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Research Paper

MECHANISM OF HEAVY METAL TOLERANCE AND IMPROVEMENT OF TOLERANCE IN CROP PLANTS

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Abstract

Heavy metal (HM) toxicity is one of the major abiotic stresses leading to hazardous effects in plants. A common consequence of HM toxicity is the excessive accumulation of reactive oxygen species (ROS) and methylglyoxal (MG), both of which can cause peroxidation of lipids, oxidation of protein, inactivation of enzymes, DNA damage and/or interact with other vital constituents of plant cells. Higher plants have evolved a sophisticated antioxidant defence system and a glyoxalase system to scavenge ROS and MG. In addition, HM's that enters the cell may be sequestered by amino acids, organic acids, glutathione (GSH), or by specifc metal-binding ligands.Physiological studies indicated crop plants absorbs heavy metals in soils and transported from roots to shoots by the xylem and after several processes of transport finally accumulates into grains.

One of the major difficulties in studies on the selection of heavy metal tolerant plants is the proper methodology that must ensure an efficient evaluation of a large number of plants, but reducing environmental contamination. Hydroponic system containing heavy metal is the efficient method for screening heavy metal tolerant genotype that is because different Plants have different capacity to remediate or sequestrate and detoxify heavy metals, and so huge variation is present for heavy metal tolerance in them which can be used to select tolerant lines or variety. Apart from selection several conventional breeding methods have been used to improve heavy metal tolerance in plants. Modern biotechnological approaches, namely marker assisted selection (MAS) and genetic engineering also contributed tremendously in improving tolerance of plant to heavy metals.

Key words: Heavy metal, abiotic stress,defense system,conventional, biotechnology.

INTRODUCTION

The development of scientific and industrial technology has provided not only a large number of benefits to the society, but also generated several undesirable environmental pollutants including heavy metals. Heavy metals are being generated by many industries, Burning Fossil fuels, Mining and Smelting, Sewage Sludge, Chemical fertilizers, Pesticide, herbicide residues etc. Soil pollution has recently been attracting public attention and the magnitude of the problem in our soils calls for immediate action (Garbisu & Alkorta 2003). Due to their immutable nature, metals are a group of pollutants of much concern.

Soil pollution with heavy metals has become a critical environmental concern due to its potential adverse ecological effects. Heavy metals occur naturally at low concentrations in soils. However, they are considered as soil contaminants due to their widespread occurrence, acute and chronic toxicity. These metals are extremely persistent in the environment. They are non-biodegradable, non thermo-degradable and thus readily accumulate to toxic levels (Chopra *et al*., 2009). Since they do not break down, they might affect the biosphere for a long time. It is known that the heavy metals form an important polluting group. They have not only toxic and carcinogenic effect but also tend to accumulate in living organisms. The irrigation of wastewater (industrial, municipal and house hold), sewage-sludge and dumped solid wastes on soils has been widespread in agricultural areas.

Heavy metals come from local sources mostly from the industries (non-ferrous industries, power plants, iron, steel and chemical industries), agriculture (irrigated with polluted water, use of mineral fertilizers especially phosphates, contaminated manure, sewage sludge and pesticides containing heavy metals), from waste incineration, burning of fossil fuels and road-traffic. Researchers have made an important contribution both in India and abroad to investigate the impact of effluent storage / irrigation / drainage on soil related with heavy metal contamination and their accumulation in the plant system and lastly in the product (Mishra and Tirpathi, 2008). Recently pollution of general environment has increasingly gathered a global interest. In this respect, contamination of agricultural soils with heavy metals has always been considered as a critical challenge in scientific community. Heavy metals are generally present in agricultural soil at low levels. Due to their cumulative behaviour and toxicity, they have a potential hazardous effect not only on crop plants but also on human health.

I. Heavy metals

Heavy metals are the stable metals or metalloids whose density is greater than 5 g/cm3, namely Pb, Cu, Ni, Cd, Zn, Hg, Cr etc and which is distinctly higher than the average particle density of soils (2.65 g/cm3). They are stable and cannot be degraded or destroyed and therefore they tend to accumulate in soils and sediments. In fact, although several metals are essential for biological systems and must be present within a certain concentration,. they are contaminant if it occurs where it is unwanted, or in a form or concentration that causes a detrimental human or environmental effect.

II. Nutrients and Heavy Metals

Some heavy metals are required by all plants as a nutrient(essentials), some are required by specific plants(Benficial) and others not at all required by plants(Non essentials).

2.1 Essentials:

These are required for all kinds of planst. For eg. Cu- The plant micronutrient copper is essential for photosynthesis, oxidative responses, and other physiological processes, Zn- it is a micronutrient needed in small amounts by crop plants, but its importance in crop production has increased in recent years. Zinc application on plants exposed to salinity stress caused a noticeable enhancement of photosynthesis (Pn), water use efficiency, mesophyll efficiency and quantum yield (Weisany *et al*., 2011)

2.2 Beneficial:

These are required by specific plant groups. Nickel- is one of the constituent element of hydrogenase enzyme and it also acts in activation of other enzymes. Bertrand and DeWolf, 1967 reported that soil-nickel application to field-grown soybean (Glycine max Merr.) resulted in a significant increase in nodule weight and seed yield. Cobalt is essential for nitrogen-fixing microorganisms, including the cyanobacteria. Cobalt has been shown to be essential for symbiotic nitrogen fixation by legumes and non legumes (Bothe *et al*., 2007).

