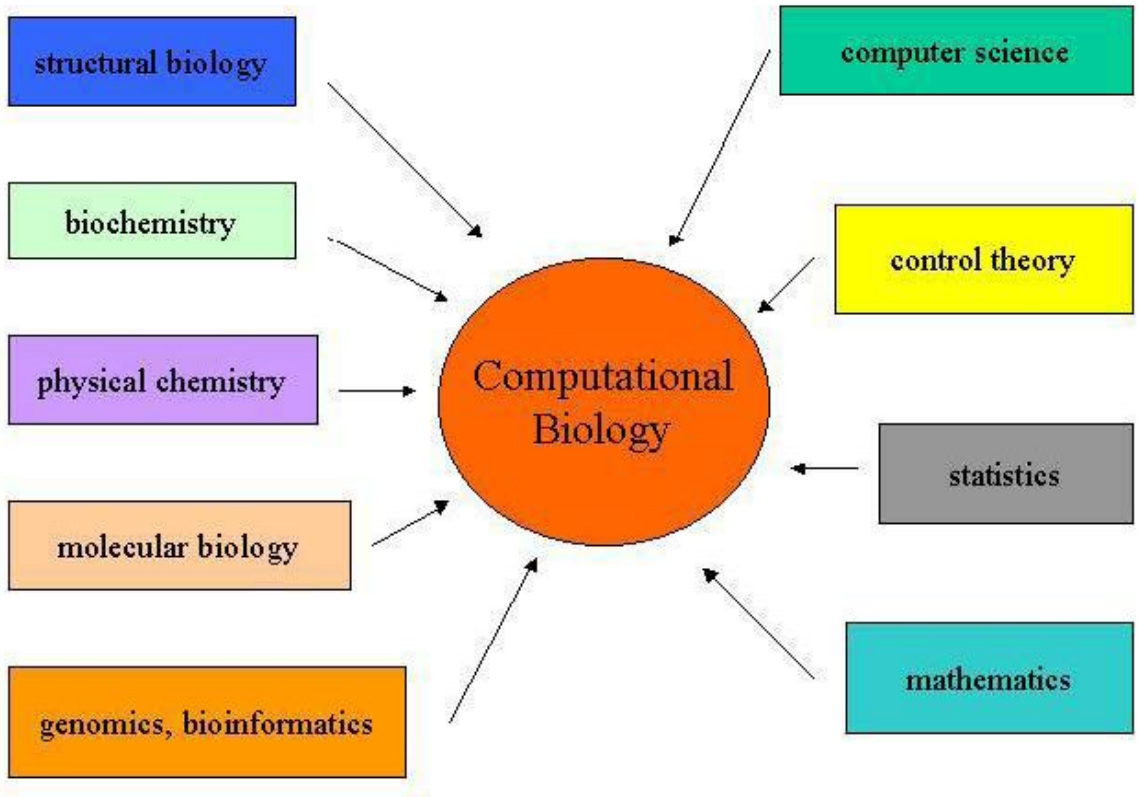
• **Computational biology:** Refers to the aspect of developing algorithmsand statistical models necessary to analyze biological data through the aidof computers.

Bioinformatics is an interdisciplinary field that develops methods and software tools for understanding biological data. As an interdisciplinary field of science, bioinformatics combines computer science, statistics, mathematics and engineering to study and process biological data.

Bioinformatics is an term used for methodology, as well as a reference to specific analysis "pipelines" that are repeatedly used, particularly in the fields of genetics and genomics. Common uses of bioinformatics include the identification of candidate genes and nucleotides (SNPs). Often, such identification is made with the aim of better understanding the genetic basis of disease, unique adaptations, desirable properties (esp. in agricultural species), or differences between populations. In a less formal way, bioinformatics also tries to understand the organisational principles within nucleic acid and protein sequences.

Bioinformatics is a Hot Field and one answer to this question is that Bioinformatics is tied to the human genome project which has generated a lot of popular interest. Various advances in molecular biology techniques (such as genome sequencing and microarrays) have led to a large amount of data that needs to be analyzed. That's where bioinformatics steps in. Bioinformatics can lead to important discoveries as well as help companies save time and money in the long run. In addition, there needs to be methods to manage large amounts of data.

One of the biggest reasons for bioinformatics being a hot field is the old supply and demand adage. **There just are too few people adequately trained in both biology and computer science to solve the problems that biologists need to have solved.**



Sequencing and assembly (terms and definitions)

- **Genome sequencing**
  - Determining the order of nucleotides in a DNA molecule
  - Identification of the molecular 'blueprints' for traits of interest (disease, agriculture, etc)
- **Genome assembly**
  - Reconstructing the complete sequence of a DNA molecule from short sequence fragments ("reads").
  - It is currently impossible to sequence a complete chromosome in one go
  - Genome annotation
  - Assigning a (possible) function to a string of nucleotides.
  - Unraveling the (molecular) mystery of life
- **Sequence:** linear order of nucleotides as they appear on the DNA molecule

- **Read:** single observation of the (partial) sequence of a DNA molecule
- **Contig:** contiguous stretch of sequence, often derived from multiple reads
- **Scaffold:** linearly ordered and oriented group of contigs.

BLAST (Basic Local Alignment Search Tool):

BLAST is a common sequence search tool that can be used for either nucleic or protein sequence. BLAST is based on Smith-Waterman algorithm, which is slow but guarantee to get the best possible alignment given input parameters.

BLAST starts with small perfect matches and tries to extend them. First the query sequence is broken down into a dictionary of "words" all possible sequences of certain size. For Proteins, the default word size is 3 amino acids. For DNA, the default word size is 11 nucleotides.

BLASTN: Query is DNA, Subject is DNA

BLASTP: Query is Protein, Subject is Protein

BLASTX: Query is Nucleic acid that is translated by the program into protein sequence, Subject database is protein. Useful for ESTs.

tBLASTn: Query is protein, Database is DNA translated into protein sequence.

tBLASTx: Query is DNA translated into protein, Subject is nucleotide translated into protein.

Query sequence must be written in FASTA FORMAT i.e single comment line that starts with a ">" followed by one or more lines of sequences.

BLAST use special database format to speed up the search operation. We can create our own database using "FORMATDB" program. Formatdb turns FASTA files into BLAST database.

By default "formatdb" program produces 3 files with the same name but different extensions. E.gath.**nhr**, ath.**nsq** and ath.**nin**. "ath" is base name and these are nucleic acid files, because the first letter of the extension is "n". (it would be "p" for proteins).

The 3 necessary parameters of "formatdb" are:

1. –i input data file (containing one or more sequences in FASTA format).
2. –n output file base name (if this parameter is not set, the input file name is used as base).
3. –p type of file: T for proteins, F for nucleic acid.

The command for formatdb would be:

 # formatdb –n <sr_db> -o T –p F –I <file.fasta multiple seq. file>

Using BLAST

The essential options are:

1. –p program to run (blastn, blastp, …….)
2. –d subject database (created with formatdb)
3. –i input sequence file (FASTA format)
4. –o output file name.

The command for BLAST would be:

 # blastall–pblastn–d <sr_db>–i<single seq. file> -o sr_out.txt

Scripting for interpretation of output i.e. The output DB has to be screened for extracting information on percent similarity, start and end sequence number and length of contigs for this open source, modern programming languages can be used like PERL, Python and Ruby, statistical analysis software like R, and database management systems like SQLite3, PostgreSQL and MySQL, these are some of the important tools in bioinformatics and biomedical research.

Pairwise Sequence Alignment is used to identify regions of similarity that may indicate functional, structural and/or evolutionary relationships between two biological sequences (protein or nucleic acid).Multiple Sequence Alignment (MSA) is the alignment of three or more biological sequences of similar length. From the output of MSA applications, homology can be inferred and the evolutionary relationship between the sequences studied. This will be studied in more detail in practical sessions.

The various tools like PERL, BLAST, FORMATDB, Sequences to structures, EMBOSS, etc will also be studied in details in practical sessions.

Recent advances in biotic and abiotic stress management

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The productivity of potato crop is constantly threatened by assorted biotic and abiotic stresses, which affects the quality and quantity of tubers, the main food storage organ. Among various biotic stresses, fungi (*P.infestans, Alternaria solani, Rhizoctonia solani*, *Verticillium dahliae*), viruses (PVY, PLRV, PVX, PVM, PVA, PVS), bacteria (*Erwinia species, Streptomyces scabies*, *Pseudomonas solanacearum*) and nematodes (*Globodera pallida* and *Globodera rostochiensis)* poses serious losses and remains as major impediment in potato production worldwide. Management of biotic stresses include both protective approaches such as breeding for resistance and therapeutic approach like chemical control. Employing durable resistant varieties is an important contrivance in the management strategy of biotic stresses. This reduces recurring chemical fungicide sprays, thus minimising the impact on environment, besides rendering economic benefits.
The sub-sections in the chapter summarises conceptual information on significant biotic stresses, detailing their area of distribution, races/pathotype, mechanism & sources of resistance, screening methodology, economic importance and various breeding strategies adopted worldwide to scuffle the diseases caused by respective pathogens, with special state of past, present and future panorama of Indian breeding programme.

Late blight

Late blight is caused by *Phytophthora infestans* which is highly variable and therefore, breeding varieties against it has been a seesaw story. Pathological variants (races) could be detected universally after the resistance genes from *S. demissum* were transferred to the commercial cultivars. In 1909, R.N. Salaman demonstrated the heritable nature of resistance to late blight in wild species *S. edinense*. Resistance to blight can occur both in foliage and tubers. However, breeders have largely neglected the latter. Broadly resistance can be grouped into two types: (i) race-specific resistance (also called vertical resistance or major gene resistance, qualitative or discontinuous resistance) and (ii) race non-specific (also called horizontal resistance or minor gene resistance, field resistance, polygenic resistance, quantitative resistance or partial resistance).

The race-specific resistance based on gene-for-gene relationship was initially identified in hexaploid (2n=6x=72), wild species *S. demissum*. It is expressed in the form of hypersensitive response of the tissue to all races of *P. infestans* that did not possess the corresponding virulence to the resistance genes (R-genes). Specific resistance is conditioned by a series of major dominant genes each of which is brought into action by distinct pathotypes; currently eleven such genes (ex-demissum) are recognized. *S. stoloniferum* has also been found to possess similar, if not identical, resistance genes.

However, the R-genes wherever deployed were defeated in due course of time. In India, the process of transfer of R-genes from *S. demissum* background was started in mid-fifties and the first set of late blight resistant varieties was released for commercial cultivation in 1968. Of these, cv. Kufri Jyoti (possessing R-genes 3.4.7) became most popular which is still grown in several parts of the country. Since then a lot of varieties carrying R-genes have been bred and deployed across the country. In cv. Kufri Jyoti, matching virulences (3.4.7) were detected immediately after 5-6 years of its cultivation both in North-eastern and North-western hills. Both frequency of matching virulences, their combinations and disease increased rapidly making it completely susceptible by 1988. In India, this problem has been avoided by making adjustments in screening methodology. The seedlings in F_1C_1 are challenge-inoculated with the a most complex race (8-9 gene complex). The seedlings showing either complete susceptibility or immunity are discarded. The selected seedlings possess both R-gene resistance coupled with a high degree of field resistance.

Race non-specific resistance is a quantitative and multifaceted trait, probably governed by many genes, it is, therefore, difficult to analyse in Mendelian ratios. Field resistance to late blight operates mainly through four factors, *viz.* infection efficiency, incubation period, colonization rate and sporulation efficiency. Many host factors, environmental aspects, edaphic, nutritional and climatic have an effect on these four components of resistance. Besides, components of field resistance to tuber blight include the depth in the soil at which the tubers are produced, the ease with which the spores are washed down from the canopy into the soil, the rapidity of periderm formation and the resistance to wounding. Although, at the phenotypic level, both types of resistances can be easily identified, yet at the genotypic level these are almost similar. Genetic analysis of resistance to late blight using DNA markers showed that major genes for resistance (R-gene) are closely linked to the factors controlling quantitative resistance suggesting that there is no real difference between qualitative and quantitative resistance to late blight as far as the nature of the genes involved. The differences observed at the phenotypic level may be the result of various allelic and non-allelic interactions. Thus a complex picture emerges, which renders both selection for and evaluation of blight resistance a slow process.

Several wild *Solanum* species possesses high degree of resistance to late blight. Species like *S. bulbocastanum*, *S. demissum*, and *S. stoloniferum* had clones, which possessed low infection frequency. Besides clones of *S. bulbocastanum* and *S. demissum* developed only small lesions, whereas clones of *S. stoloniferum* possess high degree of resistance to tissue colonization. Umaerus demonstrated that *S. demissum* is a treasure of resistance. Besides R-genes, it also possesses field resistance, which operates primarily through low infection frequency at seedling stage. In German-Dutch potato collection nothing encouraging was found in *S. tuberosum* ssp. *andigena* and the primitive cultivars except an accession of *S. phureja*. However, resistance was detected in wild diploid Mexican species like *S. pinnatisectum*, *S. bulbocastanum*, *S. polyadenium* and *S. verrucosum*. It was also detected in Bolivian and Aregentinian species, including *S. chacoense*, *S. berthaulti*, *S. microdontum* and *S. vernei*. Although, initially *S. tuberosum* ssp. *andigena* was thought to be devoid of any resistance to late blight but, there has been renewed interest in it as andigena potatoes respond to selection for resistance and produce a number of highly resistant clones. Resistance has also been reported in the Russian diploid sources, including *S. polytrichon*, *S. simplicifolium* and *S. microdontum*, which showed resistance to tuber blight as well.

Viruses

Viral diseases are an important constraint for potato crop because of their systemic distribution in the host and are mainly responsible for the degeneration of seed stocks. At least twelve viral diseases are known to infect potato crop in India and elsewhere. Among them, viruses PVX, PVY, PVS, PVA, PVM and leaf roll (PLRV) are important. Introducing resistant cultivars is one of the most efficient ways of reducing the losses caused by viruses. Resistance genes to different potato viruses have been identified in many wild potato species. Some of these genes have been incorporated in many of the recently released potato cultivars.

The nature of resistance against viruses is of several types: i) tolerance, ii) resistance to infection, iii) hypersensitivity usually giving field immunity, and iv) extreme resistance or immunity. Tolerance to viruses in potatoes is usually considered a dangerous type of resistance. Resistance to infection can be defined as the type in which only a small percentage of infection appears in the field and is governed by polygenes. Hypersensitivity and immunity are on the other hand due to mostly single dominant genes. Out of the four types of resistance, immunity gives almost complete elimination of virus and is preferable over other types. However, in recent years, more emphasis is being given to vector resistance where the resistance sought is against the vectors (aphids and other vectors), the carrier of viruses and not against viruses themselves.

There are three main groups of strains of PVY viz. PVY^O (common strains), PVY^N (tobacco veinal necrosis strains) and PVY^C (stipple streak strains). The strain-specific resistance is controlled by the resistance gene N_Y while the extreme resistance is controlled by the gene R_Y. *Ny* genes are found in large number of cultivars including Pentland Crown, Pentland Ivory, King Edward and Cana and in hybrids derived from wild species like *S. chacoense*, *S. demissum* and *S. microdontum*. The sources of *Ry* gene are *S. stoloniferum*, *S. hougasii* and *S. tuberosum* ssp. *andigena*. Accordingly the genes are named as Ry_{sto}, Ry_{hou} and Ry_{adg}. *Ry* is inherited as a single dominant gene hence easy to breed.

Mild, moderate and severe strains of PVA occur. They differ in severity of symptoms produced in potato cultivars. The gene *Na*, present in many cultivars, protects the plant from infection under natural pressure from PVA by means of a hypersensitive response. The gene *Na* is linked to gene Nx_{tbr}, which controls the resistance to PVX.

PVX strains can be separated according to their serological reaction into two main pathotypes: 1 and HB. Cockerham (1970) identified several genes conferring hypersensitivity viz. $Nx_{tbr,}$ $Nb_{tbr,}$ Nx_{chc} and Rx^n_{acl} . Similarly, genes conferring extreme resistance are known to occur in several species (Rx_{adg}, Rx_{acl} and $Rx_{(scr)}$).

Strains of PLRV differ in the severity of the symptoms they produce on potato and on test plants. Resistance to PLRV has been found to be oligogenic and has been detected in *S. brevidens* and *S. tuberosum*. Transfer of resistance genes from *S. brevidens* has been achieved through protoplast fusion but from *S. tuberosum* it has been possible only through bridge crosses. Among the hexaploid somatic hybrids derived from *S. tuberosum* and *S. brevidens* by protoplast fusion, some hybrids with high PLRV resistance were obtained.

The development of cultivars with multiple virus resistance remains a challenge for the breeders. This may be because the breeder must select for many important characters, therefore, introducing even a few genes for resistance to viruses becomes a difficult task. At CPRI, parental lines having virus resistance in duplex/triplex/tetraplex form have been developed. The progeny of triplex/tetraplex parents are being crossed with nulliplex parents to produce almost cent per cent immune clones. After the early evaluation for viral resistance, an evaluation for horticultural traits could be done at the later clonal generations.

