

Developing aluminium-tolerant crop plants using biotechnological tools

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Aluminium is considered as the main abiotic stress that causes 25–80% yield losses in various crop plants grown on soils containing excessive aluminium contents. The genetic variability among genotypes of a crop species exists for tolerance to aluminium toxicity which can be exploited either through direct phenotypic selection or hybridization followed by directional selection. Three basic genetical approaches are commonly used to improve aluminium tolerance. These are: (i) exploitation of natural genetic variation through direct selection in aluminium stress environments, (ii) mapping quantitative trait loci and subsequent marker-assisted selection, and (iii) development of transgenic plants to introduce novel genes or to change the tolerance levels of the existing genes to improve the degree of aluminium tolerance. In this article, we deal with breeding approaches including role of biotechnological tools for improving aluminium tolerance in crop plants, future challenges and opportunities.

Keywords: Aluminium tolerance, biotechnological tools, breeding approaches, genetic variability.

In India, 49 million hectares of land is affected by soil acidity of which 24 million hectares have pH below 5.5 (ref. 1). In the north-eastern region of India, more than 95% area is affected by soil acidity². The productivity potential of acidic soils is estimated to range from 25% to 80% less than normal soil³. In acidic soils, poor crop productivity and low soil fertility are mainly due to the combination of aluminium and manganese toxicities coupled with nutrient deficiencies (P, Ca, Mg and K). Among these problems, aluminium toxicity has been identified as a major growth limiting factor in acidic soils. Aluminium toxicity is a serious problem in low pH acidic soils (< 5.5). Aluminium affects about 40–70% of the world's arable land, which has potential for production of food crops⁴. It is highly toxic to plant roots⁵ resulting in poor development of the root system, susceptibility to moisture stress and nutrient deficiencies^{6,7}. The reclamation of aluminium toxicity through application of lime is an

expensive method, ineffective in the subsoil and in some cases heavy application may have a deleterious effect on the soil structure⁸. The best way of solving this problem is to develop aluminium-tolerant crop cultivars with increased aluminium tolerance.

Three basic approaches are being used to increase stress tolerance, viz. (i) exploitation of natural genetic variation through direct selection in aluminium stress environments, (ii) mapping quantitative trait loci (QTLs) and subsequent marker-assisted selection, and (iii) the generation of transgenic plants to introduce novel genes or to change the tolerance levels of existing genes to affect the degree of aluminium tolerance. This review article deals with genetics, breeding approaches and use of biotechnology for improving aluminium tolerance in crop plants, challenges and opportunities in this area of research with special focus on the recent experimentation that has led to improved aluminium tolerance.

Genetic variability

The genetic variability among genotypes or cultivars within the same species exists for tolerance to aluminium toxicity. Natural genetic variation for aluminium tolerance in crops is well documented⁶. The existence of aluminium-tolerant plants and differences in aluminium tolerance among genotypes indicate that tolerance to aluminium toxicity is genetically controlled.

Genetic variability among species^{9–11} and within species has been noted in crops such as tomato¹², wheat⁶, barley¹³, maize^{14,15}, rice^{16,17}, soybean¹⁸, pea¹⁹, cabbage²⁰, chickpea²¹, okra²², etc. Genetic variability of these crops can be exploited to develop aluminium-tolerant varieties and to explore the number of genes involved in aluminium tolerance.

Genetics of aluminium toxicity tolerance

An understanding of the genetic basis of aluminium tolerance in crop plants is a pre-requisite for a geneticist to evolve superior genotypes. Aluminium tolerance is genetically controlled²³, thus selection is possible for better

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aluminium toxicity tolerance in crop plants. Aluminium toxicity tolerance appeared to be determined by one or more major genes^{24,25}. The inheritance and genetics of aluminium resistance has been assessed mostly in cereals like wheat, maize, rice, etc. Some of the reports in these crops are inconclusive. Aluminium stress tolerance in rice is controlled by a complex multigenic system¹⁶. This is in contrast to that of aluminium tolerance which is generally monogenically controlled with tolerance determined by dominant alleles²⁶. In wheat, Kerridge and Kronstad²⁷ observed that a single dominant gene is responsible for aluminium tolerance. However, Aniol²⁸ found that tolerance to aluminium toxicity is controlled by two pairs of genes, each gene pair affecting the same character, with complete dominance of both gene pairs, but their recessive homozygotes are epistatic to effects of the other gene²⁸. Aniol²⁹ concluded that several genes are responsible for aluminium tolerance in wheat. This is consistent with Lafever and Campbell³⁰ and Campbell and Lafever³¹ who found that aluminium tolerance in wheat is not simply inherited and that the expression of aluminium tolerance is additive with high heritability. In barley, aluminium tolerance is governed by one major dominant gene^{23,32} along with multiple alleles¹³. In barley, tolerance to aluminium is controlled by a single dominant gene, designated *Pht* on chromosome 4 (ref. 32) or *Alp*³³. In maize, tolerance ability is governed by a single gene with multiple alleles³⁴. However, Sibov³⁵ found that aluminium tolerance is governed by two major genes *Alm1* and *Alm2* located on chromosomes 10 and 6 respectively. In sorghum, inheritance was polygenic with tolerance determined by dominant alleles. The ratio of general and specific combining abilities showed that additive gene effects were more important than non-additive gene effects³⁶. Singh and Choudhary³⁷ found that tolerance to aluminium toxicity is controlled by a single dominant gene and tolerance is governed by a dominant allele. In rye (*Secale cereale*), aluminium tolerance is controlled by

