Genetic improvement of Citrus for disease resistance

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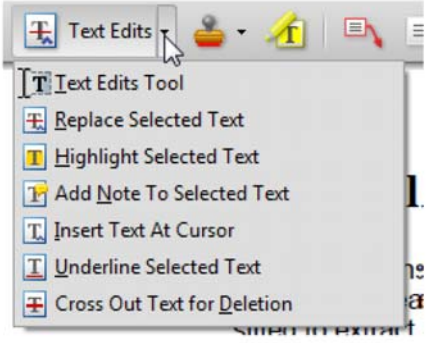

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Genetic improvement of *citrus* for disease resistance

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AQ2 Progress in the genetic improvement of *citrus* species was reviewed. Tools used for the genetic improvement of *citrus* were categorised as conventional (introduction, selection and hybridisation) and non-conventional methods (mutation, somatic cell hybridisation and genetic engineering) of improvement. Genes linked with the disease resistance were characterised and tagged through molecular marker techniques such as Sequenced Characterised Amplified Region and Cleaved Amplified Polymorphic Sequences. Disease resistance genes showed both monogenic and polygenic inheritance. Conventional methods for disease resistance improvement of *citrus* were bottleneck due to inadequate and lengthy breeding procedures. However, non-conventional methods, such as mutation breeding and protoplast fusion, have been routinely utilised for the production of disease resistant germplasm while novel genes from variable sources were used to transform *citrus* species to induce resistance against diseases. These non-conventional techniques have been shown to overcome the disadvantages of conventional breeding procedures and could be regarded as rapid methods of genetic improvement as well as helpful to overcome the interspecies barrier.

Keywords: breeding procedures; germplasm; resistance; marker

Introduction

Genetic improvement implies the creation of new genetic recombinants that have high yield potential, quality and sustainable yield due to resistance to the biotic and abiotic stresses (Rauf et al. 2010). Breeder may utilise several breeding tools to produce genetic recombinants i.e. crossing two genetically diverse genotypes through conventional means, mutation breeding to produce new alleles and advance biotechnology tool to exploit new genetic variation through somaclonal variations, transformation, *in vitro* pollination between species, genera and protoplast fusion. Once new genetic recombinants are created, the next step is to screen and select the transgressive segregant in later generations for trait of interest. The trait of interest is dictated by the breeding objectives of particular species. In *citrus*, the breeding objective may be to screen the new recombinant for traits related to yield and quality, resistance of abiotic stress such as salinity, alkalinity and biotic stresses including insects and diseases (Machado et al. 2011). The conventional breeding methods can effectively produce new genetic recombinant by exploiting intra species variation. It is at least difficult to cross the boundaries of species or genera for introgression through conventional methods i.e. hybridisation. Therefore, breeders have to rely on the non-conventional tool such as mutation breeding, transformation and

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somaclonal variation to create new genetic variation. Furthermore, selection of desirable recombinant is also cumbersome through conventional field selection methods due to substantial influence of environment and genotype × environment on low heritable traits. Advances in molecular genetics, genomics and biotechnology also led the *citrus* breeders to select the difficult and expensive traits.

Breeding for the disease resistance requires the screening and evaluation of elite germplasm including commercial scions and root stocks against diseases, to determine the magnitude of genetic variation, screening the wild germplasm and related species if adequate genetic variation in elite germplasm is absent, introgression between and within species, selection of suitable transgressive segregant for disease resistance and other traits, release of scion and root stock for commercial cultivation. This review will focus on the comprehensive breeding approaches for the *citrus* genetic improvement for disease resistance.

Genetic diversity within *citrus* germplasm

Presence of adequate genetic diversity within germplasm is pre-requisite for sustainable yield and to increase the buffering capacity of species against the disease epidemic (Rauf et al. 2010, 2012). Genetic diversity within field tends to become narrow due to breeder selection for few economically important traits through targeted breeding, inbreeding and apomictic seedling. Therefore, it is important to evaluate or characterise the germplasm, to determine the extent of genetic diversity within particular species between *citrus* species, to utilise genetically diverse parent in crossing and to release diverse recombinants.