Bacterial wilt

Bacterial wilt or brown rot, first reported in India in 1892, is the most destructive of all bacterial diseases. Incidence of bacterial wilt is wide spread in all mid hill regions of the country and pockets of Assam, Meghalaya and Maharasthra. The disease damages the crop in two different ways - premature wilting of standing crop and rotting of tubers in fields, transit and stores. It is primarily seed borne, but survives equally well in soil. Host resistance is hard to find because of lack of co-evolution of the host and the bacterium, high variability in the bacterium and instability of the host resistance.

Resistance to bacterial wilt is a partially dominant character and is more of a polygenic type. Inheritance of resistance and its expression is complex and both additive and non-additive gene actions are involved, but the latter component is more important. A gene-for-gene relationship is not applicable to bacterial wilt. Certain genes other than those 'for resistance alone' have turned out to have the novel (pleiotropic) effects in conferring the resistance once the potato plant has come into contact with pathogen under a certain set of environmental conditions. These genes were eventually called 'genes for resistance' once a certain level of resistance was detected. The major or minor status of these genes depends on the particular genotype of the pathogen, and the particular environmental conditions that influence their expression. Attempts to transfer resistance from wild *Solanum* sp. into common potato result in excessive recombination, resulting in breakdown upon intercrossing. Non-strain specificity and race-cultivar specificity are the common features required for resistance to bacterial wilt. Thus, the host genotype x pathogen genotype interaction in potato-*R. solanacearum* system seems to be artifactual. Both the host and pathogen are sensitive to environmental changes. Therefore, host genotype x pathogen genotype interaction may also be a result of host genotype x environment and / or pathogen x environment interaction.

The resistance in the clones of species like *S. phureja* mainly and a few other *Solanum* species have been exploited extensively in the South American countries, but the Indian isolates of the bacterium has proved to be strongly virulent making these sources ineffective. Resistance in *S. phureja*, the only species where it has been studied in detail, is strain- and temperature-specific and breaks down under the warm climates. Nematode injury also leads to its break down. A collection of nearly 500 clones of *Solanum* species which carry low to moderate degree of resistance i.e. *S. phureja*, *S. microdontum*, *S. canasense*, *S. stenotomum*, *S. pinnetisectum*, *S. sparsipilum*, *S. kurtzianum*, *S. jamesii*, *S. polytrichon*, *S. vernei*, *S. acaule* and *S. stoloniferum* and inter-specific hybrids between a number of above species and also resistant varieties developed so far in other parts of the world, *viz.* Prisca, Cruza, Caxamarca, Molenera and Ampola were screened against different isolates of the

pathogen. All these cultivars/cultures proved susceptible to Indian isolates except for the one clone of diploid *S. microdontum* showing a moderate level of resistance. The efforts made to transfer the useful resistance from this source into the *tuberosum* background *via* dihaploids resulted in the development of two promising meiotic tetraploids. However, in field tests these were also found to be susceptible.

Wart

Wart disease of potato caused by a phycomycetous fungus, *Synchytrium endobioticum* (Schilb) was first reported from India in 1953 in North Bengal Hills. To avoid its further spread to other parts of the country, this area was brought under domestic quarantine in 1959. This has helped in containing this dreaded disease in Darjeeling district only. The disease causes cauliflower like growths on tubers, stolons and stem bases. The heavy infection of disease causes rottage of entire produce and results in total loss of crop. Cultivation of wart immune varieties on a long-term basis is the only viable alternative. The resistance genes are available in a number of varieties of *S. tuberosum*. Besides, a number of wild species like *S. boliviense*, *S acaule*, *S microdontum, S. demissum, S. sparsipilum*, *S. polytrichon*, *S. simplicifolium, S. chacoence* f.sp. *boergerii, S. vernei* and *S. spegazzinii* are known to have resistance to the disease. Monogenic dominant mode of inheritance for at least the control of necrotic response has been proved. However, modifying genes are also present which condition the nature and extent of response. Systematic breeding programme for wart resistance started in 1964. The crosses between wart immune/resistant *tuberosum* parents result in recovery of quite high percentage of resistant clones than between resistant x susceptible parents. Using Adina x Ultimus, several late blight resistant and wart immune hybrids were developed. One of them was released for commercial cultivation under the name Kufri Sherpa in 1983, which did not become popular because of its poor keeping quality, unattractive dull white skin, round tubers with medium deep eyes. Indian cultivars, *viz*. Kufri Jyoti, Kufri Chamatkar, Kufri Muthu, Kufri Sheetman, Kufri Bahar, Kufri Khasigaro and Kufri Kumar are immune to the race of *S. endobioticum* prevalent in the Darjeeling hills. These cultivars except Kufri Jyoti also didn't establish in the area because of local preference for varieties with red skin tubers. To develop a red tuber variety, Pimpernel was used as one of the parents. One hybrid from cross SLB/Z 405a x Pimpernel was selected and has since been released as cultivar Kufri Kanchan. This variety is immune to wart and possesses high degree of field resistance to late blight.

Nematodes

About 90 species of nematodes belonging to 38 genera have been reported to be associated with potatoes. Among these, the root knot nematodes and potato cyst nematodes have been recognized as the major pests.

At least nine species of Root-knot nematode (*Meloidogyne spp.*) are known to infect potatoes. Among these, *M. incognita* is the most important throughout the world followed by *M. javanica*. The dominant root-knot nematode species affecting potato both in hills and plains is *Meloidogyne incognita* while *M. javanica* infestation is restricted to mid hills and plains.

Heavily infested plants are stunted with yellowish leaves while no visible symptoms of nematode injury are seen under low infestation levels. The galls on potato roots are small and often go unnoticed. Wart-like structures are formed due to tuber infection, which reduces the commercial value and keeping quality of tubers. The second stage juveniles (hatched out from the egg masses laid by females) infect the young roots resulting in the formation of giant cells and the formation of syncytium and development of the nematode in the roots causes formation of galls or root knots. The nematode infection on tubers is characterized by the formation of typical wart like pimples on the outer skin.

Potato cyst nematodes, *Globodera* spp. also known as Golden nematode or potato root eelworms, is considered as one of the major pests throughout the world. Quarantine or regulatory actions are imposed against them in most countries. In India, the potato cyst nematode was first detected in 1961 by Jones at Ootacamund in Tamil Nadu. The efforts to locate the source of resistance to cyst nematodes though began in 1968, a systematic breeding programme at CPRI was started in 1971. Over 2000 genotypes comprising group tuberosum, group andigena and tuber bearing *Solanum* species were screened and resistance was located in 20 accessions of 14 wild tuber bearing species *S. ehrenberjii*, *S. vernei*, *S. chacoense*, *S. phureja*, *S. demissum*, *S. gourlayi*, *S. microdontum*, *S. sucrense*, *S. tarijense*, *S. acaule*, *S. fendleri*, *S. multidissectum*, *S. oplocense*, *S. sparsipilum* and some accessions of *S. tuberosum* ssp. *adigena*. Simultaneously, efforts were also made to procure resistant breeding lines to both the species from the Netherlands and USDA. A parental line VTn^2 62.33.3 (*S. tuberosum* x *S. vernei* hybrid) received from the Netherlands, having resistance to both the species, was extensively used in crosses with late blight resistant cultivar Kufri Jyoti resulting in release of hybrid Kufri Swarna for cultivation in cyst nematode infested areas of Nilgiri hills

High temperature stress

The potato has long been considered a crop for cool and temperate climates. Higher temperatures inhibit yield by overall reduction of plant development due to heat stress or by reduced partitioning of assimilates to tubers. Tuberization is reduced at night temperatures above 20^OC with complete inhibition of tuberization above 25^OC. Exposure of potato plants to heat stress alters the hormonal balance in the plants. As a result most of assimilated carbon is partitioned to above ground vegetative parts at the cost of the tubers. Aspects of heat tolerance that are considered important and should be taken into account in breeding programme includes ability of the plants to tuberize at night temperature of 22^O C and above, low shoot/root ratio at high temperature, and early maturity of the crop.

To obtain harvestable tubers, the plants must be induced to tuberize, and must also be stimulated to partition major part of the dry matter to the below ground plant parts. Temperature has an immediate effect on assimilate partitioning. It has been established that heat sensitive cultivars accumulate more starch in leaves and the rate of transport of sucrose to tubers is low in comparison to tolerant genotypes grown under heat stress. This may be either due to lower photoassimilate synthesis or also due to utilization of substantial amount of photoassimilate by shoots itself. High temperatures are known to strongly reduce the harvest index in potato. Stolon initiation is significantly influenced by temperature; it has been found that under high temperature the stolon development is delayed. Branching and growth of stolons is stimulated but tuberization is delayed. High temperatures delay, impede

or even inhibit tuber initiation. Potato tuber formation is badly affected due to high night temperatures of above 20°C and therefore, the most important characteristic of a heat tolerant potato genotype should be its ability to tuberize under high night temperatures of above 20°C. Better foliage growth and higher LAI are also characteristics of heat tolerance. In addition, higher tuberisation in the leaf bud cuttings, low shoot/root ratio, early maturity, higher rate of photo-assimilate partitioning from leaves to tubers, higher cell membrane thermo-stability, higher stability of chlorophyll, slower rate of elongation of internodes and low GA/ABA ratio during tuberization may also be important features of a probable heat tolerant genotype. One of these attributes viz. tuberisation at high night temperatures of 22°C is being well exploited for selection of seedlings for development of heat tolerant genotype, one such genotype named as Kufri Surya has already been developed, which has potential to tuberize up to 22°C temperature and few more are in advanced stages of trial for release as a variety.

To breed heat tolerant genotypes for Indian conditions, crosses were made amongst known heat tolerant and local high yielding genotypes. The known heat tolerant genotypes used in the breeding programme were LT-1, LT-2, LT-5, LT-7, LT-8, LT-9, DTO-28, DTO-33 (received from CIP) Katahdin, Desiree and Kufri Lauvkar. The progenies were screened for heat tolerance by their ability to form tubers within one month after shifting to high temperatures. The selected genotypes were multiplied and further selected at early planting in Indo-Gangetic plains at Modipuram and Jalandhar under heat stress.

Drought Stress

Drought is a major limiting factor for potato production in the world influencing yield as well as tuber quality. Drought may occur due to erratic rainfall, inadequate irrigation techniques and lack of water supply. Even with good irrigation practices, water stress may occur because of high transpiration rates especially during mid-day, when root system cannot completely meet the transpiration requirements of the plant. Drought have chronic effects on plant growth and may lead to premature senescence of the plant. Stomata of potato leaves close at relatively lower water deficits (leaf water potential or LWP of -3 to -5 MPa) resulting in reduced transpiration, whereas, in crops like sorghum, soybean etc stomatas close at -11 to -13 MPa LWP. Adaptation to water stress may involve several physiological and morphological characters and their importance may vary according to the type of water stress experienced by the plants.

Water stress may affect potato plants through its effect on radiant energy interception, conversion of light energy into dry matter, partitioning of assimilates and tuber dry matter concentration. Water stress in known to reduce both the rate and the duration of growth of leaves along with reduction in number of leaves, therefore, the radiant energy interception is decreased. Drought has adverse effects on the functioning of photo system II, and thus the rate of photosynthesis, though conversion of light energy into dry matter content is not very sensitive to water stress. Prolonged drought reduces the activity of light harvesting complex and down regulation of carboxylating enzymes. The partitioning of dry matter is also affected by drought, though, genotypic differences do occur. The potato root system is shallow and under water stress conditions it is not able to extract water from soil

effectively as compared to cereal crops. Thus the potato plants respond by increasing root: shoot ratio in view of increasing dry matter production under drought hence the balance of growth is favoured towards root growth. The work on Indian potato cultivars has also shown that the most critical stage in crop production under water stress is the stolon initiation stage, which represses the yield by 30-65%.

Adaptation to water stress conditions may be either through drought avoidance or drought tolerance and it may involve several different physiological and morphological characters, the relative importance of which may vary according to the type of water stress experienced by the crops. Potato cultivars considered to be drought tolerant have been found either to have greater threshold of soil moisture deficit or are less sensitive to soil moisture deficit and sometimes both. Such cultivars sustain leaf expansion with increasing soil moisture deficit and also develop relatively large rooting system for better exploitation of available soil moisture. The work on recovery of leaf growth after a period of water stress in Indian potato cultivars has shown that plant response varies with the cultivars, in some cultivars minimum reduction in leaf growth occurred and the recovery from stress on re-watering enhanced the leaf growth. Some cultivars showed moderate reduction in growth and re-watering caused sufficient recovery of leaf growth, whereas, other cultivars had great reduction in growth and re-watering could not result in sufficient recovery of leaf growth. This shows that leaf growth response varies with the genotype and their tolerance limit.

Frost Tolerance

The problem of frost is known in almost every country where potatoes are grown. Temperatures below –2O C in the field can produce partial or complete loss of the crop. In temperate zones, frosts can occur during spring when the crop is establishing itself, or during autumn when the crop is maturing. Higher crop losses occur in tropical highlands and subtropical plains where frosts can occur any time during the crop growth period. In India more than 80% of the potatoes are grown during winter in plains and the crop is prone to frosts during the months of December and January. Based on the field observations, two types of frosts are often distinguished. "White frost" occurs when there is a decrease in temperature and relative humidity is high. "Black frost" occurs under low temperatures and much drier conditions, hence more damaging and severe because plant tissue is darkened immediately. Acclimation or hardening may increase the resistance to frosts in many plants. Exposure of the plants to prolonged low temperature is effective in increasing resistance to frost injury in *S. tuberosum, S. multidisectum, S. chomatophilum, S. acaule and S. commersonii*. Genetic variability exists in the genus *Solanum* with respect to frost injury. *S. acaule* has the ability to withstand extracellular ice formation up to $-5^{O}C$, which gives this species frost tolerance. This species can be used in the breeding programmes to transfer frost tolerance to *S. tuberosum*. In North India, frost occurs in most years during December and January in the plains of Punjab and Eastern UP. Cultivars Kufri Sheetman and Kufri Dewa released by the Central Potato Research Institute possess resistance to frost. High degree of frost resistance was observed in other 28 hybrids from crosses involving *S. acaule*.

Fertilizer Use efficiency

The potato is considered to be heavy feeder on nutrients and requires high inputs of NPK and water for optimum production. This not only increases the cost of production but also causes environmental pollution. The application of high rates of N and K fertilizers and irrigation water on coarse-textured soils on which the shallow-rooted crop is often grown can result in loss of N and K, which represents an economic loss to the grower and may cause environmental degradation of groundwater. A nutrient efficient potato can produce higher yields per unit of nutrient, applied or absorbed even at a limited nutrient supply (Graham, 1984). Such genotypes could reduce N fertilization and nitrate leaching (Duynisveld et al., 1988; Sharifi et al., 2007). The recovery of applied phosphorus by potato crop is not more than 15-20% (Trehan et al., 2008). Numerous studies have demonstrated the existence of considerable variation for nutrient efficiency among crop species and cultivars within species, which suggests genetic control of inorganic plant nutrition (Errebhi et al., 1999; Trehan et al., 2005). Although plant breeders seldom select for nutrient use efficiency (NUE), breeding programs that develop lines that produce high yields may result in unconscious selection of genotypes that use nutrients more efficiently (Batten, 1993).

At CPRI, studies on nutrient use efficiency have shown that potato cultivars showed wide variation in agronomic use efficiency (AUE), nutrient uptake efficiency (NUE) and physiological use efficiency (PUE) with respect to nitrogen, phosphors and potassium. Kufri Pukhraj was the most N, P and K efficient cultivar among ten cultivars tested in the absence as well as presence of green manure. The efficient cultivars gave higher tuber yield under nutrient stress (i.e. with less dose of N, P and K fertilizer) than less efficient cultivars. The main cause of higher nitrogen efficiency in the presence of green manure was the capacity of a genotype to use/absorb more N per unit green manured soil i.e. the ability of the root system of a genotype to acquire more N from green manured soil (NUE). The variation in potassium and phosphorus efficiency of different potato cultivars was due to both their capability to use absorbed K and P to produce potato tubers (PUE) and to their capacity to take up more K and P per unit soil (NUE). Breeders should combine parameters/characters (NUE and PUE) responsible for high nitrogen, phosphorus and potassium efficiency to breed multi-nutrient efficient potato cultivar. Recently CPRI has released potato variety Kufri Gaurav having higher nutrient use efficiency.