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2.3 Non-essentials:

They are not required by plants and accumulated in plant body due to their weak uptake control mechanism and **heavy metals** under this category are Cd, Hg, Cr, etc.

III HEAVY METALS CONCENTRATION IN MIXED WASTE COMPOST IN INDIA

	Lowest (mg/kg)	Highest (mg/kg)	Median (mg/kg)	Quality Control standard(mg/kg)
Zinc	82	946	252	1000
Copper	25	865	198	300
Cadmium*	0	8	0.94	5
Lead	11	647	133	100
Nickel	9	190	25	50
Chromium	14	401	69	50

*Cadmium concentration units are mg/100 kg

Note: Concentrations of all heavy metals except Cadmium are expressed in mg/kg dry mass of compost. Cadmium concentration is expressed in mg/100 kg dry mass of compost.

Heavy metals generally found in mixed waste composts are Zinc (Zn), Copper (Cu), Cadmium (Cd), Lead (Pb), Nickel (Ni) and Chromium (Cr). A study conducted by the Indian Institute of Soil Science (IISS), Bhopal found that compost produced from MSW (Metal Solid waste) in India is low grade, with high heavy metal concentrations and low nutrient value. Above table shows the range of concentration of heavy metals Zinc (Zn), Copper (Cu), Cadmium (Cd), Lead (Pb), Nickel (Ni) and Chromium (Cr) in MSW composts from 29 cities Saha *et al*., 2010

IV. POTENTIAL HAZARD OF INTRODUCING HEAVY METALS INTO AGRICULTURAL SOILS

If all heavy metal generated in India from 2011-2021 (**Table.1**) is treated in MBT facilities and the compost was used for agriculture, it would introduce 73,000 tons of heavy metals into agricultural soils (Annepu, 2012.).

V. SOURCES OF HEAVY METALS IN CONTAMINATED SOILS

Heavy metals occur naturally in the soil environment from the pedogenetic processes of weathering of parent materials at levels that are regarded as trace (<1000 mg kg−1) and rarely toxic. Due to the disturbance and acceleration of nature's, slowly occurring geochemical cycle of metals by man, most soils of rural and urban environments may accumulate one or more of the heavy metals high enough to cause risks to human health, plants, animals, ecosystems, or other media. The heavy metals essentially become contaminants in the soil environments because (i) their rates of generation via man-made cycles are more rapid relative to natural ones, (ii) they become transferred from mines to random environmental locations where higher potentials of direct exposure occur, (iii) the concentrations of the metals in discarded products are relatively high compared to those in the receiving environment, and (iv) the chemical form (species) in which a metal is found in the receiving environmental system may render it more bio-available (Wuana and Okieimen, 2011). Heavy metals in the soil from anthropogenic sources tend to be more mobile, hence bio-available than pedogenic, or lithogenic ones. Metal-bearing solids at contaminated sites can originate from a wide variety of anthropogenic sources in the form of metal mine tailings, disposal of high metal wastes in improperly protected landfills, leaded gasoline and lead-based paints, land application of fertilizer, animal manures, biosolids (sewage sludge), compost, pesticides, coal combustion residues, petrochemicals and atmospheric deposition(Kaasalainen and Yli-Halla, 2003).

VI. EFFECTS OF METALS IN PLANTS

Heavy metals interfere with the cellular structure, photosynthesis and metabolism.

6.1 Effect on cellular structure

Heavy metals cause membrane damage through various mechanisms, including the oxidation of and cross-linking with protein thiols, inhibition of key membrane protein such as H+-ATPase, or causing changes in the composition and fluidity of membrane lipids.

Accumulation of methyl glyoxal, a cytotoxic compound, was found to increase in response to heavy metal stress in plants due to impairment of the glyoxalase system that finally elicits oxidative stress by reducing the GSH content (Hossain *et al*., 2009).

6.2 Effect on photosynthesis

Heavy metals directly affect the photosynthetic machinery by binding to the various sensitive sites of the photosynthetic apparatus. In chloroplast, heavy metals disturb the architecture of thylakoid membranes, which in turn, change some light reaction processes, directly especially those associated with PSII. It is evident that excess Cu has a strong effect on chloroplast fine structure, resulting in degradation of grana stacks and stroma lamellae and an increase in the number and size of plastoglobuli and intrathylakoidal inclusions (Baszynski *et al*., 1988). Various authors gave evidences that the toxic effect of metal on phototropic organisms strongly appears to be related to the increase in the levels of lipid peroxidation and protein carboxylation as well as the production of antioxidant defense systems. Proteins and lipids found embedded in the thylakoid membranes are directly involved in photosynthesis. Metal induced oxidative damage to these essential protein and lipids might be primarily responsible for the inhibition of electron flow (Halliwell and Gutteridge, 1999).