four dominant and independent genes (*Alt1*, *Alt2*, *Alt3* and *Alt4*) located on 6RS, 3RS and 4RL and 7RS respectively³⁸⁻⁴¹. There is an increasing awareness that Al tolerance is more likely a polygenic trait. There is also need to re-assess the number of resistance genes available against aluminium toxicity. The genetics of aluminium tolerance in various crops is presented in Table 1.

Breeding approaches

Molecular breeding

The direct selection of superior aluminium tolerant genotypes under field conditions is hindered due to temporal and spatial variations in aluminium toxic soils and reliable ranking of tolerance in the field screening is difficult. Moreover, screening at field level is very expensive and time consuming when a large number of genotypes are under evaluation. Molecular breeding is a rapid method of crop improvement which permits evaluation of large number of genotypes in less time. Several molecular markers such as restriction fragment length polymorphism, random amplified polymorphic DNA and simple sequence repeats, sequence tagged sites and sequence characterized amplified regions are available to facilitate development of aluminium-tolerant genotypes more effectively. Once molecular markers are linked to QTLs governing aluminium tolerance, it would be possible to transfer tolerance to aluminium into adapted cultivars or other agronomic backgrounds through molecular breeding approach. Molecular markers linked to aluminium-tolerant gene or QTLs governing aluminium tolerance have been identified in several crop plants such as barley⁴², wheat⁴³, rye³⁹, maize³⁵, rice⁴⁴ and soybean⁴⁵. Molecular markers linked to aluminium tolerance in various crops are summarized in Table 2.

Tissue culture

The evaluation of aluminium tolerance in tissue culture may be more useful for breeding programmes, because selection is earlier and faster in tissue culture than in the field. Moreover, the selection by tissue culture can be applied to identify aluminium-tolerant plants in segregating populations. Plant cell culture provides several ways for screening plants to select aluminium-tolerant genotypes, produce and identify somaclonal variation with enhanced tolerance and study cellular responses to aluminium toxicity. Tissue culture has also been used to generate aluminium-sensitive mutants from aluminium tolerant germplasm to develop plant materials with similar genetic background for identifying and characterizing the genes involved in tolerance⁴⁶. Several studies have indicated that aluminium-tolerant plants can be identified

Table 1. Genetics of aluminium tolerance of crop plants

Crop	Gene(s) resistance	Reference
Rice	Monogenic	Ferreira <i>et al.</i> ²⁶
	Polygenic	Nguyen <i>et al.</i> ⁸⁰ Khatiwada <i>et al.</i> ¹⁶
Wheat	Monogenic	Somers and Gustafson ⁸¹ Riede and Anderson ⁴³
	Polygenic	Carver and Ownby ⁶⁶
Maize	Monogenic	Rhue <i>et al.</i> ³⁴
	Polygenic	Pandey <i>et al.</i> ¹⁴
Soybean	Polygenic	Bianchi Hall <i>et al.</i> ⁸³
Barley	Monogenic	Minella and Sorrells ¹³
Pea	Monogenic	Singh and Choudhary ³⁷
Chickpea	Monogenic	Singh and Raje ⁸³
Tomato	Polygenic	Singh <i>et al.</i> (unpublished)
<i>Arabidopsis</i>	Polygenic	Hoekenga <i>et al.</i> ⁸⁴
Common bean	Polygenic	Araujo <i>et al.</i> ⁸⁶

Table 2. Molecular mapping of some major genes and QTL for aluminium tolerance in different field crops