Quality traits

Potatoes represent a non-fattening, nutritious and wholesome food, which supply important nutrients to the human diet. Tubers contain significant concentrations of vitamin C and essential amino acids. They are also a valuable source of at least 12 essential vitamins and minerals. Besides being important in human diet, potatoes are also used as animal feed and as raw material for starch and alcohol production. Potato quality parameters change according to the specific market utilization types, and are often referred to two major categories. The first category groups "external quality", aspects comprising skin colour, tuber size and shape, eye depth. These traits are deemed very important for fresh consumption where external traits are most likely to influence consumer's choice. The second category comprises "internal quality" aspects including nutritional properties, culinary value, after-cooking properties or processing quality. Internal quality is given by traits such as dry matter

content, flavour, sugar and protein content, starch quality, type and amount of glycoalkaloids. Although quality is one of the most important characteristics of potato, it is probably the most poorly defined and least researched at the genetic level Dale and Mackay (1994). There are several factors affecting tuber quality. They include the genetic makeup of the cultivar, crop maturity, agronomic practices, environmental conditions, storage temperatures, the presence of pests and diseases Traits that are genetically controlled can be grouped as biological traits (proteins, carbohydrates, vitamins, minerals, reduced amounts of toxic glycoalkaloids), sensorial traits (flavour, texture, colour) and industrial traits (tuber shape and size, dry matter content, cold sweetening, oil absorption, starch quality).
Breeding potato for quality traits requires a continuous flow of new genes and allelic diversity into the *S. tuberosum* gene pool. The genetic improvement of this crop is hampered by its tetrasomic inheritance, high level of heterozigosity, and incompatibility barriers. However, recent advances in plant biotechnology have significantly improved the possibilities of producing novel genetic variability and efficiently perform selection, expecially when biotechnologists pool resources with breeders. Equally important is the fact that basic studies have contributed to elucidate our knowledge on the genetics, biochemistry and physiology of several quality traits, making breeding efforts less empirical and more predictable.

In potato genetic engineering techniques have been applied to produce routinely and several transformation protocols are currently available. Data published by Dunwell (2000) indicated that the potato ranks second, after corn, in the list of plant species for which field trials were carried out in the United States. In the past most potato genotypes were transformed with genes for herbicide or insect resistance. However, much emphasis is now being attached to quality traits. The production of starches with modified amylose to amilopectin ratio represents a good example of the possibilities offered by genetic engineering in improving potato quality traits. Lloyd et al. (1999) provided evidence that transgenic potato lines where the activity of ADP-glucose pyrophosphorylase (AGPase) was reduced through antisense technology had a significant reduction of amylose. They also observed that in AGPase antisense plants, amylopectin accumulated shorter chains and that the size of starch granules was reduced. By contrast, the simultaneous antisense inhibition of two isoforms of starch branching enzymes (SBE A and B) to below 1% of the wild type activity gave transgenic lines with increased amount of amylose (Schwall et al., 2000). The amylose content of their transgenic lines was comparable to that of the high-amylose corn starch reported by Shi et al. (1998).

Great attention has been attached to improve the essential amino acid composition of tubers and expecially their lysine, tyrosine, methionine and cysteine content. Chakroborty et al. (2000) transformed a potato genotype with the gene *AmA1* from *Amaranthus hypocondriacus,* encoding a protein with a nutritionally balanced amino acid composition. The amino acid profile in tubers of both types of transgenic plants showed a 1.5- to 8-fold increase in all essential amino acids to the wild type.

Genetic engineering has been recently used to improve carotenoid content of tubers. In particular, to overcome the zeaxanthin deficiency of human diet, Römer et al. (2002) down-regulated the synthesis of zeaxanthin epoxidase specifically in tubers through antisense technology and co-suppression approaches. Both strategies achieved decreased conversion of zeaxanthin to violaxanthin, in transgenic tubers with a corresponding increase of

zeaxanthin content of 4- to 130-fold. Due to the use of a tuberspecific promoter, leaf carotenoid content of all transformants was very similar to the control plants, and thus photosynthesis was not negatively affected by lack of violaxanthin.

One main constraint in the use of wild species is that, together with useful traits, they can transfer characteristics that are undesired from the commercial standpoint. In the case of *Solanum* species, traits such as long stolons, deep eyes, negative quality traits can be transmitted. As reported recently by Pavek and Corsini (2001), transmission of undesired traits has very much limited the use of potato genetic resources. Therefore, after interspecific crosses, time consuming evaluation and selection are necessary to eliminate unwanted wild-type genes and restore the cultivated improved phenotypes.

Marker-assisted selection is perhaps the most powerful approach that uses DNA markers efficiently for selection of interspecific hybridization by reducing the linkage drug in terms of time and space. The use of DNA markers can be ascribed not only to the use of markers tightly linked to target genes (positive assisted selection), but also in the use of markers specific for the wild donor parent to perform selection against the wild genome (negative assisted selection; Barone, 2004).

A very exciting development in the context of efficient selection has been the generation of a molecular-linkage map based on functional gene markers involved in carbohydrate metabolism and transport (Chen et al. 2001). Using diploid mapping populations for which molecular maps were already available, the authors performed CAPS, SCAR, and RFLP marker assays for 69 functional genes previously studied and identified (among the others *AGPase*, *SssI*, *GbssII*, *Dbe*, *UGPase*, *Ppc*, and *Cis*). This work allowed the identification of 85 genetic loci covering a considerable amount of the potato genome. The availability of this molecular-function map allowed a candidate-gene approach to be used for studying starch and other sugar-related agronomic traits in potato. Chen et al. (2001) compared the QTL map for starch content previously published (Schäfer-Pregl et al., 1998) with the molecular-function map, and various correlations between the map positions of 14 QTLs for tuber starch content and function-related loci were found.

Practical 1: DNA Isolation and Quantification

Virupaksh Patil, CM Bist and Naresh Thakur

Solutions for DNA extraction and gel electrophoresis

Preparation of solutions for DNA extraction

Chloroform : Isoamyl alcohol (24:1, v/v) - Mix 96 ml chloroform and 4 ml isoamyl alcohol and keep at room temperature in a closed container.

10 % (w/v) CTAB - Add 10 g of CTAB to approximately 70 ml of water. Dissolve the detergent by warming the solution to 65 °C. Adjust the volume to 100 ml.

0.5 M EDTA (pH 8.0) - Add 93.05 g of ethylenediaminetetraacetate. $2H_2O$ to 400 ml of H_2O. Add – approximately 10 g of NaOH pellets to adjust the pH to 8.0 (The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH). Adjust the volume to 500 ml and sterilize by autoclaving.

5 M NaCl - Dissolve 146.1 g NaCl in 400 ml water. Adjust the volume to 500 ml and sterilize by autoclaving.

Phenol : Chloroform : Isoamyl alcohol (25:24:1) - Mix 750 µl buffered phenol (pH approx. 7.8), 720 µl chloroform and 30 µl isoamyl alcohol. Mix by vortexing and keep at 4 °C in coloured container (or this could be prepared just before use).

1 M Tris - Dissolve 60.55 g Tris base in approximately 300 ml water. Adjust the pH to 8.0 by adding HCl. Adjust the volume to 500 ml and sterilize by autoclaving.

DNA extraction buffer - 100 mM Tris-Cl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2.0 % (w/v) CTAB, 0.2 % (v/v) 2-Mercaptoethanol

Protocol for Plant DNA isolation

- Grind 1-2g of frozen leaf material in liquid nitrogen to fine powder in a pre chilled pestle and mortar.
- Transfer the powder to a 50ml polypropylene tube containing 15ml of pre-warmed (65^o C) DNA extraction buffer. Mix by vortexing.
- Incubate at 65^o C for one hour with intermittent inversion. (after incubation cool the tube at RT for 5 min)
- Add 15ml chloroform: isoamylalcohol (24 : 1, v/v) and mix by inversion to emulsify.
- Spin at 12000 RPM for 20 min at room temperature.

- Pipette out gently the aqueous phase without disturbing the inter-phase to another tube. Add 2/3rd volume of isopropanol and mix by gentle inversion. (Keep at -80° C for an hour to allow the precipitation)
- Spin down the DNA pellet (@ 12,000 RPM for 10 min) or take out the DNA using a bent Pasteur pipette.
- Wash in 70% ethanol and spin at 12,000 rpm for 7 min at 4^{o}C.
- Dry the pellet and dissolve in 300 – 500 µl of sterile water or TE buffer (pH 8.0) depending upon the yield of DNA.
- Add 10 µl of RNase (10 mg / ml RNase A in 10 mM Tris-Cl, pH 7.5. Heat at 100 C using PCR machine for 15 min and cool slowly at RT. Aliquote and store at -20 C) to the final concentration of 20 µg/ml, mix gently and incubate for 1 hour at 37^{0}C.
- Transfer the DNA to 2 ml eppendorff tube and also check for quality on 0.8% gel
- Add equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and mix thoroughly to emulsify.
- Spin at 12,000 rpm for 10 minutes and carefully take out the upper aqueous phase using a 200 µl pipette without disturbing the aqueous phase. (repeat the step depending on the presence of contamints)
- Add equal volume of chloroform: isoamylalcohol (24 : 1, v/v) and mix by inversion to emulsify and centrifuge at 12,000 RPM for 10 min and carefully take the aqueous phase
- Add 1/10 volume of 3 M sodium acetate, pH 5.2 and mix thoroughly. Add two volumes of absolute ethanol (95%). Mix by gentle inversion and keep in ice (or @ -20° C) for 20 minutes.
- Spin at 12,000 RPM for 10 minutes to pellet the DNA.
- Wash the DNA pellet with 70% ethanol and spin at 12,000 RPM for 7 min.
- Dry the DNA pellet and dissolve in sterile water or TE buffer (volume should depend on the pellet size) and dissolve the pellet at room temperature. Check the quality on 0.8% Agarose gel.

Gel electrophoresis

Ethidium bromide - Add 100 mg ethidium bromide (10 mg / ml) to 10 ml of sterile water. Stir on a magnetic stirrer until the dye is completely dissolved. Wrap the container in aluminum foil or transfer the solution to a dark bottle and store at room temperature. **Caution:** Ethidium bromide is a powerful mutagen; avoid contact with skin and inhalation.

6 X Loading dye - 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water. Keep at 4 °C

25 X Tris-borate (TBE) - Weigh 270 g Tris base and 137. 5 g boric acid in a container. Add 100 ml of 0.5 M EDTA and 200 ml of H_2O (approximately). Stir in a magnetic stirrer until a clear solution is formed. Adjust the volume to 1 L and sterilize by autoclaving. (A precipitate forms when concentrated solution of TBE is stored for long period of time. Discard any batches that develop a precipitate.)

Practical 2: Genetic Fidality using SSR markers

Virupaksh Patil and CM Bist

Sample preparation for Single run sequencing using 3500 Genetic Analyzer

1. Take the appropriate quantity of DNA to be sequenced (purified and quantified)

PCR Product	Min quantity required
100 to 200 bp	1-3 ng
200 to 500 bp	3-10 ng
500 to 1000 bp	5-20 ng
1000 to 2000 bp	10-40 g
>2000 bp	20-50 ng
ssDNA	25-50 ng
dsDNA	150-300 ng
Cosmid, BAC	0.5-1.0 µg
Bacterial Genomic DNA	2-3 µg

2. Reaction mixture for the PCR in the 200 µL PCR tubes

PCR mix

	PCR (<500bp)	**Plasmid/PCR (>500bp)**	**Control**
2.5X TRR mix 0.5 µL	1.0 µL		1.0 µL
5X Buffer	1.75 µL	1.5 µL	1.5 µL
Template	as per required	as per required	1 µL
Primer (2-5p mol)	0.5 µL	0.5 µL	2.0 µL
Water	- µL	- µL	4.5 µL
Total	10 µL	10 µL	10 µL

3. Vortex the PCR tubes briefly and give a short spin
4. Keep for the PCR with fallowing program

   96 °C for 1 min
   25 cycles (with Ramp rate 1 °C/sec)
   96 °C for 10 sec
   50 °C for 5 sec
   60 °C for 4 min
   Final hold at 4 °C

Big dye terminator v 3.1 Cleanup (Qiagen kit)

5. After the PCR collect the PCR tubes and give a short spin
6. Take the spin columns (with solution) provided in the sequencing kit
7. Loosen the cap to a quarter turn
8. Then snap off the bottom
9. Keep the column in the collection tube
10. Centrifuge at 750 g for 3 min
11. Transfer the column to a clean 1.5 ml eppendorff tube (matrix bed)
12. Pour the PCR product at the centre of the matrix bed without the tip touching it
13. Centrifuge for 3 min at 750 g
14. Discard the column and Speed Vac dry the collected sample (10-15 min)
15. Resuspend the pellet in 20 µL HiDi
16. Vortex and give a short spin
17. Transfer the content to plate
18. Denature the DNA at 95 °C for 4 min (using PCR machine)
19. Snap chill on ice
20. Vortex and short spin
21. Place the septa onto the plate and place the PCR plate along with its base and the retainer on to the auto-sampler.

Some pints to be remembered:

Sequencing primers should be dissolved in low TE buffer [Tris (1mM): EDTA (0.01mM)] and the working primers (10 p mol) can be dissolved in autoclaved dd H_2O.

When 10p mol is working primer conc. If we take 0.5 µL then final conc in the PCR mix would be 5 p mol which is ideal for sequencing

2.5X + 5Y = 1 x 10, If X =1, 2.5 + 5Y = 10, 5Y = 10 – 2.5 Y = 7.5/5 = 1.5

HiDi formamide - 10 µL dissolution is ok [never freeze though repeatedly as it forms formic acid]. Make aliquots of 100 µL and keep in deep freezer finish each aliquots in one or two sequences,

Keep the polymer at 4 degrees for 1 month

Before filling the polymer, keep the polymer at room temperature for 30 to 45 min and then use

Before using polymer, 1-2 times conditioning buffer wash

RFID- Radio frequency Identification

StdSeq50_POP7 – is to be set for sequencing

Big Dye Terminator v 3.1 Cleanup (Tube method)
Make stocks of

100% Ethanol
0.5M EDTA (pH 8.0) – and freshly prepare 125mM EDTA every week from 0.5M EDTA
3M NaOAc (pH 4.6)
70% Ethanol (use the Milli-Q water for making 70%)
Milli Q Water

1. Make the master mix I (MM-I) of
   10 µL Milli-Q water | per reaction
   2 µL 125mM EDTA |
2. Make the master mix II (MM-II) of
   2 µL of 3M NAOAc | per reaction
   50 µL ethanol (100%) |
3. Add 12 µL MM-I to each reaction in the tube containing 10 µL of reaction mix and ensure that the contents are mixed
4. Add 52 µL of MM-II to each reaction tube
5. Mix well the contents and incubate at room temperature for 15 min
6. Spin at a speed of 12000g for 20 min at room temperature
7. Decant the supernatant and keep the tubes inverted on a tissue paper
8. Add 250 µL of 70% ethanol and spin at 12000g for 10 min at room temperature
9. Decant the supernatant and VacDry the tubes
10. Add 10 µL of HiDi to each the tubes, mix by pipetting up and down
11. Transfer the samples to the plates and cover with septa, denature (95° C for 5 min), snap chill (4°C for 5 min) and proceed for the sequencing

Reaction for microsatellite/SSR markers [Fragment Analysis] (Per Reaction)

DNA	As per requirement
True allele PCR Premix	4.5 µL
Sterile ddH_2O	- µL
Total Volume	7.0 µL

1. Dispense the aliquot to each PCR well and then add 0.5 µL of respective primers
2. Give a short spin
3. Cover the plate with 8 tube strips
4. Place in the PCR with the fallowing reaction program

95°C for 12 min
10 cycles of
94°C for 15 sec
55°C for 15 sec
72°C for 30 sec
20 cycles of
89°C for 15 sec
55°C for 15 sec
72°C for 30 sec
Final extension of 72°C for 10 min
Hold at 4°C forever

5. For multiplexing we can pool the PCR products in the ratio of 1:1:2 (FAM:VIC:NED)
6. Make the master mix of

HiDi-Formamide:	9 µL
GS 500 LIZ	0.5 µL
Total	9.5 µL

7. Add the 9.5 µL HiDi-LIZ mix to each PCR well and add 1.0 µL (or 2 µL) of pooled PCR product
8. Short spin, denature (95°C for 5 min) and snap chill (4°C for 5 min)
9. Make plate record for fragment analysis and start the run (**with G5 Dye set in 3500**)
10. Analyze the data using Genemapper software

Practical 3: Total RNA isolation & RT-PCR analysis

Sundaresha S. Anupama and Vandana Thakur

Material Required

- Total RNA (Minimum of 500 ng to 5µg)
- cDNA synthesis Kit
- SYBR Green/Taq Man Probe
- Real Time-PCR system

Total RNA isolation from Phenol:Chloroform Method

- 0.3 gram leaf tissues were frozen in liquid nitrozen
- Ground to fine powder in liquid nitrogen
- 10ml of extraction buffer was added (0.1 M tris HCl pH 9.0) containing 0.25 M sucrose, o.2m Nacl and 10 mM $MgCl_2$
- 750 µl of phenol (water saturated) : chloroform was added and ground (1:1) (250 µl for 0.1 gm)
- 150 µl of 0.5 M sodium was added and ground (50 µl for o.i gm tissue)
- 150 µl of 20% SDS was added and ground
- Transfered the ground tissues to the freshly sterilized eppendorf tube
- 144 µl of β- mercaptoethanol was added and shaken @ 4^0 C for 30 minutes
- Spun the tube @ 16,000 rpm for 30 minutes at the temperature of 15^0 C
- Transferred the supernatant to other tube
- Added eual volume of phenol: chloroform, vortexed and spun @ 15,000 rpm for 15 minutes at the temperature of 18^0 C. only aqueous supernatant was transferred to another tube and equal volume of chloroform : isoamylalcohol (49: 1) was added and spun @ 15,000 rpm for 15 minutes
- Transferred the aqueous phase to another tube and added 8 M lithium chloride (Li Cl_2) to get final concentration of 3 M Li Cl_2 and keept @ -70^0 C for 4 hours
- Centrifuged the tube at 16,000 rpm for 30 minutes
- discarded the supernatant and pellet was washed once with 5 ml of 2M Li Cl_2 and then with 70 % alcohol by centrifuging at 15,000 rpm for 20 minutes
- pellet was air dried and dissolved in 150 µl of DEPC water (diethyl pyrocarbonate)
- Purity and quantity was estimated spectrophotometrically (OD value 260 nm)
- Total RNA will be isolated from the collected leaf samples using RNeasy Plant mini kit or from the traditional Phenol-chloroform method (Qiagen, USA), cDNA will be synthesized from 1.0 µg of total RNA using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, USA) according to manufacturer's

instructions. cDNA will be treating with RNAse H (1µl) for 20 min at 37°C to remove any contaminating RNA.