Large collections of germplasm are characterised for various morphological traits and phenotypic diversity is estimated from the combined analyses of these traits (Rauf et al. 2012). However, morphological traits often show low polymorphism, require laborious field characterisation. Advent of molecular markers led the estimation of molecular-based diversity (Rauf et al. 2010). Molecular marker-based diversity is the rapid way of germplasm analyses, shows high level of polymorphism and wide genomic distribution (Iqbal et al. 2013). Many of the molecular markers show co-dominant inheritance which increased their usefulness. These markers could also explain the basis of genetic divergence within *citrus* germplasm. The review of the studies showed that diverse types of molecular markers have been utilised to estimate genetic divergence (Table 1). In comparison among these molecular markers, simple sequence repeats (SSR) showed the highest level polymorphism in *citrus* (Biswas et al. 2011). Jannati et al. (2009) showed that all SSR markers were highly polymorphic with average 8.27 alleles per locus while Yong et al. (2006) showed 9.85 alleles per locus for SSR markers. Studies showed that molecular markers were utilised to determine genetic distance between various *citrus* species to show degree of relatedness. The following conclusions were made from these studies: (i) There are sister species within *citrus* germplasm, (ii) Many *citrus* species originated due to hybridisation between closely related species, (iii) Accessions belonging to single species could be grouped into few distinct types, (iv) Molecular diversity is lesser than phenotypic variation observed in the germplasm, (vii) Selection for bud mutations is the cause of phenotypic variation, (viii) Heterozygosity at molecular level is indicative of rare sexual recombination followed by self-pollination. In order to manipulate heterosis or transgressive segregation, hybridisation between the distinct types may be carried out.

Table 1. Summary of various studies showing genetic diversity within *citrus* germplasm.

Cultivar species	Marker	Diversity	References
Trifoliate orange [*Poncirus trifoliata*	38 probe-enzyme (RFLPs) and (ISSR) 11 primers	Low genetic divergence within *citrus* germplasm. "Monoembryonic" and "Simmons" were most diverse accessions and had zygotic origin	Fang et al. (1997)
31 acid *citrus* species and cultivars	60 RAPD**	Sour oranges showed higher diversity than "Yuzu" and its relatives	Abkenar and Isshiki (2003)
370 sexually derived *Citrus* accessions	24 SSR	Most of the species originated from spontaneous hybridisation. Fortunella included in *citrus* genus and poncirus was sister genus	Barkley et al. (2006)
122 accessions of pummelled, *citrus* grandis Osbeck	31 SSR	SSR-based molecular diversity clustered 122 accessions into 7 groups	Yong et al. (2006)
13 species and 5 hybrid	40 RAPD primers	Jatti-Khatti was genetically diverse. Molecular diversity coefficient (0.40). Jafa and Blood red showed the highest similarity index	Baig et al. (2009)
Citrus species	(AFLP), (S-SAP), (SAMPL) and (SSR) markers	SSR marker showed the highest molecular diversity than other molecular marker system	Biswas et al. (2011)
201 local accessions belonging to four facultative apomictic species	20 nuSSR markers, 4 mt markers	Tunisian root stock was genetically diverse due to allelic diversity. Allelic diversity arises due to mutations	Snoussi et al. (2012)

**Restriction Fragment Length Polymorphism (RFLP); Randomly Amplified Polymorhic DNA (RAPD); Sequence-Specific Amplified Polymorphism (SSAP); Selectively Amplified Microsatellite Polymorphic Loci (SAMPL), simple sequence repeats (SSR), Amplified fragment length polymorphism (AFLP).

Genetic variation within *cirus* germplasm for disease resistance

First step to start any breeding programme for the introgression of diseases resistance is to screen the elite, cultivars and to determine the source of resistance and magnitude of genetic variation within germplasm for disease resistance. If the desired resistance is absent in the elite cultivars, than obsolete cultivar or related species are exploited for the source of resistance. However, the introgression from related and wild species is difficult due to ploidy, genetical and phenological divergence among the species. Furthermore, breeder has to combine high yield and quality with disease resistance to directly utilise the genetic recombinant as cultivar which further makes introgression more difficult, since alien introgressions often show linkage drag i.e. incorporation of undesirable genes that reduces the yield and quality along with gene of interest.