Heavy-metal ions would reduce the efficiency of photosynthesis by inhibiting the key enzymes (ribulose-1,5- bisphosphate carboxylase, phosphoenol-pyruvate carboxylase) of the Calvin cycle. Photosystem II (PSII) is extremely sensitive to Cd, and its function is inhibited to a much greater extent than that of PSI (Mallick and Mohan, 2003). The CO_2 assimilation rate, leaf stomatal conductance and chlorophyll fluorescence were inhibited by Cd stress (Duan *et al.*, 2010).

6.3 Effect on metabolism

Several plant species are able to accumulate and withstand large quantities of heavy metals in their tissues without dramatic alterations in their growth usually observed in plants. Such metal accumulating plants are tested and used for remediation of contaminated soils and waters. Heavy metalS (Ni, Cd) can produce oxidative stress as they can work as free radicles .The concentration of Zn, Ni and Cd differentially affected the specific activities and the protein levels of ammonia assimilating enzymes glutamine synthetase (GS), glutamate synthase (Fd-GOGAT) and glutamate dehydrogenase (GDH). Response and tolerance of metal stress of plants depends on the concentration level and on essentiality of the metal

VII. CELLULAR MECHANISMS OF HEAVY METAL TOLERANCE IN PLANTS

Plant tolerance to a particular HM is governed by an inter-related network of physiological and molecular mechanisms and understanding of these mechanisms and their genetic basis is an important aspect to developing plants as agents of phytoremediation (Dalcorso et al., 2010). Different plant species may have evolved different mechanisms to tolerate excess HMs, and even within the one plant species more than one mechanism could be in operation. Plants have both constitutive and adaptive mechanisms to withstand excess HMs. Physiological, biochemical, and molecular approaches continue to be employed to identify the underlying mechanisms of HM accumulation, tolerance, and adaptive mechanisms to cope with HM stress. Some adaptive mechanisms evolved by tolerant plants include immobilization, plasma membrane exclusion, restriction of uptake and transport, synthesis of specific HM transporters, chelation and sequestration of HMs by particular ligands (PCs and MTs), induction of mechanisms contrasting the effects of ROS and MG (such as upregulation of antioxidant and glyoxalase system), induction of stress proteins, the biosynthesis of Pro, polyamines, and signalling molecule such as salicylic acid and nitric oxide (Yang *et al*., 2005).

7.1. Restriction of Uptake and Transport of HMs

7.1.1 Exclusion of HMs from the Plants*:* Uptake of HMs by plants involves root interception of HM ions, entry of HM ions into roots, and their translocation to the shoot. Once an HM is bioavailable to the plant, the entry of HM ions inside the plant, either through the symplast (intracellular) or through the apoplast (extracellular), depends on the type of HM. Most HM ions enter plant cells by an energy-dependent process via specific or generic HM-ion carriers or channels (Zhu et al., 2011). One mechanism (avoidance strategy) of preventing or lessening the toxic effects of HMs is by preventing excess HMs entering the plant. There are two main ways in which a plant could do this, either by precipitating or by complexing HMs in the root environment. Plants can precipitate HMs by increasing the pH of the rhizosphere or by excreting anions such as phosphate. Root exudation of phosphate in maize have been detected in response to Al stress in the Al-tolerant cultivar (South American 3) with no toxicity symptoms whereas sensitive cultivars (Tuxpeno and South American 5) showed symptoms (Pellet *et al*., 1995). Additionally, root exudation of malate in sorghum (*Sorghum bicolor* L.) and exudation of citrate in maize have also been reported in response to Cd stress . These findings support the idea that the HM-binding capabilities of root exudates may be an important mechanism for stabilizing HMs in the vicinity of the root thus making them unavailable to the plant and lessening the experienced toxicity. Zhu and co-worker in 2011 showed that the oxalate secreted from the root apex helps to exclude Cd from entering tomato (*Lycopersicon esculentum* L.) roots, thus contributing to Cd resistance in the Cd resistant tomato cultivar (Micro-Tom).

7.1.2. Cellular Exclusion of HMs: Cellular exclusion of HMs is an important adaptive strategy for HM tolerance in plants. A large fraction of HMs in plant roots are found in the apoplastic space. For example, at equal external Al concentrations, a sensitive wheat cultivar had more symplastic Al than the tolerant cultivar suggesting an exclusion mechanism . HM transporter proteins are potentially involved in the exclusion of toxic HM ions from the symplastic to the apoplastic space (Tice *et al*., 1992).