- qRT-PCR will be performing by using Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). Reaction consisted of 150 ng of cDNA and 120 nM of each gene-specific primer in a final volume of 15 µL. Amplification was carried out for three technical replicates for each sample, including negative controls.

ABI PRISM HT 7900 (Applied Biosystems, USA) will be used for the following thermal cycles: 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s, and 60°C for 1 min. Expression levels were assessed based on the number of amplification cycles needed to reach a common fixed threshold (cycle threshold ΔCT) in the exponential phase of PCR. For relative quantification/expression, the $2^{-\Delta\Delta CT}$ method between conditions in qRT-PCR will be applied.

The expression will be calculated as follows:

***C*t (sample) = ∆*C*t (gene of interest)− ∆*C*t (endogenous control)**
∆∆*C*t = ∆*C*t (sample) −∆*C*t (calibrator)
Relative expression (R.E.) = (1 + PCR efficiency)− ∆∆*C*t

Note: PCR efficiency = (10(−1/slope of standard curve)) − 1
As standard curve the CT values were plotted against the logarithm of template concentration.
R.E+S.D. = (1 + PCR efficiency) (− ∆*C*t −S.D. d*C*t).

Practical 4: Genomic Library Preparation for WGS shotgun sequencing

Virupaksh Patil, Sundaresha S, Youvika Singh and Sadhana

A) DNA fragmentation by Nebulyzation:

1. Start with **500 ng** of sample DNA in a 1.7 ml microcentrifuge tube.
2. Add TE Buffer to a final volume of **100 µl**.
3. Using sterile gloves, affix a Nebulizer Condensor tube around the Aspiration tube. [To ensure proper function, make sure to push the Condensor tube all the way down around the base of the Aspiration tube, being careful not to rotate the Aspiration tube, and press the vented cap into the Nebulizer top]
4. Set the assembled Nebulizer top, with the aspiration tube pointing upwards; making sure that the inside parts do not contact any contaminated surfaces (counter top, hands).
5. Pipet the **100 µl** DNA sample in the Nebulizer cup.
6. Add **500 µl** of Nebulization Buffer, pipet up and down to mix.
7. Apply **30 psi** (**2.1 bar**) of nitrogen for **1 minute**.
8. Disconnect the tubing and remove the cup from the hood.
9. Remove the Nebulizer top from the cup.
10. Add **2.5 ml** of PBI Buffer.
11. Pipet up and down to mix.
12. Purify the nebulized DNA sample on a column from the Qiagen MinElute PCR Purifi cation kit, as follows, [with all centrifugation steps carried out at 13,000 rpm in a tabletop centrifuge]
    a. Load **750 µl** of the nebulized DNA at a time into **a single** column.
    b. Centrifuge for **15 seconds** and discard the flow-through.
    c. Repeat steps a and b three more times, using the same column.
    d. Centrifuge for **1 minute**. Discard all the flow-through.
    e. Add **750 µl** of PE Buffer, centrifuge for **1 minute**, and discard the flow-through.
    f. Centrifuge **1 minute**, rotate the column 180º, centrifuge **1 minute**.
    g. Elute in new tube with **16 µl** of TE Buffer by centrifuging for **1 minute**.
    h. Transfer the sample to a 200 µl PCR tube.

B) Fragment End Repair

1. In a 1.7 ml microcentrifuge tube, prepare the End Repair mix, as follows.
   2.5 µl RL 10× PNK Buffer
   2.5 µl RL ATP
   1 µl RL dNTP
   1 µl RL T4 Polymerase
   1 µl RL PNK
   1 µl RL Taq Polymerase
   9 µl Total volume
2. Pipet up and down to mix, and add the **9 µl** of End Repair mix to the DNA sample
3. Vortex for 5 seconds, then spin for 2 seconds in a mini centrifuge.
4. Run the End Repair program on a thermocycler, with the heated lid on:

25ºC for 20 min
72ºC for 20 min
4ºC on hold

5. While the program is running, you can prepare the Agencourt AMPure beads as described in Section

C) AMPure Bead Preparation

1. Vortex the AMPure bead bottle for 20 seconds, or until the beads are completely resuspended.
2. Aliquot **125 µl** of AMPure beads in a 1.7 ml microcentrifuge tube.
3. Place the tube on the Magnetic Particle Concentrator (MPC) (ISC).
4. When the beads have completely pelleted on the side of the tube, carefully remove and discard all supernatant, without disturbing the beads. **For ISC only:** add **73 µl** of TE Buffer to the beads and vortex 5 seconds.
5. Add **500 µl** (ISC) of Sizing Solution to the beads, vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
6. Keep the tube **on ice**, until you reached Section 3.5.
7. Prepare 5 ml of 70% ethanol, by adding 3.5 ml of 100% ethanol to 1.5 ml Molecular Biology Grade Water, and vortex.

D) Adaptor Ligation

1. Once the End Repair program has completed add **1 µl** of RL Adaptor or of RL MID Adaptor to the reaction tube.
2. Add 1 µl of RL Ligase to the reaction tube.
3. Vortex 5 seconds, then centrifuge for 2 seconds in a mini centrifuge.
4. Incubate at **25ºC** for **10 minutes**.

D) Small Fragment Removal

1. Add the sample to the AMPure beads prepared. Vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
2. Incubate at **room temperature** for **5 minutes**.
3. Place the tube on the MPC.
4. When the beads have fully pelletted on the wall of the tube, carefully remove and discard the supernatant.
5. Add **100 µl** (ISC) of TE Buffer. Vortex for 5 seconds (ISC)
6. Add **500 µl** (ISC) of Sizing Solution. Vortex for 5 seconds (ISC)
7. Incubate at **room temperature** for **5 minutes**.
8. Place the tube on the MPC.
9. When the beads have fully pelleted on the wall of the tube, carefully remove and discard the supernatant.
10. **Repeat steps 5 to 9, once.**
11. Keeping the tube on the MPC, wash the beads twice, as follows:
    a. Add **1 ml** (ISC) of 70% ethanol.

b. Completely remove and discard the ethanol.

12 Keeping the tube on the MPC, uncap the tube and air dry the pellet at **room temperature** for **2 minutes**.

12. Remove the tube from the MPC. Add **53 µl** of TE Buffer. Vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
13. Place the tube on the MPC, wait for the beads to pellet on the wall of the tube and transfer **50 µl** of the

SUPERNATANT, containing the library, to a new, labeled 1.7 ml microcentrifuge tube. Make sure **not to carry-over any beads** in this process as they will cause incorrect readings during library quantitation

E) Library Quantitation

1. In tube 1, prepare a 2.5 × 109 molecule/µl solution of the RL Standard by mixing **90 µl** of the RL Standard (orange cap) with **90 µl** of TE Buffer.
2. Fill the remaining 7 tubes (tubes 2 to 8) with **60 µl** of TE Buffer.
3. Transfer **120 µl** from tube 1 into tube 2.
4. Vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
5. Change pipet tip and transfer **120 µl** of tube 2 into tube 3.
6. Vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
7. Proceed with the same serial dilution (transferring **120 µl** of one tube into the next, vortexing for 5 seconds, and changing pipet tip between each dilution) for the remaining 5 tubes.

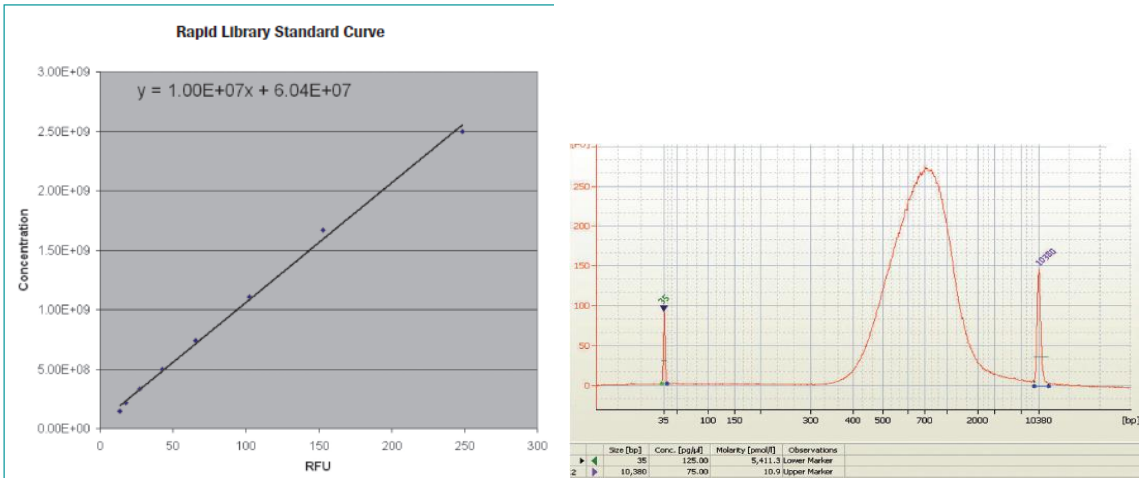

Fig: Example for the standard curve with RL standard and biooanlyser graph

Practical 5: Protoplast isolation and Fusion

Jagesh K. Tiwari, Poonam, Sapna and Nilofer

Protoplast Isolation

1. *In vitro* plant material: Three-week-old *in vitro*-grown microplants, raised from single nodal cuttings are used to isolate mesophyll protoplasts under sterile conditions
2. Microplants growth condition: 16 h photoperiod/40μmol m^{-2} s^{-1}/20°C.
3. Pre-isolation (protoplast) incubation: Cultivate *in vitro* plants at 20°C for 48 h under a 16-h photoperiod in the dark (covered with black muslin cloth) prior to protoplast isolation to integrate cell cycles.
4. Protoplast isolation: Mince young leaf tissues (1.0-2.0 g) in a Petri dish (Ø = 90 × 15 mm) containing **Protoplast Digestion Solution (PDS)**: 10.0 ml digestion solution for 1.0 g leaf tissue.
5. Incubation (for protoplast): 16 h/ dark/ 25°C/ optional: gyratory shaking at 40-50 rpm; not exceeding 50 rpm.
6. Post-isolation handling:
   - Add 0.3 M KCl (filter-sterilize) (FS) to the digestion medium containing released protoplasts (protoplast suspension) in a 1:1 ratio (PDS:KCl).
   - For example, add 15.0 ml of 0.3 M KCL to 15.0 ml digestion medium/ solution followed by gentle shaking of PDS containing released protoplast
   - Filter the suspension through a 40-µm nylon mesh, and collect in 10 ml centrifuge tubes; 60µ can be used, but the debris and/ or undigested tissues will be much.

Protoplast purification

- Centrifuge the filtrate at 50 ×g (60 RCF) for 5 min, and then resuspend the pallet in 10 ml of 0.6 M sucrose (filter sterilized).
- Layer 1.0 ml of 0.3 M KCl onto this protoplast suspension, and centrifuge at 50 g for 5 min.
- Recover the live protoplasts (green) from the sucrose: KCl interface, and dilute with 10.0 ml of 0.3 M KCl.

- Centrifuge at 50 ×g for 5 min to pellet the protoplast. Resuspend the pellet in 0.5 M mannitol (sterile) to a final density of 1×10^6 protoplasts ml^{-1} for electrofusion.

Protoplast Digestion Solution (PDS) Preparation

	Chemicals	Strength	Medium volume (500 ml)
1.	MS Macronutrients		
	KNO_3	0.95 g/L	475.0 mg
	KH_2PO_4	0.085 g/L	42.5=43.0 mg
	$MgSO_4.7H_2O$	0.185 g/L	92.5=93.0 mg
	$CaCl_2.2H_2O$	0.660 g/L	330.0 mg
2.	MS Micronutrients (To be used as half strength)	× 1000	250 µl
3.	MS Fe-EDTA(MS stock 5) (To be used as full strength)	× 100	2.5 ml
4.	MS Vitamins (MS stock 6) (To be used as half strength)	× 1000	0.5 ml
5.	Myo-Inositol	100 mg/L	50.0 mg
6.	Polyvinylpyrrolidone (PVP) [AVM 10,000]	5.0 g/L	2.5 g
7.	MES (FW 195.24 for MES hydrate)	5.0 mM	488.0 mg
8.	D-Glucose (FW 180.16)	0.1 M	9.0 g
9.	D –Mannitol (FW-182.17)	0.4 M	36.434 g
10.	Cellulase 'ONOZUKA' RS or Cellulase ONOZUKA' R-10	1.0 %	Not to be added here
11.	Macerozyme R-10	0.5 %	Not to be added here

Notes

- **pH 5.7** to be adjusted using 0.1/1.0 N NaOH.
- Prepare the above digestion medium without digesting enzymes, adjust the pH and filter-sterilize.
- Store the medium in aliquots (20-25 ml) at -20°C.
- Before using, thaw the medium, add enzymes at required amount to medium aliquots, centrifuge to dissolve and check/adjust the pH (5.7), if required.
- Filter-sterilized (0.2 µm filter) and use.

1.3 Other Solutions

	Chemicals	Strength	Medium volume (500 ml)
1.	KCl (FW 74.55)	0.3 M	11.183 g
2.	Sucrose (FW 342.30)	0.6 M	102.69 g
3.	D-Mannitol (FW 182.17) (pH 7.0)	0.5 M	45.543 g
4.	**Solution 2 (pH 7)**		
	$CaCl_2.2H_2O$ (MW 147.02 g)	10 mM	220.53 mg
	D-Mannitol (FW 182.17 g) (pH 7)	0.5 M	150 ml
5.	**Solution 3 (pH 7)**		
	$CaCl_2.2H_2O$ (MW 147.02 g)	50 mM	1.1026 g
	D-Mannitol (FW 182.17 g) (pH 7)	0.5 M	150 ml
6.	**Sodium Alginate solution**		
	Na-Alginate (alginic acid sodium salt)	2.8%	2.8 g
	D-Mannitol (FW 182.17 g) (pH 7)	0.5 M	100 ml
7.	**NaOH (FW 40)**	1N	4.0 g/100 ml

Notes:

- Autoclave all solution at 121℃ for required duration depending on the volume, and then filter-sterilize using a 0.2 µm filter.
- Use 1N NAOH to prepare 0.1N NAOH and filter-sterilize the solution in small aliquots.
- pH adjustment of Mannitol (pH 7) may take longer time, adjust with 0.1N NaOH.
- Na-Alginate solution may take longer time while dissolving solution.

Protoplast Electrofusion

Protocol:

1. Electrofusion medium: 0.5 M mannitol (FS)/ pH 7.0-7.3/adjust pH with sterile 0.1 N NaOH.
2. Symmetric fusion: 1:1 of each species under laminar work station
3. Electrofusion settings (under laminar):

Method 1:

CHAMBER:	BTX Microslide Model 453/3.2mm gap
Alignment amplitude:	16 V
Alignment time:	25-30 s
Alignment field strength:	5V cm^{-1}
Electroporation amplitude:	260 V

DC pulse width:	60 µs
Number of pulses:	2
Electroporation field strength:	812V/cm

Method 2:

CHAMBER:	BTX Microslide Model 453/3.2mm gap
Alignment amplitude:	32 V
Alignment time:	25-30 s
Alignment field strength:	100V/cm
Electroporation amplitude:	260 V
DC pulse width:	60 µs
Number of pulses:	1
Electroporation field strength:	812 V/cm

Method 3:

CHAMBER:	Disposable cuvette P/N620 (2mm gap)
Alignment amplitude:	16 V
Alignment time:	25-30 s
Alignment field strength:	50 V/cm
Electroporation amplitude:	260 V
DC pulse with:	60 µs
Number of pulse:	2
Electroporation field strength:	812 V/cm

Notes:

- In a disposable cuvette, fuse protoplasts in an aliquot of 400 µl and allow to stand the fusion products for at least 30 min for post-fusion recovery.
- Post-fusion AC pulse can be employed maximum up to 9 s compress the fusion product.