In order to evaluate the germplasm, screening is done by artificially or naturally inoculating the germplasm with the pathogen. Screening for particular diseases has its own requirements and methods (Table 2). After inoculation, host plants are evaluated and characterised on the basis of severity disease symptoms. Phenotypic characterisation

Table 2. Screening methods against economically important diseases.

Disease	Screening Method	References
Citrus bacterial spot	Young leaves were punctured with 10–26 guage syringe needle and 10 µL of bacterial suspension containing (10^8 cfu/ml) was placed over wound and incubated under florescent light of 14 h. Disease rating (0–3) was carried out after 7 days	Graham and Gottwald (1990)
Citrus canker *Xanthomonas axonopodis* pv. citri	Disease severity was calculated by dividing the no. of infected leaves over total no. of leavesThe level of resistance or susceptibility was determined by 0–9 grades	Atiq et al. (2007)
Citrus greening	6–8 month old seedlings were with tow-three bud wood inoculated with Ca. L. *asiaticus*'-infected budwood, confirmed for the pathogen with PCR. The plant were kept in secure green house with temperature 28–32 °C.	Folimonova et al. (2009)
Citrus canker (*Xanthomonas citri* subsp. *citri*, *Xcc*) and *citrus* bacterial spot (*Xanthomonas alfalfae* subsp. *citrumelonis*, *Xac*)	Bacterial inocula of known concentration were injected into immature leaves of resistant and susceptible types. Inoclulated leaves were placed on agar (0.5%) plates at 28 °C under 12 h photo-period. The resistant type kumquat showed hypersensitive reaction after 3 dpi while susceptible type showed water soaked area developed after 3 dpi and canker lesion developed after 7–14 dpi	Francis et al. (2010)

of the germplasm is difficult and confusing due to delayed appearance of symptoms in different genotype. PCR traditional or q-PCR is an effective way to detect the pathogen at low concentration or multiplication within host species after specific period of infection. Molecular techniques have greatly increased the progress of the resistance breeding or the disease epidemiology (Iqbal et al. 2013).

In vitro screening methods could also be utilised for the mass selection of the germplasm against particular disease. The plant organ (ex-plant) or seedlings are cultured on the tissue culture media under aseptic condition after inoculation with pathogen. The ex-plant is evaluated for various traits i.e. necrotic area of explant, callus growth rate, callus weight gain, proportion of dead part of callus, percentage of somatic embryos, regeneration of embryos into plant let, etc. (ŠVábová & Lebeda 2005). However, due to over growth of pathogen under optimum condition, pathogen killed the ex-plants but successful experiments have been conducted with live pathogen (Bowman 1996). Bowman (1996) inoculated the *Phytophthora parasitica* along with seedling of *citrus* (*Poncirus trifoliata*) grown under *in vitro* condition. Resistant and susceptible genotypes were identified from the discolouration of stem. The germplasm was characterised into resistant, intermediate and susceptible. Bas and Koc (2006) screened the callus of Kutdiken lemon against the *Phoma tracheiphila*, and identified one resistant callus which was used to regenerate plant line "Kutdiken lemon 20b".

Alternatively, deleterious phyto-chemicals or patho-toxin may be used for the *in vitro* screening of the germplasm (Buitatti & Ingram 1991). Phyto-toxin involved in the pathogenesis is called patho-toxin. For *in vitro* screening, non-host toxins that interfere with the plant defence responses have been shown to be useful.

Genetics of disease resistance

There are two types of traits regarding the number of genes and effects of environment. Polygenic traits are controlled by numerous genes and show considerable effect of environment. Inheritance of such type of traits is complex and show continuous variation. Genotypes show considerable genotype × environment (G × E) for these types of traits when studied under multiple environment. Gene controlling a particular polygenic trait also shows various type of interaction such as additive (cumulative effect of minor genes), dominance (intra allelic interactions) and epistasis (inter allelic interaction). Selection for these traits is also difficult due to effect of environment as well due to dominance and espistasis. Special Quantitative trait loci (QTL) mapping techniques have been devised to study these traits. These traits cannot be introgressed through novel techniques such as transgenic breeding and require significant efforts of plant breeder for improvement.