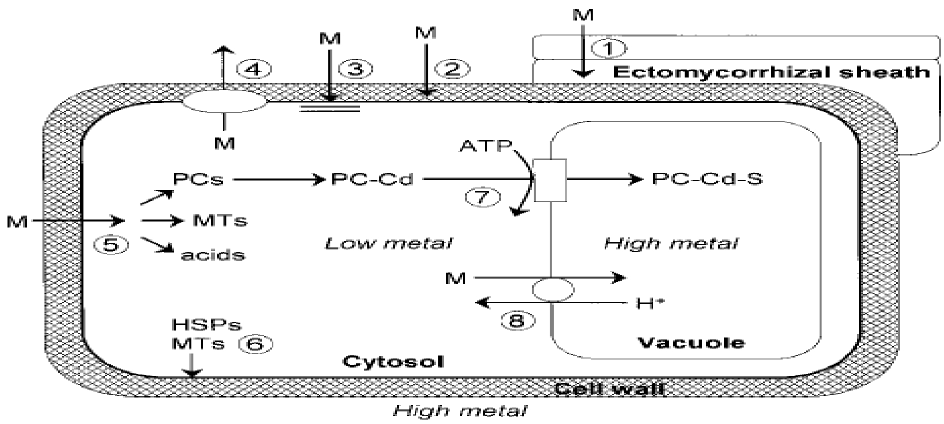

Fig.1: Cellular mechanism of metal toxicity

7.2. HM Complexation at the Cell Wall-Plasma Membrane Interface.

The cell wall-plasmamembrane interface accumulates large portions of HMs and it is therefore believed that this could be the potential site of HM tolerance. In Italian ryegrass (*Lolium multiflorum*), 60% of Cu in the roots was bound by the cell wall and plasma membrane (Iwasaki *et al*., 1990.). Plant cation exchange capacity (CEC) is largely determined by the exchange sites in cell walls. Sensitive wheat cultivars have much lower cell wall CECs than tolerant cultivars indicating that tolerant cultivars use a high CEC to complex HMs at the cell wall and prevent entry to the cell. However, the role of the cell wall in HM tolerance remains to be clarified.

7.3. Complexation and Compartmentation of HMs within the Plant Cell

7.3.1. Intracellular Sequestration or Compartmentation within Vacuoles: Once an HM has entered the cell, a plant uses various strategies to cope with its toxicity. One such strategy consists of transporting the HM out of the cell or sequestrating it into the vacuole, thereby removing it from the cytosol or other cellular compartments where sensitive metabolic activities takes place (Dalcorso et al., 2010). Therefore, the central vacuole seems to be a suitable storage reservoir for excessively accumulated HMs. In fact, two vacuolar proton pumps, a vacuolar proton-ATPase (V-ATPase) and vacuolar protonpyrophosphatase (V-Ppase), energize vacuolar uptake of most solutes. Uptake can be catalyzed by either channels or transporters. The application of powerful genetic and molecular techniques has now identified a range of gene families that are likely to be involved in transition HM ion uptake into cells, HM vacuolar sequestration, HM remobilization from the vacuole, xylem loading, and unloading of HMs. Some well-characterized HM transporter proteins are zinc-regulated transporter (ZRT), iron-

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regulated transporter (IRT) like protein ZIP family, ATP-binding cassette (ABC) transporters, the P-type metal ATPases, the natural resistance-associated macrophage protein (NRAMP) family, multidrug resistance-associated proteins (MRP), ABC transporters of the mitochondria (ATM), cation diffusion facilitator (CDF) family of proteins, copper transporter (COPT) family proteins, pleiotropic drug resistance (PDR) transporters, yellow-stripe-like (YSL) transporter and Ca^{2+}: cation antiporter (CAX).

7.3.2. Formation of Metal Complex by Phytochelations: Chelation of HMs in the cytosol by high affinity ligands is potentially a very important mechanism of HM detoxification and tolerance in plants under HM stress. Plants make two types of peptide metal binding ligands: phytochelatins (PCs) and metallothioneins (MTs). Recent advances in the understanding of different aspects of biosynthesis and function of PCs are derived predominantly from molecular genetics approaches using model organisms. PCs are synthesized from GSH the metal binds to the constitutively expressed enzyme γ-glutamylcysteinyl dipeptidyl transpeptidase (PC synthase), thereby activating it to catalyze the conversion of GSH (glutathione) to phytochelatin (Nouairi *et al*., 2009). The biosynthesis of PCs is induced by many HMs, including Cd, Hg, Ag, Cu, Ni, Au, Pb, As, and Zn; however, Cd is by far the strongest inducer. PC-Cd complexes are accumulated in the vacuole through the activity of ABC transporters, thus limiting the circulation of free Cd^{2+} inside the cytosol. Additionally, plants are not able to metabolize or eliminate Cd. Rather, they adopt the strategy of making Cd-GSH and Cd-PCs complexes to sequester Cd within vacuoles efficiently and also to transport Cd over a long distance through xylem and phloem vessels.

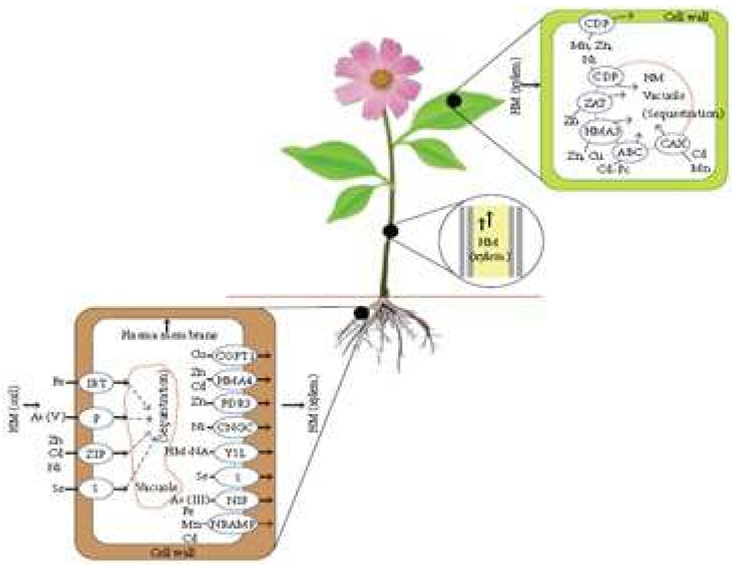


Fig.2: Diagrammatic representation of uptake and transport of heavy metals in plants through metal transporters