Regeneration of Post-Fusion (Protoplast) Products

Protocol:

1. Dispense 50 µl Na-alginate 2.8%, prepared in 0.5 M mannitol in each box of 'castor rack' and mix well the 50 µl post-fusion products with sodium alginate to form film layer. (1:1 ratio i.e. 50 µl each protoplast and Na-alginate)

2. Add ~5 ml Solution # 3 in each box and incubate at RT inside laminar for 30 min followed by add ~5 ml Solution # 2 and incubate same for 1.0 h

3. Remove Solution # 2 & # 3 by Pasteur pipette and add 5 ml **VKMG (VKM Glucose)** liquid medium and tightly wrapped the 'castor rack' with parafilm.

4. Incubate the castor racks (containing post-fusion products mixed with sodium alginate into VKMG) under dark at 25 °C for regeneration of microcalli (upto 4-6 months, depends upon the species).

5. The first cell division of the protoplast usually occur at 3-4 days after fusion, examine the growth and development of post-fusion products cultures under microscope at regular intervals. Cell aggregates (microcalli) are formed at 4-6 months after the fusion.

6. Before transfer the microcalli onto solid medium, dissolve the sodium alginate film surrounding post-fusion products/microcalli in **Dissolving Solution**.

7. After dissolving sodium alginate, wash microcalli in **Washing Solution**.

8. **Dissolving solution**→ MS_{13K} Medium (Without hormones + Coconut water + Gelrite) + 20 mM or 50mM Na-Citrate dehydrate (pH=7.0)

9. **Washing solution**→ MS_{13K} Medium (Without hormones + Coconut water + Gelrite) (pH=5.8)

10. Finally transfer microcalli onto **MS_{13K} Medium** (solid) in a Petri dish for development of micro-shoots/plants.

11. Transfer of microshoots/plants onto the **MS Medium** for *in vitro* regeneration and multiplication of putative somatic hybrids' plantlets.

VKM Medium Preparation

1. Macroelements

Chemical	Medium volume: 1 lit
KNO_3	1480 mg/L
KH_2PO_4 (FW 136.09)	68 mg/L
$MgSO_4.7H_2O$ (FW 246.48)	984 mg/L
$CaCl_2.2H_2O$ (FW 147.02)	735 mg/L

No need to prepare stock solution, add directly during medium preparation

2. VKM Stocks

Chemical	Medium volume: 1 lit
VKM stock-I	1 ml/L
MS stock-5	10.0 ml/L
VKM stock II	10.0 ml/L
VKM stock III	10.0 ml/L
VKM stock IV	10.0 ml/L
VKM stock-V	10.0 ml/L
VKM stock-VI	400 µl/L

3. Complex additives

Chemical	Medium volume: 1 lit
Casein Hydrolysate	250 mg/L
Coconut water (**store at -20°C**)	20 ml/L

Add directly during medium preparation. Coconut water to be added inside laminar

4. Growth hormone

Chemical	Medium volume: 1 lit
2,4-D (FW 221) (0.2 mg/L) (store at 0°C)*	200 µl/L
α-NAA (1.0 mg/L) (store at 0°C)*	1000 µl/L = 1 ml/L
Zeatin (trans) (FW 219.2) (0.5 mg/L) (store at -20°C)**	500 µl/L

*Separate stock of 1 mg/ml (dissolved in NaOH and water)
**Dissolved Zeatin-vial, supplied by manufacture, in 100 µl KOH and make up water volume to prepare stock of 1 mg/ml. (e.g. 10 mg/10 ml)

5. Sugar: Glucose (0.5M) - 90.1g for 1 litre medium

Note:

- Adjust medium pH: 5.6-5.7 with 0.1 M KOH.
- VKM medium is used for the division and growth of protoplast.
- Please note, that-VKM medium must be filter-sterilized using 0.2µ membrane filter (cellulose acetate/cellulose nitrate + 0.4µ pre filter (glass fiber).
- NO AUTOCLAVING

VKMG (VKM Glucose) medium

- VKM medium must be filter-sterilized using 0.2 µ membrane filter (cellulose acetate) + 0.4 µ prefilter (glass fibre)

- Weigh 9.01 g of 0.5 M Glucose in 100 ml VKM and adjust pH 5.6-5.7 with 0.1 M KOH

VKM STOCKS

Composition of VKM Stock for preparation of VKM Medium

a) VKM Stock-I

MICRO ELEMENTS	mg/L	Stock (×1000)
H_3BO_3 (FW 61.83)	3.0	300 mg/100 ml
$MnSO_4.H_2O$ (FW 169.02), OR $MnSO_4.4H_2O$, OR $MnSO_4$ (anhyd)	8.0 10.0 7.0	800 mg/100 ml 1000 mg/100 ml 700 mg/100 ml
$ZnSO_4.7H_2O$ (FW 287.54)	2.0	200 mg/100 ml
KI (FW 166.01) (Store in amber colour	0.75	75 mg/100 ml

bottle)		
$Na_2MoO_4.2H_2O$ (FW 241.95)	0.25	25 mg/100 ml
$CuSO_4.5H_2O$ (FW 249.68)	0.025	2.5 mg/100 ml*
$CoCl_2.6H_2O$ (FW 237.93) (store in amber colour bottle)	0.025	2.5 mg/100 ml*
1.0 ml VKM Stock-I for 1.0 L medium, Store at 2-8ºC		

* Prepare 1 mg/ml separate stock of each and add 2.5 ml to prepare VKM Stock-I

b) MS Stock-5

Chemical	mg/L	Stock (×100)
$FeSO_4.7H_2O$ (FW 278.01)	27.8	278 mg/100 ml
$Na_2EDTA.2H_2O$ (FW 372.24)	37.2	373 mg/100 ml
10.0 ml MS Stock-5 for 1.0L medium		

c) VKM Stock-II

Sugar & Sugar alcohol	mg/L	Stock (×100)
d-Mannitol (FW 182.17)	250.0	2.5g/100 ml
d-Sorbitol (FW 182.17)	250.0	2.5g/100 ml
Sucrose (FW 342.3)	250.0	2.5g/100 ml
Fructose (FW 180.2)	250.0	2.5g/100 ml
Ribose (FW 150.1)	250.0	2.5g/100 ml
Xylose (FW 150.1)	250.0	2.5g/100 ml
Mannose (FW 180.2)	250.0	2.5g/100 ml
Rhamnose (FW 182.2)	250.0	2.5g/100 ml
Cellobiose (FW 342.3)	250.0	2.5g/100 ml
m-Inositol (180.16)	100.0	1.0g/100 ml
Glucose*	90,000 (90 g)	9.0 g/100 ml
10 ml VKM Stock-II for 1.0 L medium, Store at 2-8°C		

*Glucose not to be added in the stock; to be added while preparing the medium at the end.

- **Filter-Sterilize (FS)** this stock and to be added inside the laminar work station (while preparing the medium)

d) VKM Stock-III

Organic acid	mg/L	Stock (×100)
Sodium-pyruvate (FW 110.04) (Pyruvic acid-sodium salt)	20.0	200 mg/100ml
Citric acid (FW 192.12)	40.0	400 mg/100ml
Fumaric acid (FW 116.07)	40.0	400 mg/100ml
Malic acid (FW 134.09)	40.0	400 mg/100ml
10ml VKM Stock-III for 1.0 lit medium, Store at 0ºC		

- Filter-Sterilize this stock and to be added inside the LFCA Work station (while preparing the medium

e) VKM Stock-IV

Vitamin	mg/L	Stock (×100)
Calcium-D-pontothenic acid (FW 283.3)	1.0	10 mg/100ml
Choline chloride (FW 139.63)	1.0	10 mg/100ml
Ascorbic acid (FW 176.12)	2.0	20 mg/100ml
p-Aminobenzoic acid (FW 137.14)	0.02	0.2 mg*
Nicotinamide	1.0	10 mg/100ml
Pyridoxine-HCl (FW 205.6)	1.0	10 mg/100ml
Thiamine-HCl (FW 337.27)	10.0	100 mg/100ml
Biotin (FW 244.3) (Vit H)	0.01	0.10 mg**
10 ml VKM Stock-IV for 1.0 lit medium, Store at 0ºC		

- Filter-Sterilize and to be added inside the LFCA Work sterilize (while preparing the medium)

* 200 µl from 1mg/ml separate stock of p-Aminobenzoic acid.
** 100 µl from 1mg/ml separate stock of biotin in KOH/NaOH.

f) VKM Stock-V

Vitamin	mg/L	Stock (×100)
Vitamin A	0.01	0.1 mg
10 µl from stock of 10 mg/ ml OR 100 µl from stock of 1 mg/ml for 100 ml VKM Stock-V. Dissolve in 100% ethanol only		
Vitamin B_{12}	0.02	0.2 mg
200.0 µl from 1mg/ml for 100 ml VKM Stock-V		
Vitamin D_3	0.01	0.1 mg
10 µl from stock of 10 mg/ ml OR 100 µl from stock of 1 mg/ml for 100 ml VKM Stock-V. Dissolve in 100% ethanol only		
10ml VKM Stock-V for 1.0 lit medium, Store at -20ºC		

g) VKM Stock-VI

Vitamin	mg/L	Stock
Folic acid (FW 441.4)	0.4 mg	1 mg/ml
400 µl VKM Stock-VI for 1.0 lit medium, Store at 0°C in amber colour bottle		

- This stock must be kept in an amber colour vial/or wrap Al-foil.

MS_{13K} Medium Preparation

1. Macroelements

Chemical	mg/L	Medium Volume (1000 ml)
NH_4NO_3	1650	1650 mg/1000 ml
KNO_3	1900	1900 mg/1000 ml
$CaCl_2.2H_2o$	440	440 mg/1000 ml
$MgSO_4.7H_2O$	370	370 mg/1000 ml
KH_2PO_4	170	170 mg/1000 ml

Add directly during medium preparation

2. Microelements

Chemical	mg/L	Stock (×1000)
H_3BO_3	6.2	620 mg/100 ml
$MnSO_4.H_2O$	16.9	1690 mg/100 ml
$ZnSO_4.7H2O$	10.59	1059 mg/100 ml
KI	0.83	83 mg/100 ml
$Na_2MoO_4.2H_2O$	0.25	25 mg/100 ml
$CuSO_4.5H_2O$	0.025	2.5 mg/100 ml*
$CoCl_2.6H_2O$	0.025	2.5 mg/100 ml*
1 ml for 1.0 lit medium		

* Prepare 1 mg/ml stock and add 2.5 ml to prepare Microelements stock

3. Iron –EDTA (or MS stock-V)

Chemical	mg/L	Stock (×100)
$Na_2EDTA.2H_2O$	37.2	373 mg/100 ml
$FeSO_4.7H_2O$	27.8	278 mg/100 ml
10.0 ml for 1 lit medium		

4. Vitamins (or MS stock-VI)

Chemical	mg/L	Stock (×1000)
Nicotinamide	0.5	50 mg/100 ml
Pyridoxine-HCl	0.5	50 mg/100 ml
Thiamine-HCl	0.1	10 mg/100 ml
Glycine	2.0	200 mg/100 ml
1.0 ml for 1 lit medium		

5. Choline chloride

Chemical	mg/L	Stock
Choline chloride	8.0	1 mg/ml

- 8 ml from stock of 1 mg/ml for 1 lit medium

6. Amino acids

Chemical	mg/L	Stock (×100)
Arginine-HCl	6.24	62.4 mg/100ml
Asparaginic acid*	4.8	4.8 mg/100ml
Cystein**	1.2	12.0 mg/100ml
Glutamic acid	11.2	112.0 mg/100ml
Histidine	2.08	20.8 mg/100ml
Isoleucin	8.2	82 .0mg/100ml
Leucin	12.48	124.8 mg/100ml
Lysine	12.48	124.8 mg/100ml
Methionine	10.4	104 .0mg/100ml
Phenylalanine	4.0	40 .0mg/100ml
Proline	4.0	40.0 mg/100ml
Threonine	10.4	104.0 mg/100ml
Tryptophan	3.2	32.0 mg/100ml
Valine	10.4	104.0 mg/100ml
10.0 ml for 1 lit medium		

- Asparagine, isoleucine, tryptophan and valine take time to dissolve so use warm water

7. Myo-Inositol: 100 mg for 1 lit medium

8. Sucrose: 30.0 g for 1 lit medium

9. Coconut milk/Water: 50 ml for 1 lit medium

10. Growth Hormones (to be added by sterile - filtration)

Chemical	mg/L	Stock (1mg/ml stock)
IAA	0.1 mg/l	100 µl/ 1 lit
Zeatin-riboside	2.0 mg/l	2 ml/ 1 lit

11. Gelrite: 2.5 g for 1 lit medium

- Prepare MS13K medium by mixing amount/volume of SN 1 to 11
- Adjust pH: 5.8; Autoclave: 121℃ for 20 min
- After autoclave, add the required amount of growth hormones by sterile filtration
- This medium is used for Callus regeneration and growth of post-fusion products.

Solubility Parameters of Growth Hormones

SN	Hormones	Solubility	Stock solution	Storage
1.	2,4-D (2,4 dichlorophenoxy acetic acid)	At 60ºC in water bath (ddH_2O)/ 100% EtOH/ 1N KOH or NaOH	20 mg/20 ml	0ºC
2.	α-NAA (Naphthalene acetic acid)	At 60ºC in water bath (ddH_2O)/ 1N KOH or NaOH	20 mg/20 ml	0ºC
3.	Zeatin mix isomer*	1N KOH or NaOH	10 mg/10 ml	-20ºC
4.	Zeatin riboside (ZR)*	1N KOH or NaOH	10 mg/10 ml	-20ºC
5.	BA (N^6Benzyl adenine)	1N KOH or NaOH	20 mg/10 ml	0ºC
6.	GA_3	100% EtOH/1N KOH or NaOH	20 mg/10 ml	0ºC
	*Directly dissolve in the vial supplied by manufactures			

Solubility Parameters of Vitamins

SN	Vitamins	Solubility	Stock solution	Storage
1.	4 (para) amino benzoic acid (PABA) (Vit Bx/Vit H1)	At 60ºC in water bath (ddH_2O)	20 mg/20 ml	2-8 ºC (0ºC)
2.	Foliac acid (Vit M)	1N KOH	20 mg/20 ml	2-8 ºC (0ºC)
3.	Biotin (Vit H/VitB_7)	At 60ºC in water bath (ddH_2O)/ 1N KOH	20 mg/20 ml	2-8 ºC (0ºC)
4.	Vit A (Retinol acetate)*	100% EtOH (@ 25 mg/ml of EtOH)	20 mg/2 ml	-20ºC
5.	Vit B_{12} (Cyanocobalamine)	At 60ºC in water bath (ddH_2O)/ 1N KOH	20 mg/20 ml	2-8 ºC (0ºC)
6.	VitD_3 (Cholcalciferol)*	100% EtOH (@ 10 mg/2ml of 100% EtOH)	20 mg/2 ml	2-8 ºC (0ºC)
7.	Pyrodoxin-HCl (Vit B_6)**	ddH_2O	20 mg/20 ml	2-8 ºC (0ºC)
8.	Thiamine-HCl (Vit B_1)**	ddH_2O	20 mg/20 ml	2-8 ºC (0ºC)
9.	Nicotinamide (Vit B_3)**	ddH_2O	20 mg/20 ml	2-8 ºC (0ºC)
10.	Nicotinic acid (Niacin)** (Vit B_3)	ddH_2O	20 mg/20 ml	2-8 ºC (0ºC)
	*May encounter difficulties in dissolving			
	** Weigh directly while preparing the vitamins stock			

MS Medium Preparation

1. MS Stock- 1

Chemical	**Strength** (× **50)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
NH_4NO_3 1650 mg/L	20 ml MS Stock 1 for 1L medium	8.250 g	20.625 g	41.250 g	82.500 g
KNO_3 1900 mg/L		9.500 g	23.750 g	47.500 g	95.000 g

2. MS Stock- 2

Chemical	**Strength** (× **100)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
$MgSO_4.7H_2O$ 370 mg/L	10 ml MS Stock 2 for 1L medium	3.700 g	9.250 g	18.500 g	37.000 g
$MnSO_4.H_2O$ 16.9 mg/L		169 mg	423 mg	845 mg	1690 mg
$ZnSO_4.7H_2O$ 8.6 mg/L		86 mg	215 mg	430 mg	860 mg
$CuSO_4.5H_2O$ 0.025 mg/L		0.25 mg (1.0 ml)	0.625 mg (2.5 ml)	1.25 mg (5.0 ml)	2.5 mg (10.0 ml)
Dissolve 25mg $CuSO_4.5H_2O$ in 100 ml dH_2O and then add required volume (within parenthesis) to MS 2 Stock					

3. MS Stock- 3

Chemical	**Strength** (× **100)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
$CaCl_2.2H_2O$ 440 mg/l	10 ml MS Stock 3 for 1L medium	4.400 g	11.000 g	22.000 g	44.000 g
KI 0.83 mg/L		8.3 mg	21.0 mg	41.5 mg	83.0 mg
$CoCl_2.6H_2O$ 0.025 mg/L		0.25 mg (1.0 ml)	0.625 mg (2.5 ml)	1.25 mg (5.0 ml)	2.5 mg (10.0 ml)
Dissolve 25mg $CoCl_2.6H_2O$ in 100 ml dH_2O and then add required volume (within parenthesis) to MS 3 Stock					

4. MS Stock- 4

Chemical	**Strength (× 100)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
KH_2PO_4 170 mg/L	10 ml MS Stock 4 for 1L medium	1.700 g	4.250 g	8.500 g	17.000 g
H_3BO_3 6.2 mg/L		62.0 mg	155 mg	310 mg	620 mg
$NaMoO_4.2H_2O$ 0.25 mg/L		2.5 mg (1.0 ml)	6.25 mg (2.5 ml)	12.5 mg (5.0 ml)	25.0 mg (10.0 ml)
Dissolve 250mg $NaMoO_4.2H_2O$ in 100 ml dH_2O and then add required volume (within parenthesis) to MS 4 Stock					

5. MS Stock- 5

Chemical	**Strength (× 100)**	**100 ml**	**250 ml**	**500 ml**	**1000 ml**
$FeSO_4.7H_2O$ 27.8 mg/L	10 ml MS Stock 5 for 1L medium	278 mg	695 mg	1390 mg	2780 mg
$Na_2EDTA.2H_2O$ 37.3 mg/L		373 mg	933 mg	1865 mg	3730 mg
Store in amber colour bottle					

6. MS Stock- 6 (Vitamins)

Chemical	**Strength (× 1000)**	**100 ml**
Thiamine-HCl (0.1 mg/L)	1 ml MS Stock 6 for 1L medium	10.0 mg
Pyridoxine-HCl (0.5 mg/L)		50.0 mg
Nicotinic acid (0.5 mg/L)		50.0 mg
Glycine (2.0 mg/L)		200.0 mg
Store at 0°C		

<u>Weigh and add directly</u>:

- Myo-Inositol: 100 mg/L
- Sucrose: 20.0 g/L
- pH: 5.8
- Gelrite: 2.0 g/L
- Autoclave-sterilize: 121 °C for 20 min

Practical 6: MultiNA-Microchip based Electrophoresis System for DNA/RNA Analysis

Dalamu and Reena Sharma

MultiNA is easier, faster and more sensitive electrophoresis than agarose gel, enabling high-speed analysis, high separation performance and reproducibility.