Crop	Gene/ QTLs	Designation and chromosome location	Contribution	Mapping populations	Marker type	Reference	
Alfalfa	QTL	–	–	F ₂ and backcross lines	SSR, RFLP	Sledge <i>et al.</i> ⁸⁶	
Barley	Gene	<i>Alt</i> (4H)	Single major gene	Wheat-barley chromosome addition lines 67 F ₂	AFLP and SSR	Raman <i>et al.</i> ⁴²	
	Gene	<i>Alp</i> (4H)	Single major gene	48F ₂	RFLP	Tang <i>et al.</i> ⁸⁷	
Maize	Genes	<i>Alm1</i> (10S) <i>Alm2</i> (6)	24.2 7.67	56 inbred lines	RFLP	Sibov <i>et al.</i> ³⁵	
	QTLs	QTL1 (2) QTL2 (6) QTL3 (6) QTL4 (8) QTL5 (8)	10.9% 5.3% 15.6% 7.4% 8.6%	168 F _{3:4}	RFLP SSR	Ninamango-Cardenas <i>et al.</i> ⁸⁸	
	QTLs	<i>QAIRr1.1</i> (1) <i>QAIRr3.1</i> (3) <i>QAIRr7.1</i> (7) <i>QAIRr8.1</i> (8) <i>QAIRr9.1</i> (9)	9.0% 24.9% 22.5% 20.8% 9.9%	171F ₆ RILs	RFLP and SSR	Nguyen <i>et al.</i> ⁸⁹	
Rice	QTLs	<i>qALRR-1-1</i> <i>qALRR-1-2</i> <i>qALRR-2</i> <i>qALRR-3</i> <i>qALRR-4</i> <i>qALRR-7</i> <i>qALRR-8</i> <i>qALRR-9</i> <i>qALRR-10</i> <i>qALRR-12</i>	24.1% 18.5% 13.4% 12.8% 20.1% 10.3% 28.7% 19.3% 17.7% 19.7%	146 DH lines	RFLP AFLP SSR	Nguyen <i>et al.</i> ⁴⁴	
	QTLs	QTLs 2 weeks (1) (3) (12) QTLs 4 weeks (1) (9) (12)	19% 9% 10% 15% 9% 20%	159F ₉ RILs	AFLP RFLP	Wu <i>et al.</i> ⁹⁰	
	QTLs	QTL (1) QTL (2) QTL (6)	11.1% 7.3% 8.7%	183 Backcross lines	RFLP	Ma <i>et al.</i> ⁹¹	
	QTLs	QTL (1) QTL (9) QTL (11)		71 F ₇ RILs		Xue <i>et al.</i> ⁹²	
	Rye	Gene	<i>Alt1</i> (6RS)	Dominant	F ₂	RAPD and SCARs	Gallego <i>et al.</i> ³⁹
		Gene	<i>Alt3</i> (4RL)	Single	F ₆ RILs	AFLP	Miftahudin <i>et al.</i> ⁴⁰
	Soybean	QTLs	–	–	40F ₂	SSR	Tasma <i>et al.</i> ⁴⁵
		QTLs	–	–	F ₄	RFLP	Bianchi-Hall <i>et al.</i> ⁸²
	Wheat	Gene	<i>Alt_{BH}</i> (4DL)	85%	101F ₅ RILs	RFLP	Riede and Anderson ⁴³
		Gene	<i>Alt_{BH}</i> (4DL)	Single dominant	91 F ₅ RILs	RFLP, SSR and AFLP	Milla and Gustafson ⁹³

by comparing growth of callus in an acidic medium with and without added aluminium. This suggests that similar mechanisms of aluminium tolerance are active in both cell cultures and whole plants.

Several tolerant crop plants have been obtained from somatic callus and microspore cultures⁴⁷. The tissue culture induced somaclonal variation is being used for improving aluminium tolerance in rice⁴⁸, wheat⁴⁹, tomato⁵⁰ and many other crops^{51–54}. The aluminium tolerance exhibited at cellular level is also maintained by adult plants as reported in tomato⁵⁵, sorghum⁵² and alfalfa⁵⁶. In alfalfa, callus derived from acid-tolerant cultivars has been observed to have greater ability to grow on acidified medium⁵⁷. Parrot and Bouton⁵⁶ reported that alfalfa expressed aluminium tolerance at the callus stage and consequently the selection by tissue culture could be applied to identify aluminium-tolerant plants. Ojima and Ohira⁵¹ developed aluminium-tolerant cell lines of carrot by exploiting the cells to Al-EDTA. Plants regenerated from selected calli were tolerant to aluminium due to secretion of organic acids.