On the other hand, mono or oligogenic traits are under control of single or few genes. These traits do not show the effect of environment, simply inherited and show discrete variation. Most of resistances are under the control of this type of traits. Therefore, resistance can be readily introgressed into germplasm through both conventional and non-conventional techniques. However, such type of resistance is easily broken down due to continuous evolution of new pathogen having ability to defeat the resistant genes. Special molecular markers have been developed to facilitate the differentiation of resistant and susceptible plant types and to tag the region of genome harbouring the genes related to resistance. Summary of these studies are presented in Table 3 which

Table 3. Summary of the studies showing cloning and characterisation of genes related to disease resistance in *citrus*.

Host	Marker	Host/candidate gene results	References
Poncirus trifoliata	Eight dominant and two co-dominant SCAR markers	Co-dominant SCAR markers identified two genomic region linked with CTV resistance	Deng et al. (1997)
Poncirus trifoliate *Citrus medica*	7 RAPD BSA analysis	RFLP used to map resistance gene linked to CTV	Mestre et al. (1997)
Poncirus trifoliata	CAPS	3 DNA fragment associated with *Citrus* tristeza virus resistant gene (CTV) and one nematode resistance gene (Tyr1)	Deng et al. (2000)
C. paradisi × *Poncirus trifoliata*	SCAR	Poly genic control of resistance for nematodes. QTL analysis explained 53.6% phenotypic variance	Ling et al. (2000)
Sunki mandarin *Citrus sunki* (susceptible) × *Poncirus trifoliata*	–	Inheritance was polygenic control, low heritability ($h^2 = 18.7\%$) and two QTL linked to the *Phytophthora* gummosis were identified	Siviero et al. (2006)
Poncirus trifoliata	282-kb region was mapped	Harbouring disease resistant gene with seven member for CTV	Yang et al. 2003

Sequence characterised region (SCAR), BSA (Bulk segregant analysis), Characterised amplified polymorphic regions (CAPS), Randomly amplified polymorphic region (RAPD), *Citrus* triteza virus (CTV), QTL.

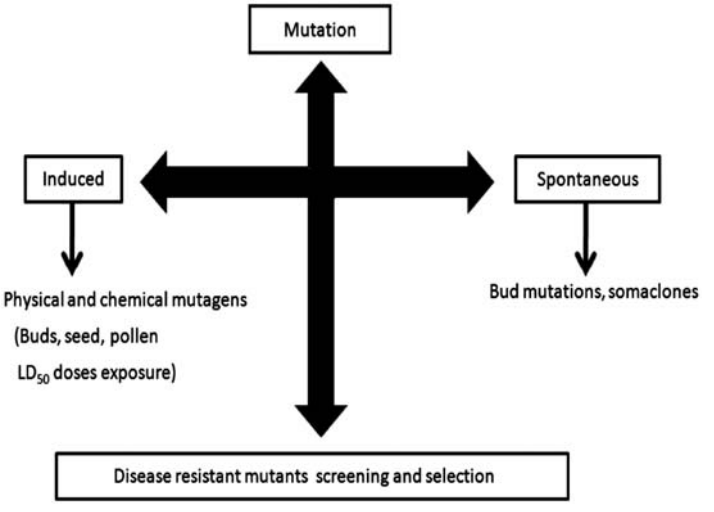

Figure 1. Schematic view for selection of disease resistant mutants.

showed that genes related to disease resistance were ranged from monogenic to polygenic type of inheritance.