Features of MultiNA:

1. Enhanced precision for size estimation.
2. High resolution and high repeatability.
3. Minimizes running cost and time.
4. Automatic analysis once the samples and reagents are loaded.
5. Facilitates either automatic or manual reanalysis of data.
6. High throughput with parallel processing with four microchips at a time. This reduces the analysis time of 96 samples to 124 minutes and a total of 108 samples can be loaded in one go.
7. Ease to use with the combination of instrument control software and a sophisticated GUI viewer ensuring analytical operation to be very simple.
8. Wide range of applications like genotyping, microbiological analysis, infectious disease analysis, RNA analysis and food analysis.
9. High speed and ease of operation from rinsing of the microchips, filling of the separation buffer, loading the sample and electrophoretic separation up to data analysis.
10. Increased sensitivity due to built-in fluorescence detector which is approximately 10 times more sensitive than conventional ethidium bromide staining.

Inputs of MultiNA:

1. **Microchip**: Microchip comprised of extremely fine flow channels and electrode patterns created in a quartz substrate using Micro Electro Mechanical Systems technology. A maximum of four microchips can be simultaneously used with the instrument. A chip has a maximum capacity of analysing 3000-3500 samples. Separate chips are to be used for DNA and RNA analysis.
2. **DNA and RNA Kit**: A kit comprise of separation buffer, marker solution, buffer bottle (for dispensing separation buffer) and a vial (for dispensing marker).

Type of kits

i. DNA-500 Kit (25 to 500 bp):
ii. DNA-1,000 Kit (100 to 1,000 bp)
iii. DNA-2,500 Kit (100 to 2,500 bp)
iv. RNA Kit 28S rRNA (5.0 knt):

3. **Dye:** SYBR Gold dye for DNA analysis and SYBR Green II for RNA analysis are used.
4. **Ladders:** The ladder varies with the kits, 25 bp DNA ladder for DNA-500 Kit, ϕX 174 DNA/Hae III for DNA-1000 Kit, pGEM for DNA-2500 Kit and RNA 6000 ladder for RNA Kit.

5. **Chip cleaning kit:** Cleaning kit facilitates removal of fluorescent dye and reagents adsorbed on the wall of the microchip and improve the separation process and efficiency of microchip.

Storage of microchip and consumables:

Separation buffer at 2-8 °c, marker, ladder and dye at -20 °c and microchip at ambient room temperature.

Steps involved in on-chip DNA 500 kit analyses:

1. **Preparation of sample:** PCR assay of the samples.
2. **Thawing of the MultiNA reagents**: Reagents (separation buffer, markers) and fluorescent dye are kept at room temperature for 15- 30 minutes before using.
3. **Creation of the analysis schedule:** PC-based MultiNA control software is started and an analysis schedule is registered on the MultiNA instrument control software according to the samples. Mean while the reagents are prepared as following.

4. Preparation of MultiNA reagents:

i. Preparation of fluorescent dye: SYBR Gold dye is prepared in the ratio of 1:99 of dye: TE buffer. The mixture is to be protected from light and to be used within 3-4 days of preparation.
ii. Preparation of ladder: The 25 bp ladder is prepared in the ratio of 1:49 of ladder: TE buffer so that the final concentration is 20 ng/ µl. The quantity of ladder to be used is 10-12 µl irrespective of the number of samples to be analysed.
iii. Preparation of separation buffer: The quantity of separation buffer varies with the number of samples to be analysed. The ratio for preparation is for every 495 µl Separation buffer, 5 µl SYBR Gold dye mixture (1:99 dye: TE buffer) is to be added. The mixture is to be vortexed properly taking care that no air bubbles forms and protected from light. The mixture is prepared in the buffer bottle supplied with the kit.
iv. Preparation of marker solution: Marker solution are available readymade. The required quantity of marker varies according to the number of samples. The solution is loaded in the vial supplied with the respective kit.

5. Loading of the instrument

If analysis is being conducted for the first time or if the microchip is being replaced, set the microchip(s) in the instrument. Set the ladder, sample, separation buffer and marker at the position registered in the analysis schedule. Replenish the wash water and empty the waste liquid bottle.

6. Analysis

Close the top cover and press the Start button displayed in the MultiNA instrument control software. The further steps *viz*., separation buffer filling, sample loading, electrophoresis detection, microchip washing and data analysis are handled automatically by the instrument.

7. Analysis results

Press the View Data File button in the MultiNA instrument control software. The Viewer opens to allow viewing of the results for samples already analyzed.

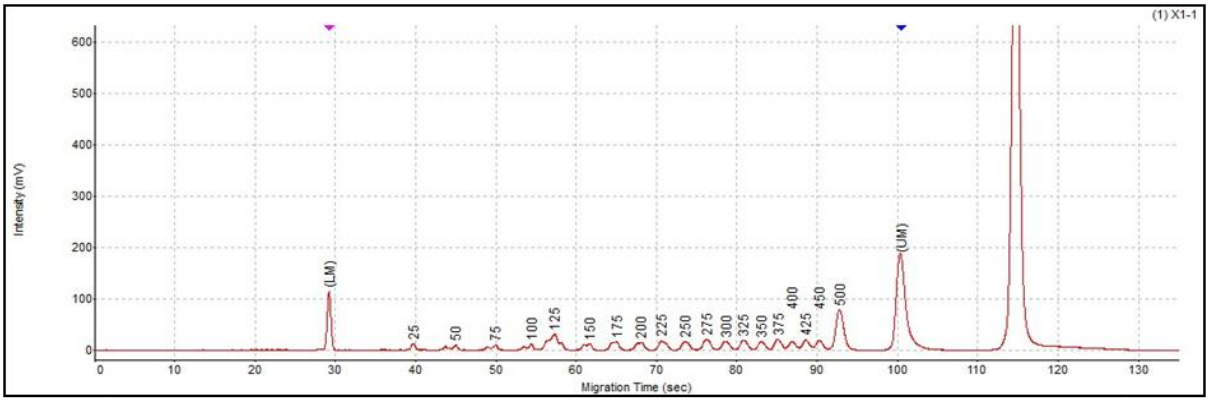

Fig. 1 25 bp ladder for DNA 500 kit (total 19 peaks ranging from 25-500 bp)

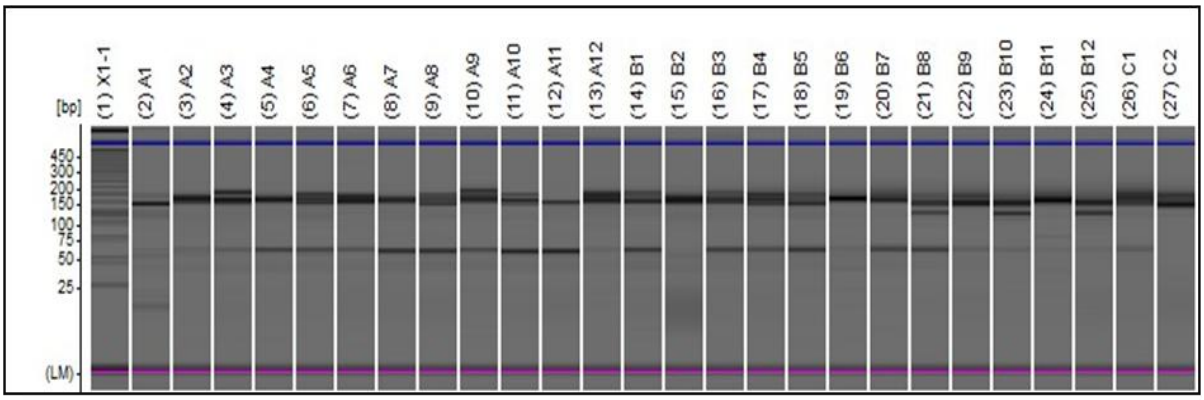

Fig.2 Analysis schedule of potato samples; lane1: 25 bp ladder; lane 2-27: potato samples

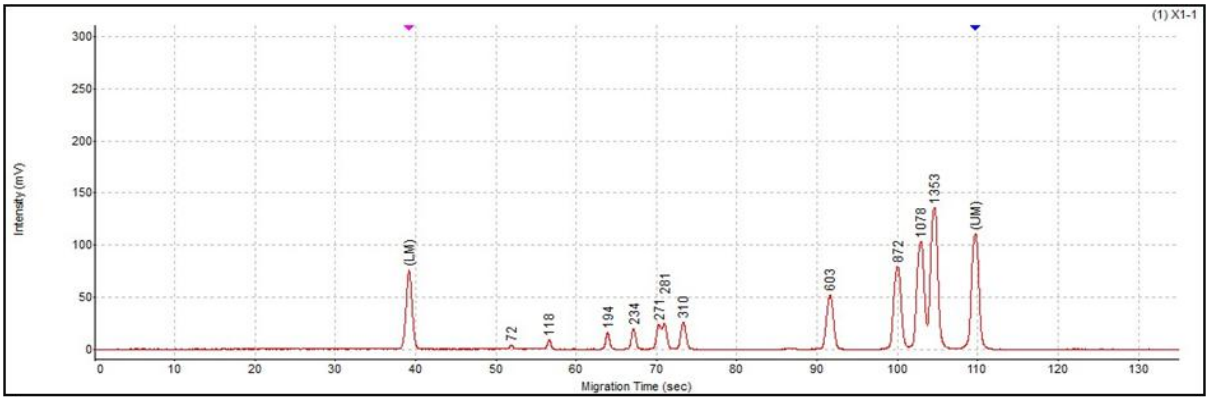

Fig. 3 ϕX 174 DNA/Hae III for DNA-1000 Kit (total 11 peaks ranging from 72-1353 bp)

Practical 7: Pathogen diagnostics using ELISA, Electron microscopy and molecular tools

A. Jeevalatha, Baswaraj, R and Ravinder Kumar

(A) Enzyme Linked Immuno Sorbant Assay

Principle

The basic principle of an ELISA is to use an enzyme to detect the binding of antibody (Ab) antigen (Ag). The enzyme converts a colorless substrate to a colored product, indicating the presence of Ag:Ab binding. In a positive test, the substrate solution turns colored, whereas a negative test remains colorless. The color intensity, which is proportional to virus contents, can be measured by spectrophotometer.

Materials Required

General materials

Polystyrene microtitre plates, Virus infected and non-infected samples, Virus specific antiserum and Enzyme-labeled anti-rabbit immunoglobulins (IgG).

Buffers and other chemicals: PBS = Phosphate - buffered saline, pH 7.4

NaCl	8.0g
KH2PO4	0.2g
Na2HPO4	2.9g
KCl	0.2g

Adjust pH with either NaOH or HCL and make up to 1L.

Coating or bicarbonate buffer, pH 9.6

Na2CO3	1.59g
NaHCO3	2.93g

Make up to 1L with distilled water

Sample Buffer pH 7.4

Na2HPO4	0.02M
NaH2PO4	0.02M

Adjust the pH and add 0.15M NaCl

Extraction buffer/ Conjugate buffer pH 7.4

Sample buffer + 0.05% Tween 20 + 2% polyvinl pyrrolidon (PVP) + 0.2% Egg albumin + 1M Urea.

Substrate buffer pH 9.8

Diethanolamine	97 ml
MgCl2	100mg

Adjust pH 9.8 (with 6N HCl) and make upto 1L with distilled water.

Protocol for DAS-ELISA

It was standardized by Clark and Adams (1977) and is the most common one with slight modifications. Therefore, the procedures involving DAS-ELISA with alkaline phosphatase, horse radish peroxidase and penicillinase are detailed hereunder:

Protocol: The test is performed in micro-ELISA plates as follows,

- Add 100-200 µl purified diluted r-globulin in coating buffer to each well of the microtitre plate and incubate at 37^{o}C 3 hrs.
- Wash by flooding wells with PBS- Tween-20. Leave at least 4-5 min and repeat washing five times. Later empty the plate.
- Add 100-200 µl aliquots of the test sample to well and leave for 3 hrs at 37^{o}C or overnight at 4^{o}C.
- Wash plate 5 times as earlier.
- Add 100-200µl aliquots of enzyme labelled r-globulin conjugate to each well and incubate for 3 hrs at 37^{o}C.
- Wash plate 5 times again.
- Add 100-200 µl of freshly prepared substrate (p-nitro-phenol phosphate) @ 0.6 mg/ml in substrate buffer. Incubate at room temperature for 10 min to 1 hrs.
- Stop reaction by adding 20 µl 3M NaOH to each well.
- Assess results by Visual observation or by measurement of absorbance at 405'nm.

(B)Electron microscopy

Principle

In TEM, electron beam is used as a source of illumination where extremely small negatively charged electrons given off by a heated tungsten filament can be accelerated by high voltage to produce a coherent electron beam and can be focused by an electric field. The electric field of a doughnut-shaped electromagnet surrounding the electron beam acts just like the glass lens, which focuses the light beam on the specimen. A similar electromagnet is used as the objective lens to form a highly magnified image of the specimen; and one or two electromagnetic lenses further magnify and project the image onto a fluorescent viewing screen or light sensitive sensor such as a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed in real time on a computer monitor.

Both particles and ultrathin sections are held in the electron beam on carbon-coated grids. These grids are thin enough to be transparent to the electrons. The grid is held in a movable holder for observation in the TEM.

Material required

Clean glass Petridis, Para film or dental wax, filter paper, copper grids of 400 mesh coated with carbon, Sorenen's phosphate buffer, micropipette with disposable tips, clean and dry

tweezers with very tips, negative stain for example: 2% UA in DDW, eppendorf tubes of 0.5ml capacity, wooden tooth picks and antiserum etc.

Specimen Preparation for TEM

Leaf dip method- This method was originally described by Brandis (1957) and this method is mainly used for the detection of viruses particularly for those viruses that remain in very high concentration in leaf tissues, for example *Tobacco mosaiv virus* (TMV).

Protocol

- Grind an infected leaf of about 2-3 mm diameter in phosphate buffer, 0.07M, pH 6.5.
- Place 10 µl of homogenate on parafilm or waxed slide in a wet Petriplate.
- Place carbon coated copper grids (film side downward) on the surface of the droplet, ensuring that the grid surface is wet and allow it for 2-5 minutes.
- Pick up the grid by its edge with fine forceps and wash the grid with 10-15 drops of double distilled water (DDW) to remove the sap.
- The grids are stained with 2% aqueous uranyl acetate (UA). The excess stain is immediately drained off by using Whatman filter paper.
- The grids are observed under TEM to study the virion morphology.

Immunosorbent electron microscopy (ISEM)

It is a procedure in which EM support film is first coated with a layer of antibody which serves to trap the virus preparation. ISEM is used for two main purposes as follows:

1. To trap increased number of particles on grid, by reducing the amount of host material.
2. To estimate degree of serological relationship between viruses by making use of trapping response.