7.3.3. Complexing by Metallothioneins*:* MTs are low molecular weight (4–8 kDa), Cys-rich, HM-binding, gene-encoded polypeptides that can bind HMs via the thiol groups of their Cys residues. Although the precise physiological function of MTs has not yet been fully elucidated, proposed roles include (a) participation in maintaining the homeostasis of essential transition HMs, (b) sequestration of toxic HMs, and (c) protection against intracellular oxidative damage.

7.4. Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) act as molecular chaperones in normal protein folding and assembly but may also function in the protection and repair of protein under stress conditions. Induction of HSPs by several transitions HMs (Zn, Cu, Cd, Hg, Al, Cr) has been reported. Increased accumulation of a large HSP (HSP70) was reported in response to Cd (Ireland *et al*., 2004).

7.5. Reactive Oxygen Species Production in Plant Cells

Higher plants produce ROS during different metabolic processes in cellular organelles. However, during HM stress, their rate of production is dramatically elevated. Organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, such as Chl, mitochondria, and peroxisomes, are the predominant sources of ROS production in plant cells (Apel and Hirt, 2004). The Chl is the prime source of ROS having the capacity to produce high amounts of superoxide (O^{2-}) through the Mehler reaction and H_2O_2, essentially during the reduced rate of photosynthetic carbon fixation, a typical situation during abiotic stresses (Takahashi and Murata, 2008) . It is generally accepted that the water oxidizing system of PS II is affected by HM (Cd) by replacing the Ca^{2+} and Mn^{2+} ions in the PS II reaction centre; thereby inhibiting the reaction of PS II leads to the uncoupling of the electron transport in the Chl. Cd was also found to inhibit the electron flow on the reducing side of PS I (Siedlecka and Askі, 1993). The negative effects of HM can also be observed in the carboxylating phase of photosynthesis. The main targets of the influence of HM are two key enzymes of CO_2 fixation, ribulose 1,5-bisphosphate carboxylase (RuBisCO) and phosphoenol pyruvate carboxylase (PEPC). Cd^{2+} ions lower the activity of RuBPC and damage its structure by substituting for Mg^{2+} ions, which are important cofactors of carboxylation reactions, and may also shift RubisCO activity towards oxygenation reactions and the glycolate that is produced moves from the Chl to peroxisomes, where it is oxidized by glycolate oxidase (GO) forming H_2O_2 (Takahashi and Murata, 2008).

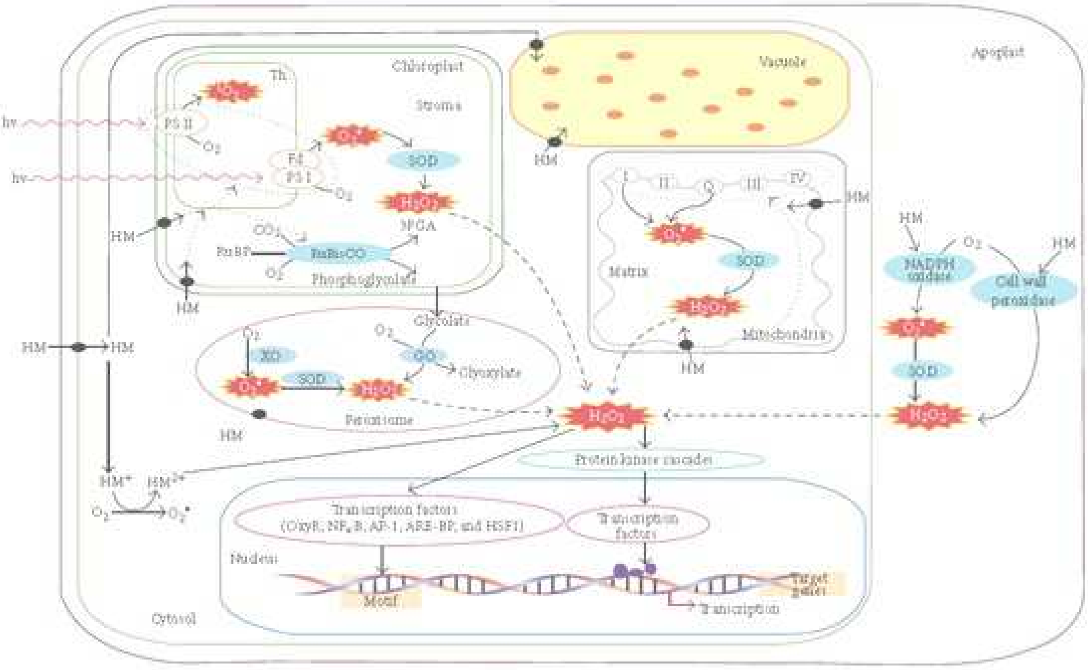


Fig.3: Heavy metal-induced ROS production in different organelles of plant cells and ROS-induced signaling in defense gene expression. Yellow circles in the vacuole designate the deposit of HMs.