Mutation breeding

Citrus genetic improvement is itself restricted due to various factors such as long juvenility period, poly-embryony, self- and cross incompatibility and apomixes. Therefore, mutation breeding hold great promise for the expansion of genetic variability and introduction of new alleles within the species (Jain 2005). Mutation could broadly be categorised as induced or spontaneous (Figure 1). Breeder may select the spontaneous bud mutation for the desired traits. Spontaneous mutation or tissue chimaera may be found at the frequency of 0.009%–0.271% in various *citrus* species (Bowman et al. 1991). Tissue chimaera can lead to the isolation of disease resistant mutation and occurrence of polyploidy mutants. Such type of chimaera tissues or bud could be obtained by searching the orchards having minimal control for the pest and disease through the use of pesticides. In *citrus,* several examples have been present which show successful utilisation of physical and chemical mutagen for induction of induced mutation for various useful traits. Physical and chemical mutagen induces several types of mutations such as point mutation, structural aberrations, translocations and polyploidy. It has been shown that gamma rays have been intensively used in *citrus* improvement programme. LD_{50} for the gamma rays in *citrus* has been found to be in the range of 40–100 gy depending on the species and plant organ. Scion (bud wood), seeds, foral stage embryos, immature seed and *in vitro* material of *citrus* may be treated with the gamma rays. In *citrus* mutation, breeding programme has been intensified for the development of seedless *citrus*. However, little efforts have been carried for induction and screening of disease resistant mutants in *citrus*. Gulsen et al. (2007) used five dosage of gamma radiation (0, 3, 5, 7, 9 kiloradin) to the bud stick Kutdiken' lemon and evaluated 358 mutant for Mal secco (caused by *P. tracheiphila* (Petri). The mal secco tolerant plants were obtained from 5 and 7 krad irradiation. Three mutants from 5 krad irradiation were high yielder while 7 krad irradiation showed alteration in tree morphology and induced early maturity.

Hybridisation/introgression *citrus* for disease resistance

If the desired level of resistance is absent in the elite or obsolete cultivars, then wild germplasm is the potential source of resistance for particular disease. It has been noted that wild germplasm still hold the resistant alleles due to least human influence over the related and wild species of *citrus*. However, introgression is bottlenecked due to significant genetic divergence among the cultivated and wild germplasm. In *citrus*, conventional crossing is also difficult due to occurrence of poly embryonic seedling, apomixes and long juvenility period. However, biotechnological tools such as protoplast fusion could facilitate the introgression.

It is difficult to make introgression among the species with different ploidy levels and structural (shape, size) and genetical chromosomal divergence. The crossing of such type of species through conventional hybridisation methods produces sterile hybrids. The odd somatic chromosome number of hybrids results in univalent to multivalent chromosome formations during first meiotic division. The species with such type of chromosome behaviour are called as cross incompatible species. The somatic chromosome number of interspecific hybrid can be doubled to restore the pairing within each genome through the use of chemical such as colchicine. However, it is difficult to make cross between the species with high cytological and genetical divergence, since alien embryos are aborted during their earlier development phases. Protoplast fusion facilitates the fusion of somatic cell rather than haploid gametes in normal reproduction to

Table 4. Summary of development of resistant *citrus* germplasm through protoplast or somtic cell hybridisation.

Parental species	Hybridisation	Results	References
Diverse crossing between 12 *citrus* species	Tetraploid somatic hybrid plants	Eight new *citrus* somatic hybrids have been propagated and entered into field trials	Louzade et al. (1992)
Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck] × "Femminello" lemon [*C. limon* (L.)	Somatic hybrid and the cybrids	Lower mortality in the asymmetrical lemon cybrids to the disease *Phoma tracheiphila*	Tusa and Del Bosco (2000)
Diverse crossing between 5 *citrus* species	Five new somatic hybrids with potential for improved disease resistance	Somatic hybrids had tolerance to blight and *citrus* tristeza virus (CTV)	Mendes et al. (2001)
Sweet orange × lime	Successful somatic hybridisation	Lime-like fruit hybrid had disease resistance for witches' broom disease of lime	Khan and Grosser (2004)
Diverse crossing between 14 *citrus* species	Triploid hybrids were recovered through embryo culture.	A total of 650 hybrids (mostly triploid) were transferred to soil	Viloria and Grosser (2005)
Mandarin (*C. reticulata* Blanco) + pummelo (*C. grandis* L. Osbeck)	Leaf protoplasts protoplast fusion	Ten resistant/tolerant Sting nematode (*Belonolaimus longicaudatus* Rau) pummelo seedlings were selected from the 800 pummelo seeds	Grosser et al. (2007)