Transgenic breeding

Production of aluminium-tolerant genotypes through genetic engineering is considered an alternative approach to increase crop production in acidic soils. Two approaches, viz. expression of genes to increase organic acid production and expression of aluminium induced plant genes have been applied for improving aluminium tolerance in tobacco, papaya, rice, *Arabidopsis* and alfalfa. The selective reports on aluminium-tolerant transgenics are presented in Table 3.

De la Fuente *et al.*⁵⁸ transformed tobacco and papaya plants that overexpressed a citrate synthase gene (*CSb*) derived from *Pseudomonas aeruginosa* in their cytoplasm. This gene showed high citrate synthase activity, enhanced citrate efflux and greater tolerance than non-transformed lines. The transformed lines of tobacco expressing *CSb* had up to 10-fold greater internal citrate in their root tissues whereas in papaya, citrate level in the roots was only 2–3 folds. Increased production of citric acid was shown to result in aluminium tolerance in both the species.

In another experiment, functions of the BnALMT1 and BnALMT2 (*B. napus* aluminium-activated malate transporter) protein were studied by heterologous expression in cultured tobacco. Such transfection system showed an enhanced capacity for malate efflux but not citrate efflux when exposed to aluminium. Transgenic tobacco cells grew significantly better than control cells. This indicated that expression of BnALMT1 and BnALMT2 increased the resistance of these plant cells to aluminium stress⁵⁹. In another study, citric acid *Arabidopsis thaliana* was transformed to overexpress citrate synthase isolated from carrot mitochondria. The transformants showed up to 3-fold increase in citrate synthase activity and 1.6-fold increase in citrate secretion compared with controls⁶⁰. Aluminium tolerance in these plants was increased slightly. Citrate synthase gene has been identified⁵⁸ and many yet to be discovered before targeted genetic modifications can be effectively designed. Thus, the use of citrate synthase gene may prove to be effective strategies for the production of aluminium-tolerant crop species without undesirable effects on other agronomic traits. The production of transgenic plants with an increased

Table 3. Aluminium tolerance in transgenic plants expressing genes involved in chelation of aluminium tolerance genes

Crop	Gene/protein/enzyme	Reference
Genes		
Wheat	Citric acid/Alt locus	Delhaize <i>et al.</i> ⁶⁸
Wheat	<i>Wali</i> 1-5	Snowden and Gardner ⁶⁹
Soybean	<i>Sali</i> -4a/ <i>Sali</i> 3-2	Ragland and Soliman ⁷⁰
<i>Arabidopsis</i>	ALR	Degenhardt <i>et al.</i> ⁷¹ Larsen <i>et al.</i> ⁷²
Alfalfa	Malate dehydrogenase	Tesfaye <i>et al.</i> ⁶¹
Carrot cells	Citrate synthase	Koyama <i>et al.</i> ⁷³
Protein		
Wheat	51 kDa	Basu <i>et al.</i> ⁷⁴
Wheat	23 kDa exudates polypeptide	Basu <i>et al.</i> ⁷⁵
Alfalfa	18.7 kDa	Campbell and Jackson ⁷⁶
Wheat	TAL-18	Cruz-Ortega and Ownby ⁷⁷
<i>Arabidopsis</i>	AtBcB	Ezaki <i>et al.</i> ⁶³
Enzymes		
Wheat/rye	NAD ⁺ kinase	Slaski ⁷⁸
Wheat	1-3 β -Glucanase	Cruz-Ortega <i>et al.</i> ⁷⁹

capacity to produce and/or excrete organic acids that chelate and detoxify aluminium in the rhizosphere is an appealing strategy to produce aluminium-tolerant plants.

Overexpression of plant genes for two enzymes involved in organic acid synthesis, phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) enhanced organic acid synthesis and secretion which resulted in greater aluminium tolerance in alfalfa⁶¹. Selected transgenic plants with a 1.6-fold increase in MDH-specific activity showed a 4.2-fold increase in citrate, oxalate, malate, succinate and acetate in root tissues compared to the control (untransformed line). A transformed line containing the PEPC transgene with a 2-fold increase in PEPC activity had increased amounts of malate compared to the control. In acidic solution culture assays, plants expressing the MDH or PEPC transgene showed enhanced root elongation compared with the control untransformed line⁶¹. In such an assay, transgenic lines of alfalfa increased 2–3-fold greater root growth in the presence of 20 μM aluminium level than the growth rate of the untransformed control. When subjected to culture with 100 μM AlCl_3 , transgenic lines continued to grow, albeit at a reduced rate whereas the untransformed control plants did not show root growth⁶¹.