ROS are normally scavenged immediately at their sites of production by locally present antioxidants. However, when this local antioxidant capacity cannot cope with ROS production, H_2O_2 can leak in the cytosol and diffuse to other compartments. Importantly, beyond their harmful effects on cells, ROS (especially H_2O_2) have been proposed to act as signals in stress response and modulate the activation of stress responsive pathway's proteins and genes (Mittler *et al*.,2004) . As H_2O_2 is immediately produced under HM stress, it is probably a key molecule triggering signal transduction and HM tolerance in plants.

HM toxicity is attributed to three mean reasons: (a) stimulation of ROS and MG production by auto-oxidation and the Fenton reaction or by modification of the antioxidant defense system and the glyoxalase system, (b) direct interaction with proteins due to their affinities for thioyl-, histidyl-, and carboxyl-groups, causing the HMs to target structural, catalytic, and transport sites of the cell, and (c) displacement of essential metal ions from specific binding sites, causing function to collapse. The possible sequential events of ROS-induced damage development in sensitive plants in response to HM stress are summarized in Figure.

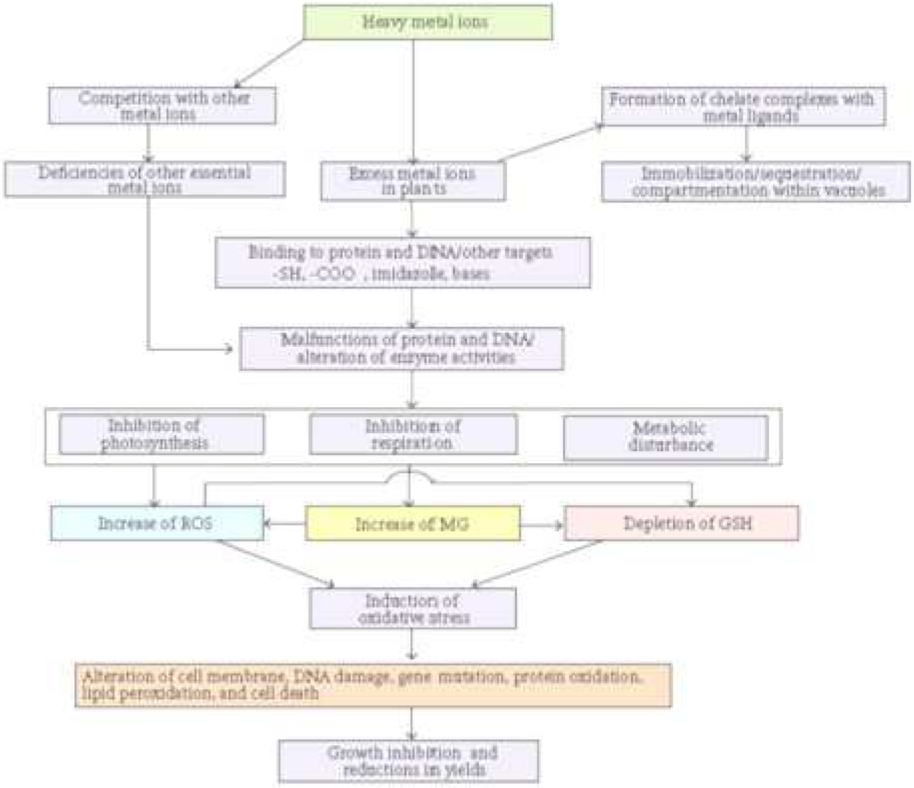


Fig.4: Possible biochemical and molecular mechanisms of heavy metal-mediated ROS induction and damage to the development of higher plants.

VIII. PHYSIOLOGY OF RICE METAL TRANSLOCATION

Rice absorbs Cd2+ in soils, and after several processes of transport Cd finally accumulates into grains. Cd is rapidly transported from roots to shoots by the xylem after absorption (Uraguchi *et al*., 2009). Substantial Cd is detected in the xylem sap and shoot tissues 1 hr after Cd treatment to roots, and this activity of root-to-shoot translocation by the xylem is the determinant for shoot Cd accumulation level. On the other hand, in the panicle neck, phloem is the major Cd transport route into grains (Tanaka *et al*., 2007).

Cd is rapidly translocated from roots to shoots through culms and Cd tends to be retained in nodes. And after 7 hr of Cd treatment, Cd is preferentially deposited into panicles rather than into leaf blades. These suggest that nodes are the important tissue for redirecting Cd transport from roots probably by transferring Cd from xylem to phloem. In addition to Cd absorbed from roots, remobilization of Cd in leaf blades is also likely to contribute to grain Cd accumulation. They suggest that a substantial amount of Cd accumulated in leaf blades before heading is remobilized and transported into grains during the ripening stage.