avoid several hindrances such as poor pollen germination at the stigma, stylar tissue incompatibility and finally, the abnormal distribution and pairing of chromosome in wide crosses. Protoplast fusion of somatic cell develops an amphidiploids cell or allotetraploid showing that chromosome pairing will not be disturbed due to presence of homologue for each chromosome in each type of genome. Furthermore, somatic cell hybridisation permanently locks the heterosis due to genetic divergence of two genomes. Somatic cell hybrids may be grouped as symmetric or asymmetric depending on the contribution of the two parental species for their genome. If both species contributed equal number of chromosome, it may be regarded as symmetric hybrids, while in asymmetric hybrid contribution is not equal. In *citrus,* once few plants are regenerated after somatic cell hybridisation, they may be easily multiplied through vegetative propagation and disease resistant somtic hybrids may be utilised as root stock. These wide somatic hybrids may be identified by morphology, cytology, isozyme, Randomly amplified polymorphic region (RAPD) and Restriction Fragment Length Polymorphism (RFLP) analyses. Several disease resistant root stocks have been recommended for general cultivation (Gua & Deng 2001). Guo and Deng (2001) noted that more than 60 sexually compatible or incompatible intergeneric somatic hybrids between *citrus* and its various related wild genera have been produced via somatic cell hybridisation. Summary of studies carried out for the development of somatic hybrids has been shown in Table 4.

Transgene engineering

Genetic engineering has also been exploited to produce transgenic disease resistant stock. Exploitation of this technique could help to introduce gene potentially from any genera or phylum through vector. Various transgenes have been exploited to induce resistance in *citrus* species (Table 5). *Agrobacterium* strain (EH-105) has been utilised

Table 5. Summary of the development of transgenic for introgression of resistance against various *citrus* diseases.

Pathogen	Host	Transgene	References
CTV (*Citrus tristeza* virus)	42 trangenic mexican lime *Citrus aurantifolia* Swing	Coat protein gene of CTV P25 Coat protein gene	Dominguez et al. (2000, 2002)
Phytophthora citrophthora	Transgenic orange *Citrus sinensis* L.	PR-5 gene	Fagoaga et al. (2001)
Phytophthora nicotianae	Rangpur lime	Bacterio-opsin (*bO*) gene	Azevedo et al. (2006)
Low conidial germination and fungal growth of *Phoma tracheiphila*	Femminello siracusano' lemon	*chit42* gene	Gentile et al. (2007)
Botrytis cinerea	Femminello siracusano' lemon	*chit*42	Distefano et al. (2008)
Xanthomonas axonopodis pv. *citri*	Transgenic orange *Citrus sinensis* L.	*hrp*N gene	Barbosa-Mendes et al. (2009)
Xanthomonas axonopodis pv. *citri*	Sweet orange cultivars Hamlin, Natal, Pera and Valencia	Rice *Xa21* R-gene	Mendes et al. (2010)
Xanthomonas citri pv. *citri*	Grapefruit and certain sweet orange	*NPR1* gene	Zhang et al. (2010)

to transform explants cell using binary vector (pBI 121/*CTV-C*) in auxin-rich environment (Dominguez et al. 2000). Transformation frequency has been variable depending upon specific citrus species. For instance, Mendes et al. (2010) reported transformation efficiency of 18·6% for Hamlin, 8·3% for Natal, 3% for Pera and 11·3% for Valencia in sweet orange varieties. Transgenic plants were confirmed using marker genes such as β-glucuronidase assays while test such as Sothern analysis (Dominguez et al. 2000) was used to confirm the number of copies of transgene integerated into genome. Moreover, reverse transcriptase analysis and northern analysis were used to evaluate the level of expression of transgene in the host species (Gentile et al. 2007).

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