Trapping: Float the carbon coated grids (carbon side down) over drop of diluted antiserum (1:1000) in phosphate buffer (pH 6.5, 0.07 M). The drop can be put on a piece of parafilm and leave for1 h at 37°C.

- Wash the grid for 10-15 n phosphate buffer.
- Drain briefly and then place them over a drop of 10µl leaf extract and leave for 30 min. at room temperature.
- Remove the grid and wash with approximately 10-15 drops of DDW.
- Stain with 2-4 drops of 2% freshly prepared uranyl acetate solution.
- Dry the grid by draining.
- Examine the grid under the TEM at 20,000 magnification.
- Count the number of particles from 10 different areas of treated and control grids.

Decoration

It is a process whereby virus particles are first individually observed to grid and grids are floated over specific antibody, layer of specific antibody halo is seen to have absorbed the stain and became electron dense. Milne and Luisoni (1977) first introduced the decoration method to the Plant Virology.

- Virus particles are adsorbed to the grid.
- Grid is carefully washed with phosphate buffer.
- Put the grid over a drop of diluted antiserum (1:50) at room temperature for 30 minutes.
- Wash the grid with 10-15 drops of DDW and stain with 2% uranyl acetate and allow to dry.

Expected results

1. In leaf dip preparation only few particles will be visible as against several particles trapping.
2. The particles will show an antibody halo on the surface of the virus particle.
3. The intensity of decoration depends on the concentration of the antibodies.

(C) Polymerase Chain Reaction detection of *Potato leafroll virus*

Extraction of Total RNA

- Use only midrib of the leaf / petiole for extraction of RNA
- RNA extraction using Total RNA extraction kit from sigma or any other kits
- Check concentration and quality of RNA by spectrophotometer.
- cDNA synthesis using first strand cDNA synthesis kit from fermentas –follow manufacturer protocol

PCR primers

Primer name	Primer sequence	Amplicon size
PLRV-CP-FP1	CTAACAGAGTTCAGCCAGTGGTTA	492bp
PLRV-CP-RP1	CGGTATCTGAAGATTTTCCATTTC	

PCR reaction mix

Water-	Make upto 20 µl
10 X Taq buffer A(with 15mM Mgcl2)	2.0 µl
2mM dNTPs-	0.5µl
10 mM Forward primer-	0.5 µl
10 mM Reverse primer-	0.5 µl
Taq DNA polymerase-	0.5 µl
cDNA-	2.0 µl

PCR cycle conditions

94 °C for 5min
94 °C for 1 min
64 °C for 1min } 35 cycles
72 °C for 1min
72 °C for 10 min.

- Check the PCR products in 1 agarose gel

(D)Rolling circle amplification-Polymerase chain reaction

Extraction of DNA

- CTAB method (or) Commercially available kit which gives good quality DNA
- Check concentration of DNA by spectrophotometer.

Rolling circle amplification

The viral DNA is amplified using the TempliPhi™ amplification kit (GE Healthcare) as follows: 80 ng of total DNA (1 or 2 µl) is added to 5 µl of the sample buffer, heated to 95 °C for 3min to denature the DNA, chilled on ice, and combined with 5 µl of reaction buffer plus 0.2 µl of enzyme mix. The reaction mixtures are incubated for 18 h at 30 °C, followed by inactivation of the enzyme at 65 °C for 10 min.

Polymerase chain reaction

PCR reaction mix

Water-	Make upto 20 µl
10 X Taq buffer A(with 15mM Mgcl2)	2.0 µl
2mM dNTPs-	0.5µl
10 mM Forward primer-	0.5 µl
10 mM Reverse primer-	0.5 µl
Taq polymerase-	0.5 µl
RCA product-	1-4.0 µl of diluted RCA product

PCR cycle conditions

94 °C for 5min
94 °C for 1 min
62 °C for 1min } 35 cycles
72 °C for 1min
72 °C for 10 min.
Hold at 4 °C

Check the PCR products in 1 to 1.5 % gel and the product size- **491bp**

(E) Realtime PCR assay

i) SYBER green method

Reaction mix

2X Maxima^{TM} SYBR Green/ROX qPCR Master Mix	10 µL
Forward Primer	300nM
Reverse Primer	300nM
DNA Template	20 ng
Water	~ to 20 µL

PCR cycle included initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 15 s and 60°C for 60 s. Record the fluorescence resulting from the binding (intercalation) of SYBR Green I into the amplicons at the end of the elongation step of every cycle. Do a melt curve analysis to evaluate the amplicons.

ii) Fluorescent probe method (Taqman probe)

Prepare the following reaction mixture, carry out PCR reactions Step one plus realtime PCR machine (Applied biosystems), observe for fluorescent signal and compare C_T values.

Reaction mixture

Milli Q water	6.5 µl
Taqman universal master mix	10.0 µl
Forward primer	0.5 µl
Reverse Primer	0.5 µl
Taqman probe	0.5 µl
Template (cDNA)	2 µl

Cycle conditions

50 °C for 2 min
95 °C for 10 min
95 °C for 15 sec
60 °C for 1 min } 40 cycles

Practical 8: Screening Techniques for Late Blight Resistance

Sanjeev Sharma
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Production of *P. infestans* inoculum

P. infestans inoculum is multiplied using tuber slice method. Tubers of any susceptible potato cultivar are surface sterilized with ethyl alcohol and cut into 1 cm thick slices using a sharp sterilized knife. Slices are placed in plastic petri dishes and inoculated with *P. infestans* by scratching the mycelium on slice surface with sterilized needle and incubated in air tight plastic boxes lines with moist foam sheet at 18±1^{o}C for a week in the dark. A thick white growth of sporangiophore with plenty of *P. infestans* sporangia would cover the tuber slices surface within 5-6 days.

Zoosporangial germination

Tuber slices containing *P. infestans* zoosporangia are gently dipped in sterilized distilled water to dislodge the zoosporangia from tuber slice surface. This zoosporangial suspension is then kept at 12±2^{o}C for 60-90 min for releasing zoospores. The zoospore suspension is calibrated to a desired level (6x10^{4} zoospores/ml) using haemocytometer for each isolates. This zoospore suspension is utilized for inoculating the leaves or tubers for screening the material for foliage/tuber resistance.

Screening for Foliage Resistance

Detach leaf method: Plants of genetic material to be screened are raised under glass house in the pots. On the 45th days after planting, fourth leaves from the top are plucked from each clone and placed in plastic trays on perforated plastic separators (Umaerus and Lihnell, 1976). Five leaves of each clone are collected and placed in the tray. Leaves of a susceptible variety are kept to serve as control. These leaves are inoculated with 20 ul zoospore suspension using an autopipette. *P. infestans* zoospores suspension is prepared and calibrated as described above. The trays are incubated at 18±1^{o}C for 5 days. High humidity in the trays is maintained with the help of moist foam sheet. Observations are recorded on the 6th day of inoculation measuring the leaf area infected. The accessions are categorized into highly resistant, resistant, moderately resistant and susceptible on the basis of following scale.

Lesion area (cm2)	Grade
Up to 1.0	Highly resistant (HR)
1.1 to 2.5	Resistant (R)
2.5 to 6.0	Moderately resistant (MR)
>6.0	Susceptible (S)

Whole plant method: Whole plant screening is done in the screening chambers having controlled temperature, humidity and fluorescent light. Plants of genetic material to be tested along with a susceptible cultivar are raised in pots in the glass house. Forty days after planting, the pots are transferred to the screening chamber. Close the chamber and put on

the humidifier for 1-2 hrs. so that leaf surface is completely wet. Prepare zoospore suspension of most complex races and spray the plants with inoculums. Keep the humidifier off for a night and then incubate the plants at 18±1^{o}C with 90% humidity. After 6 days, score the plants using the following scale:

% Area infected	Score
Trace of infection	9
10	8
11-25	7
26-40	6
41-60	5
61-70	4
71-80	3
81-90	2
Collapsed	1

(Malcolmson, 1976)

Tuber resistance

Tuber slice method: In this method 3 tubers of each accessions to be tested, are surface sterilized with ethyl alcohol and cut into 1 cm thick slices aseptically by a sterilized knife. One slice from each tuber is kept in a sterilized petri dish. Three slices of a susceptible variety are also placed in a petri dish to serve as control. Tuber slices are inoculated with zoospore suspension using filter paper discs (0.3 cm^2) dipped in the zoospore suspension prepared and concentration adjusted as per methods given above. The petri dishes are kept in plastic trays lined with moist foam sheet and incubated at 18±1^{o}C for 5 days in dark. Length and breadth of the lesions are measured and lesion area calculated as per following formula:

Lesion area = π/4 x ab, where a= length and b= breadth of the lesion

The accessions are categorised in 4 groups as per scale given under foliage resistance by detach leaf method.

Whole tuber test: After 7-16 days of harvest, give superficial injury to the tubers. After 24 hrs of injury place the tubers on wet filter paper in seed boxes. Incubate the tuber (i) By spraying the zoospore suspension of *P. infestans* (Lapwood, 1967 and Bjor (1987); (ii) By placing the soaked filter paper discs on the lanticels or eyes (Lacey, 1967). After inoculation, close the box to avoid drying and incubate at 18±1^{o}C. After two weeks score the tubers individually on the following scale (Bjor, 1987).

Score	0	1	2	3	4	5
% of the surface with symptoms	0	5	5-10	10-25	25-30	>50

Field Screening: Field screening is generally done on a large population of plants. For this purpose disease should be recorded in each cultivar right from its appearance till the maximum build up of the disease at regular (weekly) intervals. This data may be used to calculate the Vander Plank 'r' (apparent infection ratio Van der Plank, 1963). AUDPC (Area

under disease progress curve, (Wilcoxon, 1975, Shaner and Finney, 1977) or mean values (Simmonds and Wastie, 1987). However, out of them AUDPC has been considered more reliable for categorizing the cultivars according to their resistance grades.

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Practical 9: Isolation, identification and inoculation techniques for *Ralstoniasolanacearum* and *Streptomyces* species

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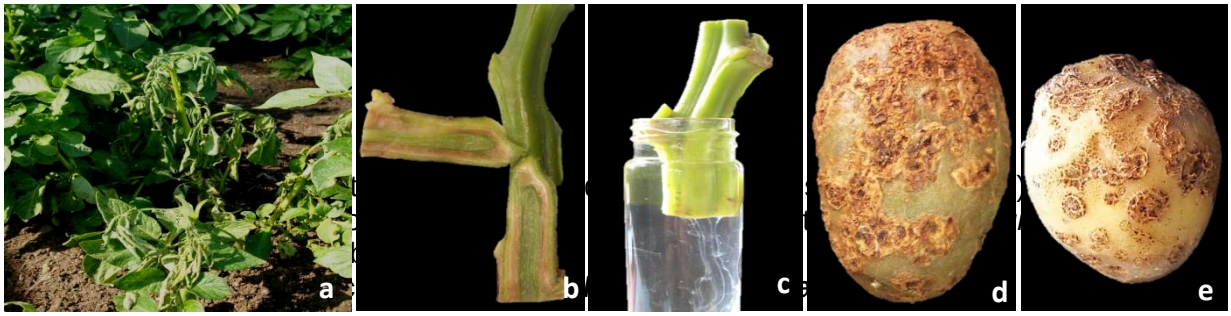
Collection of bacterial wilt/ scab infected plants/ tubers

Potato plants/ tubers infected with typical symptoms of bacterial wilt/ common scab disease (Fig 1, 2a)are obtained from various potato growing regions. At each collection site, tubers (brown rot/scab infected) or stem pieces (6 to 10 cm) of wilt infected plants showing typical brown rot/scab/wilt symptoms are collected, labelled properly and brought to laboratory immediately for isolations.



Potato tubers/ stem pieces are washed thoroughly in running tap water for 5 to 7 minutes to remove soil adhered to their surfaces and air dried. The samples (stem pieces and tubers) are then surface disinfected with 70% ethanol, peeled, subsampled and macerated in sterile distilled water. Macerates are streaked on Kelman'striphenyltetrazolium chloride (TZC) agar medium (Kelman, 1954) with slight modification (Peptone, 10 g litre^{-1}; glucose/ glycerol, 2.5 g/ 5 ml litter $^{-1}$; Casamino Acids, 1 g litre^{-1}; yeast extract, 1 g litre^{-1}; agar, 15 g litre^{-1}; TZC, 50 mg litre^{-1}; pH 7.0-7.1). Plates are incubated at 28±2 ^{o}C for 48 to 72 hrs. This medium is useful for distinguishing *R. solanacearum* among other bacteria during isolation, and for distinguishing virulent (wild type) colonies from avirulent mutant ones during purification of cultures. Bacterial colonies developing the typical irregular mucoid colonies are again transferred to fresh TZC medium for further purification. Well separated typical wild type *R. solanacerarum* colonies (Fig. 2b) are further transferred to medium modified by exclusion of TZC for multiplication of inoculum free of formazan pigment (which is slightly bacteriostatic).Two loop full of bacterial culture are then transferred in 2 ml of double distilled sterile water and the cultures stored at 20±2 ^{o}C.

TZC stock solution: One gram of 2,3,5 triphenyltetrazolium chloride is dissolved in 100 ml of distilled water, placed in a light proof capped bottle, autoclaved for only 8 minutes or sterilized by filtration and stored refrigerated. To each 200 ml of melted, somewhat cooled Kelman's basal medium i.e. Casamino Acid Peptone Glucose/Glycerol Agar (CPG) medium, one ml of TZC stock solution is added to give a final concentration of 50 mg litre^{-1}

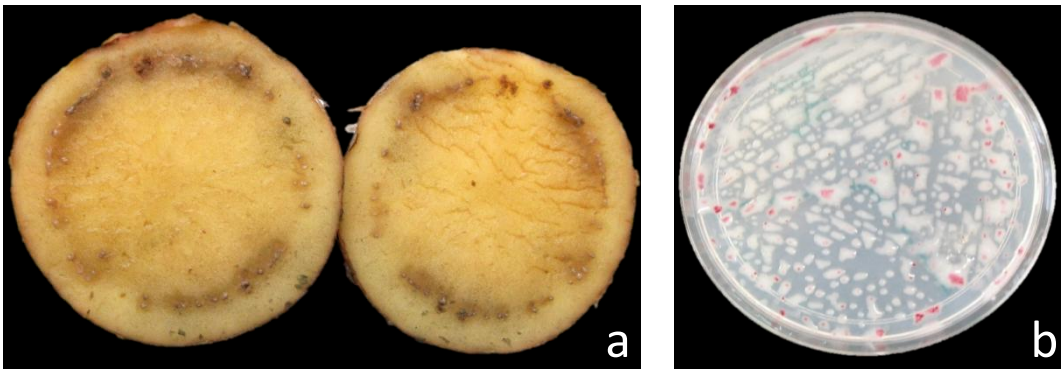


Fig 2: Grey-brown discoloration of vascular tissues and bacterial ooze in potato tuber infected by *R. solanacearum* (a); typical *R. solnacearum* colonies on TZC agar medium (b)

Characterization of *R. solanacearum* isolates into biovars

Biovars of *R. solanacearum* strains are determined by standard procedure (Hayward 1964). The following basal medium is used for biovar identification: $NH_4H_2PO_4$, 1.0 g; KCl,0.2 g; $MgSO_4.7H_2O$, 0.2 g; Peptone, 1.0 g; 1% (wv^{-1}) aqueous solution of bromothymol blue, 0.3 ml; agar, 1.5 g; distilled water, 1litre.

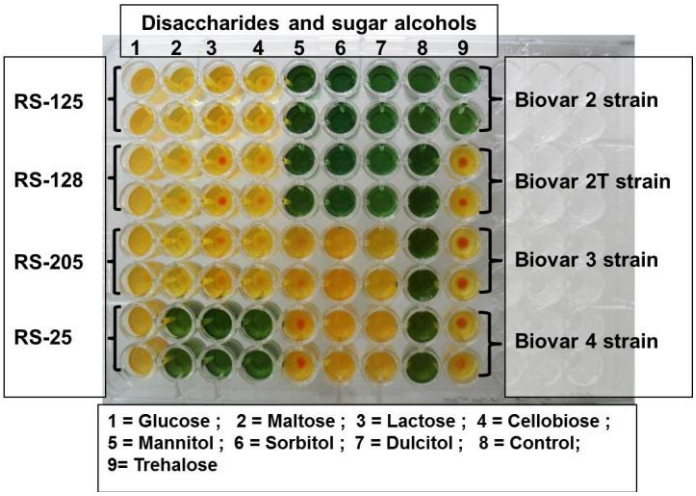


Fig 3: Micro-titre plates showing results of biovar test with *R. solanacearum* strains

The pH of the medium is adjusted to 7.1 with 40% (wv^{-1}) NaOH solution before addition of the agar. Five millilitres of a 10% (wv^{-1}) pre-sterilized solution of the sugars (lactose, maltose, cellobiose) and sugar alcohols (mannitol, sorbitol and dulcitol) are added to 45 ml of molten cooled basal medium separately. 200µl of these media were then dispensed into each well of 96-well micro-titre plates. Hayward's medium without a carbon source and un-inoculated wells serves as control. Each well is inoculated with 3 µl of a 2 x 10^9 CFU ml^{-1} cell suspension prepared from overnight CPG broth culture. The cultures are incubated at 28±1 ^oC and examined at 3, 7 and 14 days for change of pH (yellow colour; Fig 3). Positive cultures change the culture medium from green to yellow. Each test is replicated three times. The biovar classification is done as described in Table 1.