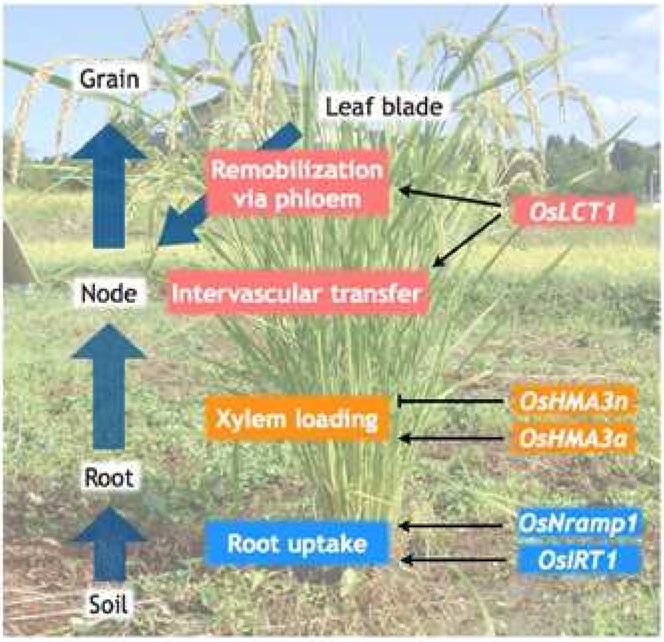


Fig.5: Translocation of heavy metal by different transporter

These physiological studies indicate four major transport processes for rice Cd accumulation: (1) root Cd uptake, (2) root-to-shoot translocation by xylem flow, (3) redirection at nodes and (4) remobilization from leaves

Uptake by roots

OsIRT1 and OsIRT2 have an influx activity of Cd^{2+} as well as Fe^{2+} in yeasts, suggesting that OsIRTs play some role in root Cd uptake especially after release of pounded water during intermittent water management. They suggested that under flooded paddy soils, OsIRTs might be induced by lower levels of available iron and after water release induced OsIRTs might contribute to uptake of Cd which was much available in aerobic conditions. When OsIRT1 was overexpressed, Cd accumulation in roots and shoots was increased under MS medium containing excess Cd, but this phenotype was not observed in the field condition (Lee and An, 2009). These suggest that OsIRT1 is potentially involved in root Cd uptake but its contribution is largely affected by the environmental (soil) conditions.

Xylem loading and root-to-shoot translocation

In *A. thaliana*, the P1B-type ATPase AtHMA2 and AtHMA4 regulate root-to-shoot translocation of Cd and Zn Following the identification of the genes for xylem Cd transport in *A. thaliana* and *A. halleri*, OsHMA3 has been identified as a regulator for xylem Cd transport in rice by mediating vacuolar sequestration of Cd in root cells (Ueno *et al*. 2009). Compared to AtHMA4 and AhHMA4, OsHMA3 has some unique features. All these HMAs mediate Cd efflux transport, but OsHMA3 reportedly does not transport other metals such as Zn, whereas AtHMA4 and AhHMA4 functions in both Zn and Cd transport. Subcellular localization also differs between OsHMA3 and others. OsHMA3 is suggested to be localized to the vacuolar membrane, but AtHMA4 and AhHMA4 are localized to the plasma-membrane.

Phloem transport into grains

A transporter gene involved in phloem Cd transport (Uraguchi *et al*., 2011) named OsLCT1 expression was higher in leaf blades and nodes during reproductive stages. Especially in node I, the uppermost node, OsLCT1 was mainly expressed in diffuse vascular bundles which connected to panicles. Cd levels in grains and phloem exudate from leaf blades were substantially reduced in RNAi plants compared to control plants, although Cd concentration in xylem sap did not differ. These results suggest that OsLCT1 in leaf blades functions in Cd remobilization by phloem, and in node I, OsLCT1 is likely to play a part in intervascular Cd transfer from enlarged large vascular bundles to diffuse vascular bundles, which connect to the panicle. This is the first identification of a transporter for phloem Cd transport in plants.

TRADITIONAL BREEDING APPROACH FOR TOLERANCE TO HEAVY METAL TOXICITY

No crop varieties have been released as a product of selection programme consciously designed for heavy metal stress. Exi*s*ting metal tolerant varietie*s* have been developed by breeder where natural selection was operating (Srikanth *et al*., 1995). Proper methodology that must ensure an efficient evaluation of a large number of plants, but reducing environmental contamination. Rapid screening for selection of heavy metal-tolerant plants in hydroponics is an efficient method of screening germplasms without environmental hazard. *Cd23* M3 mutant line of tomato has been identified as tolerant to cadmium using this selection procedure.