Transgenic rice was more tolerant to aluminium than the wild type because root tips of transgenic rice accumulated less aluminium than those of wild type. Aluminium-induced oxalate exudation from roots occurred at increased rates in the transgenic line. Overexpression of C4-PEPC drastically increased PEPC activity in the leaves of transgenic rice and resulted in enhanced aluminium tolerance in transgenic rice causing higher organic acid concentration in leaves and roots⁶².

The expression of aluminium-induced genes in transgenic *Arabidopsis* plant could ameliorate aluminium stress and/or oxidative stress⁶³. More resistant transgenic plants could be produced by a combination of four genes⁶⁴. A *Arabidopsis* blue-copper-binding protein gene (*AtBCB*), tobacco glutathione S-transferase gene (*parB*), a tobacco peroxidase gene (*NtPox*) and a tobacco GDP-dissociation inhibitor gene (*NtGDI1*) conferred a degree of resistance to aluminium toxicity through different mechanisms. Two of these genes, *AtBCB* may suppress aluminium absorption and *NtGDI1* promotes a release of aluminium in the root tip region; whereas *parB* and *NtPox* enhance the enzyme activities which diminish oxidative damage caused by aluminium.

Challenges

The aluminium tolerance in transgenic experiments as described here has been mostly assessed using a limited number of plant species in laboratory. However, the level of aluminium tolerance of crops ultimately needs to be

evaluated in field conditions. The evaluation of field performance under aluminium stress conditions is difficult because of field heterogeneity in aluminium toxic soils which hinders the reliability of the response of genotypes⁶⁵. Under such situations it is necessary to create and maintain the desired levels of aluminium in soils. Screening in soils representative of the targeted production area, where soil acidity is a yield limiting factor provides a critical intermediate step in selection of tolerant genotypes after preliminary screening in nutrient solution but before more lengthy and costly screening under natural field conditions⁶⁶.

Although there have been many successes in developing aluminium-tolerant transgenics such as rice⁶², papaya and tobacco⁵⁸, there is urgent need to validate this success in other crops. An extensive quantum of genetical and breeding work on primary transgenic has to be carried out before the expression of the transgene is stabilized, so that a specific cultivar that is acceptable to local farmers can be bred. There is need to support on a large-scale basic research leading to identification, isolation and cloning of novel aluminium stress tolerance related genes from Indian germplasm. For production of aluminium-tolerant genotypes, there is need for a greater number of laboratories which deal solely with the production of aluminium-tolerant transgenic crops. The success achieved in developing aluminium-tolerant transgenic cultivars by some foreign countries needs to be explored in the coming years in India. It is suggested that there should be special emphasis and integrated approach on research work aiming at production of aluminium-tolerant transgenic by the Government of India with sufficient funding. This is needed especially for the developing countries like India where the population is rising and available resources are depleting.

Molecular markers which are associated with aluminium-tolerant genes need to be identified and pyramided in agronomically superior genotypes. There have been several successes in improving aluminium tolerance through markers-assisted selection in barley, wheat, maize and rice plants. We now need to begin introducing tolerance genes into other crops through marker-assisted selection.

Organic acids have been strongly implicated in aluminium tolerance, a logical approach is to manipulate the biosynthesis and efflux of organic acids. The mechanisms controlling organic acid secretion need to be studied and the genes responsible for citrate and oxalate secretion identified and cloned. Limited progress has been made in understanding aluminium-tolerance mechanisms based on organic acid efflux, much about the molecular mechanisms underlying the activation of anion-channels by aluminium is yet to be learnt. There is also need to substantial increase in the efflux of organic acids for these approaches to have practical application in agriculture.

Opportunities

Although progress in improving stress tolerance has been relatively slow, there are several opportunities and reasons for optimism. Over the past 15 years, there has been the development of a number of functional tools that can allow us to dissect many of the fundamental questions associated with stress tolerance. These include: (i) development of molecular markers for gene mapping and the construction of associated maps; (ii) development of EST libraries; (iii) the complete sequencing of plant genomes, including *Arabidopsis*, rice and maize; (iv) the production of T-DNA or transposon tagged mutagenic populations and (v) the development of several forward genetics tools that can be used in gene function analysis (TILLING)⁶⁷. Thus, we need to focus on looking at the comparative effects and interaction of specific transgenes within a defined genetic background and determine the efficacy of these approaches in the field.

Basic understanding of mechanisms under aluminium stress conditions will open new avenues for genetic engineering for aluminium tolerance in various crops. Examples of transgene-mediated aluminium tolerance listed in Table 3 show that a gene from one species can be used in another to improve the performance of transgenic plants under aluminium stress.

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