Isolation of genomic DNA

Bacterial growth from a well separated colony on TZC agar is used to inoculate 1.5 ml of CPG broth in 2.0 ml Eppendorf tubes. The cultures are grown at 28^{o}C for 48 hours with vigorous shaking. Total genomic DNA is extracted by rapid method as described by Chen and Kuo (1993). 1.5 ml of saturated culture is harvested with centrifugation for 3 min at 12,000 rpm. The cell pellet is re-suspended and lysed in 300 µl

Table 1: Differentiation of *R. solanacearum* strains into biovarsbased on the ability to utilize disaccharides and oxidize hexose alcohols producing acid when positive (+)

Biochemical Test	**Biovar**				
	1	2	3	4	5
Utilization of					
Cellobiose	-	+	+	-	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
Oxidation of					
Dulcitol	-	-	+	+	-
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-

of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) by vigorous pipetting. To remove most proteins and cell debris, 100 µl of 5M NaCl solution is added andmixed well, and then the viscous mixture is centrifuged at 12,000 rpm for 10 min at 4°C. After transferring the clear supernatant into a new vial, an equal volume of chloroform is added, and the tube is gently inverted at least 50 times when a milky solution is completely formed. Following centrifugation at 12,000 rpm for 3 min, the extracted supernatant is transferred to another vial and the DNA is precipitated with 100% ethanol, washed twice with 70% ethanol, dried in speed-vacuum, and re-dissolved in 50 µl of TE buffer. If required, RNA could be removed by adding RNAase in the lysis step for 30 min at 37°C.

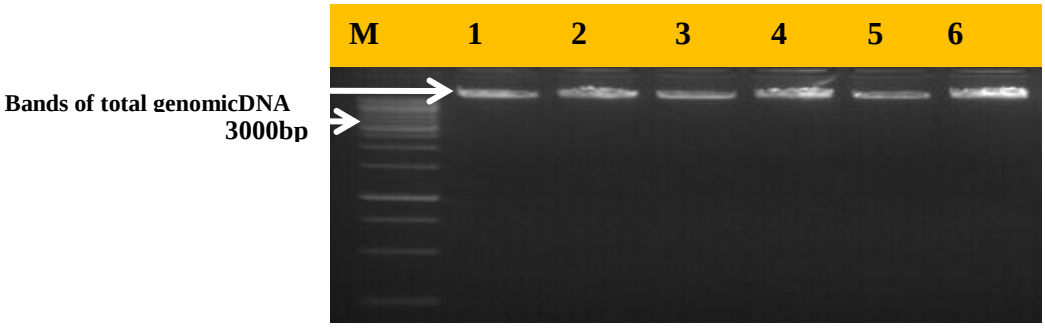


Fig 4: Bands of total genomic DNA

Table 2: Stock solutions for preparation of lysis buffer for DNA extraction

Sr. No.	Name of stock solution	Required concentration	Volume (µl) required for 10 samples @ 300µl lysis buffer per sample
1.	Tris-acetate (1.0M)	40mM	120
2.	Sodium acetate (1.0M)	20mM	60
3.	EDTA (0.1M)	1mM	30
4.	SDS (10%)	1%	300
5.	Distilled water	**-**	2490
	Total		3000
6.	NaCl (5.0M)	100µl (added after lysis to remove most proteins and cell debris)	

Purity check and quantification of DNA

For purity check and quantification, 5µl template DNA is loaded with 2µl of 6X loading dye on 1.0% agarose gel in Tris–Acetate EDTA buffer with 0.04 µl ml^{-1} of ethidium bromide at 100V for 1hr and visualized under ultraviolet (UV) light. DNA samples showing intact bands are selected for PCR amplification.

Characterization of *R. solanacearum* isolates into phylotypes

Phylotype identification of each isolate is done as described (Fegan and Prior 2005; Prior and Fegan 2005). Multiplex PCR (Pmx-PCR) is carried out in 25 µl final volume of reaction mixture, containing 1×Taq Master Mix (PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, 50 mMKCl, 10 mM Tris-HCl and 1.25U of Taq DNA polymerase.), 6 pmoles of the primers Nmult:21:1F, Nmult:21:2F, Nmult:22:InF, 18 pmoles of the primer Nmult:23:AF and 4 pmoles of the primers 759 and 760 (Opina*et al*., 1997). The following cycling programme is used in a thermal cycler: 96°C for 5 min and then cycled through 30 cycles of 94°C for 15 s, 59°Cfor 30 s and 72°C for 30 s, followed by a final extension period of 10 min at 72°C.

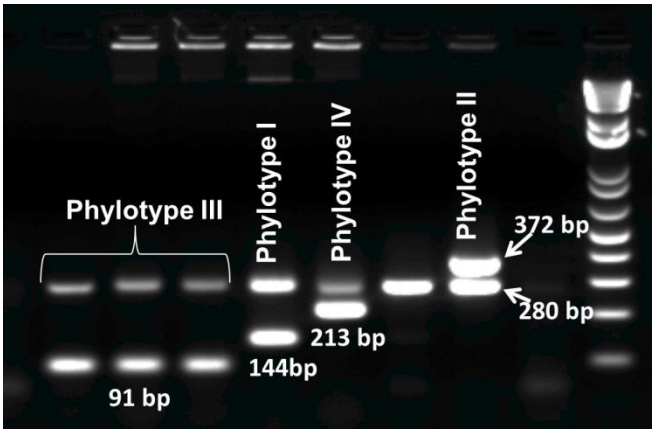


Fig 5: Pmx-PCR using phylotype-specific primers showing PCR products of 280bp (i.e. *R. solanacearum*) amplicons for all isolates, 91bp (Phylotype III, lane 1-3), 144bp (phylotype I, lane 4), 213bp (Phylotype IV, lane 5) and 372bp (phylotype II, lane 7) amplicons

A 5 µl aliquot of each amplified PCR product is then subject to electrophoresis on 1% agarose gel, stained with ethidium bromide and visualized on a UV-trans-illuminator. This Pmx-PCR produces the following phylotype-specific PCR products: a 144-bp amplicon from phylotype I strains; a 372- bpamplicon fromphylotype II strains; a 91-bp amplicon from phylotype III strains; and a 213-bp amplicon from phylotype IV strains (Fig 5) and *R. solanacearum* specific PCR product of 280-bp.

Table 3: List of primers used for multiplex PCR

S No.	Primer	Primer sequence	Expected band size	Remarks
Primers for multiplex PCR				
1.	Nmult:21:1F	5′-CGTTGATGAGGCGCGCAATTT-3′	144 bp	Phylotype I (Asiaticum)
2.	Nmult:21:2F	5′-AAGTTA TGGACGGTGGAAGTC-3′	372 bp	Phylotype II (Americanum)
3.	Nmult:22:InF	5′-ATTGCCAAGACGAGAGAAGTA-3	213 bp	Phylotype IV (Tropical)
4.	Nmult:23:AF	5′-ATTACGAGAGCAATC GAAAGATT-3′	91 bp	Phylotype II (African)
5.	Nmult:22:RR	5′-TCGCTTGACCCTATAACGAGTA-3		Amorce reverse unique
6.	759R	5'-GTCGCCGTCAACTCACTTTCC-3'	280 bp	Universal *R. solanacearum* specific primers
7.	760F	5'-GTCGCCGTCAGCAATGCGGAATCG-3'		

Inoculation

Ralstoniasolanacearum isolates are inoculated to susceptible cultivars of potato (cv. K. Jyoti), tomato (cv. Pusa Rubi), brinjal (Pusa Purple Long) etc. for pathogenicity test or screening of varieties. The pathogenicity test or screening of varieties is conducted on potted plants (healthy plants grown in sterilized soil) in glass/ green house (temp. 20-30^{o}C) using 3-5 replicates for each isolate/ cultivar. A bacterial suspension of an isolate is prepared in distilled sterilized water using 48 h old growth on casamino acid peptone glucose (CPG) agar medium. The plants are inoculated by stem stab method when they are 15-20 cm tall. Usually 3rd of 4th axil bud from the top is inoculated by injecting/ placing a droplet of suspension (15-20 µl) on injury. Plants stabbed with sterilized distilled water serve as control. The inoculated plants are incubated at 20-30^{o}C temperature and observed for wilt appearance till 40 days of inoculation.

Isolation and maintenance of *Streptomyces* isolates

Potato tubers are washed thoroughly in running tap water for 5 to 7 minutes to remove soil adhered to their surface and air dried. The tubers are surface disinfected with 70% ethanol and small tuber pieces containing scab lesions are cut out and surface disinfected for one minute in 1% sodium hypochlorite solution. After several rinses in sterile distilled water, the tissues (about 1g) are ground in a pestle mortar in 5 ml of sterile water. Serial dilutions are plated onto starch casein agar (SCA) medium (Soluble starch 10g, casein 0.3g, potassium

nitrate (KNO3) 2g, di-potassium hydrogen ortho-phosphate (K2HPO4) 2g, magnesium sulphate (MgSO4*7H2O) 0.05g, calcium carbonate (CaCO3) 0.02g, ferrous sulphate (FeSO4*7H2O) 0.01g, agar 20g, distilled water to make the final volume 1 litre, pH 7.2). Plates are incubated at 28±1 °C, and individual characteristic powdery (mycelial) bacterial colonies are picked and serially transferred until a pure culture is obtained. Potato dextrose agar medium is used for routine sub culturing of *Streptomyces* strains. Strains are maintained as spore suspension in 20% glycerol at –20°C.

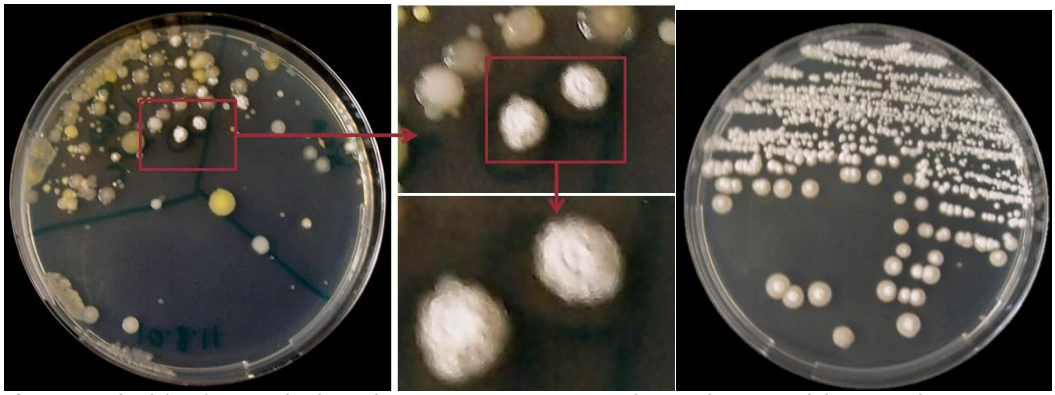


Fig 6: Typical leathery colonies of *Streptomyces* on starch casein agar giving powdery appearance and pure culture of *Streptomyces* (right)

Extraction of genomic DNA

Bacterial growth from a well purified culture on SCA medium is used to inoculate 1.5 ml of starch casein broth in 2.0 ml Eppendorf tubes. The cultures are grown at 28±1^{o}C for 48-72 hours with vigorous shaking (200 rpm). Genomic DNA is isolated by a versatile quick-prep method for genomic DNA of Gram-positive bacteria with some modifications. Mycelia (1–2 ml) grown in a starch casein broth shake culture are centrifuged, rinsed with TE and re-suspended in 0.4 ml SET buffer (75 mMNaCl, 25 mM EDTA, 20 mM Tris, pH 7.5) (Table 4). Lysozyme is added to a concentration of 1 mg ml^{-1} and incubated at 37^{o}C for 0.5–1 hour. Then 0.1 volume 10% SDS and 0.5 mg Proteinase K ml^{-1} are added and incubated at 55^{o}C with occasional inversion for 2 hours. One-third volume 5 M NaCl and 1 volume chloroform are added and incubated at room temperature for 0.5 hour with frequent inversion. The mixture is centrifuged at 4500 g for 15 minutes and the aqueous phase is transferred to a new tube using a blunt-ended pipette tip. Chromosomal DNA was precipitated by the addition of one volume 2-propanol with gentle inversion, washed twice with 70% ethanol, dried in speed-vacuum, and re-dissolved in 100 µl of distilled water. The dissolved DNA is treated with 20 mg RNaseA ml^{-1} at 37^{o}C for 1 hour. Samples are extracted in the same volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 2.5 volume cold ethanol and 0.1 volumes 3 M sodium acetate. The pellets are washed with 70% ethanol, dried and dissolved in 50 µl TE or distilled water.

Table 4: Stock solutions used for preparation of lysis buffer for DNA extraction

Sr. No.	Name of stock solution	Required concentration	Volume required for 10 ml of SET buffer
1.	NaCl (5.0M)	75 mM	150 µl
2.	EDTA (0.1M)	25 mM	2500 µl
3.	Tris (2.0 M)	20 mM	100 µl
4.	Distilled water	**-**	7250 µl
	Total		10,000 µl

Purity check and quantification of DNA

For purity check and quantification, 5µl template DNA was loaded with 2µl of 6X loading dye on 1.0% agarose gel in Tris–Acetate EDTA buffer with 0.04 µl ml^{-1} of ethidium bromide at 100V for 1hr and visualized under ultraviolet (UV) light. DNA samples showing intact bands were selected for PCR amplification.

Sequencing of the 16S rRNA gene

The 16S rRNA gene was amplified from DNA of *Streptomyces* isolates by PCR using the primers 16S-1F (5'-CATTCACGGAGAGTTTGATCC-3') and 16S-1R (5'AGAAAGGAGGT GATCCAGCC-3') (Takeuchi *et al*., 1996). Amplification is carried out in 50 µl of reaction volume containing 10 mM Tris HCl (pH 9.0), 50 mMKCl, 0.1% Triton X100, 1.0 to 2.0 mMMgCl , 250 µM each dNTP, 10 pmol each primer, *Taq*DNA polymerase at 5 U/µl, 20 to 40ng DNA template, and MilliQ water to bring the volume to 50 µl. The thermal cycler is set to the following conditions: initial denaturation at 95°C for 3 min; followed by 40 cycles of denaturing at 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min; and ending with a 4°C hold.

PCR products of 1,500 bp(expected size) are electrophoreses in 1% agarose gel in Tris–Acetate EDTA buffer with 0.5 µl ml^{-1} of ethidium bromide at 105V for 1 hour and visualised and photographed under ultraviolet (UV) light.

For sequencing of PCR products, amplified products are further purified using QiagenMinielute PCR purification kit following manufacturer's guidelines. The concentration of each product is estimated by gel electrophoresis with a low DNA mass ladder(Fermentas 1 Kb) and diluted with ultra-pure water to give a final concentration of 10-20 ng $µl^{-1}$ for sequencing. PCR products are then cycle sequenced using forward primer in thermal cycler (Gen-AmpR PCR System 9700 of M/S Applied Biosystem). The cycle sequenced products are further purified. DNA samples then sequenced by an Applied Biosystem (Foster City, CA, USA) Model, AB310 Genetic Analyzer).

The 16S rRNA gene sequences analysis are performed using off-line software CLUSTAL X, Org. CLUSTAL W or X is a multiple sequence alignment program which calculates the best match for the selected sequences, and lines them up so that similarities and differences can be seen. Sequence data of all the 48 strains are manually edited and compared with the sequences from NCBI through BLAST programme. Sequences are aligned with the

reference sequences from NCBI and phylogenetic analysis is done using CLUSTAL X software (Thompson *et al.*, 1994).

Pathogenicity test

Pathogenicity of *Streptomyces* strains is examined through Tuber Slice Assay as described by Hao*et al*., (2009).Potato tubers (cv. KufriChandermukhi) free from surface diseases are washed and sterilized for 1 min with 10% bleach (0.0625% NaClO). A tissue disk (20 mm in diameter by 7 mm in height) is bored from the tuber and placed on moist filter paper in a petri plate (100mmx 15mm). Pathogenicity of *Streptomyces* strains is examined as described by Loria*et al*. (1995). *Streptomyces* sp. inoculum is prepared from the cultures grown on oatmeal agar medium for 5 to 7 days. Agar plugs from *Streptomyces* cultures (3 mm-diameters) are placed at the centre of the potato tuber disks replicating thrice. Agar plugs from oat meal agar placed on potato tuber slices served as control. The disks were incubated in a moist closed container at 28±1°C in the dark and observed for necrosis of tuber slices.

Fig 7: Pathogenicity assay on tuber slices of cv. KufriChandermukhi: necrotic halos produced by the plugs from 5 days old oat meal agar cultures of *Streptomyces* strains, slices without halo are control.