Rice is a staple food for nearly half of the world's population and is the largest source of dietary intake of toxic Cd in many countries. Cd is not phytotoxic at the low concentrations that are of concern for human health (Chaney, 1998). So to identify low Cd content brown rice Ueno and co-worker conducted a study on core collection of rice that accounted for 90% of the genetic diversity and 80–90% of the phenotypic diversity of the original population. Large variation in shoot Cd concentration (13-fold between the lowest and highest Cd-accumulating accessions) was found. They have compared Cd content between Indica and Jponica Rice. Overall, indica accessions (Badri Dhan) showed higher Cd concentrations in the shoots than Japonica accessions (She War). These data provide valuable information that may be used to select and breed low or high Cd accumulating cultivars in the future.

Charlson and co-workers in 2004 coducted a detail study to see the relation between cyst nematode and chlorosis. Iron-deficiency chlorosis (IDC) and soybean cyst nematode (SCN) result in yield and income losses for soybean growers in the U.S. Breeding programs are identifying soybean genotypes with resistance to IDC using calcareous soils infested with SCN, where SCN might interfere with evaluation. Four groups of genotypes were used in the study. One group was comprised by ten F_2derived lines, a second group was SCN-resistant genotypes, third group was formed by lines with varying degrees of IDC resistance. From this study they have concluded that there was no association between field chlorosis scores and SCN infestation for either population individually, neither for the two populations over the two years, nor for the data pooled over populations, parents, and the five IDC controls for each individual year. Nutrient solution chlorosis scores of the four parents plus the five IDC controls were not correlated to SCN field infestation for either year.

Genetics of tolerance to iron chlorosis in rice was studied by Hoan and coworkers, 1992 in eight crosses involving parents distinctly in their level of tolerance. Tolerance to Fe chlorosis was dominant over susceptibility. They were controlled by two sets of genes with complementary gene action. Gene Ic_1 is the basic and in complementation with Ic_3 and it confers tolerance. Likewise Ic_2 with Ic_4 confers tolerance. $Ic_1$and Ic_2 are non-allelic and in the absence of their complementary gene Ic_3 and Ic_4 ineffective. Tolerant ARC 1037 and Cauvery assigned as **$Ic_1Ic_2Ic_3Ic$.** The susceptible ARC 5723 has been assigned as **$Ic_1Ic_2Ic_3Ic_4$** and IET 9828 and IET7614 as **$Ic_1Ic_2Ic_3Ic_4$**.

Pollen culture offers an efficient experimental system, for genetic manipulation. The good complementary characteristics of two parents can be combined in pollen plants and in one generation the dihaploid plants can be developed. Several workers emphasised the importance of anther culture in rice and reported studies on callus formation and plant regeneration. From a cross of *indica* x Basmati rice, anther culture in F_1 hybrids resulted in isolation of genotypes having desirable agronomic characteristics (Rohilla *et al*., 1997).

Patil and coworkers in 1998 under took a study on three hybrids of rice with an objective to isolate genotype having tolerance to iron chlorosis and improved grain quality. Callus formation and plant generation was observed only in the hybrid Prabhavati x Basmati 370 (**Table-2**), which was also very low. The spontaneously diplodised plants were studied for their trueness and their progenies were studied for the important characteristic Variability was quite evident among the different progenies for the characters studied. Promising cultures were evaluated in multi location trials for three years. One of the cultures Parag 401 has been released for cultivation under upland irrigated conditions. It is superior to the parent Prabhavati and check Sugandha for yield and quality characters having tolerance to iron chlorosis.

Based on the performance of the callus ,haploids were derived from anther culture. Haploid plants are diplodised and the diplodised plants were screened for yield and tolerance to chlorosis. Selected lines were evaluated in multi locational trial. Among all the line tested ACR 401 (Parag 401) was selected as a tolerant to iron chlorosis.

MUTATION **BREEDING**

Mutation breeding is the process of exposing seeds to chemicals or radiation in order to generate mutants with desirable traits to be bred with other cultivars. Plants created using mutagenesis are sometimes called mutagenic plants or mutagenic seeds. Ishikawa and coworker in 2010 studied the effect of ion beam radiation on rice crop and screened the low Cd content (lower than the wild type) lines.

Irradiated seeds of the most popular Japanese temperate japonica rice cultivar, Koshihikari, with accelerated carbon ions were produced. Three low-Cd mutants (lcd-kmt1, lcd-kmt2, and lcd-kmt3) were identified in initial screening for grain Cd concentration from among 2,592 M2 plants grown in Cd-polluted soil. The grain Cd concentration in the three mutants was <0.05 $mg \cdot kg^{-1}$, compared with an average of 1.73 $mg \cdot kg^{-1}$ in the WT Koshihikari parent. The root and shoot Cd concentrations were significantly lower in allM3 lcd-kmt mutants than in the WT, when the seedlings were exposed to Cd in hydroponics. The concentrations of iron (Fe), zinc (Zn), and copper (Cu) in shoots and roots did not differ significantly between the lcd-kmt mutants and the WT. However, the manganese (Mn) concentration in the shoots was significantly lower in the mutants (73.6–79.7 $mg \cdot kg^{-1}$) than in the WT (1,004 $mg \cdot kg^{-1}$). There was no difference in plant growth among the WT and lcd-kmt1 or lcd-kmt2 mutants, but the growth. of lcd-kmt3 was reduced under the sufficient Mn level in hydroponics. These results suggest that Cd might be transported via the Mn pathway into the roots.

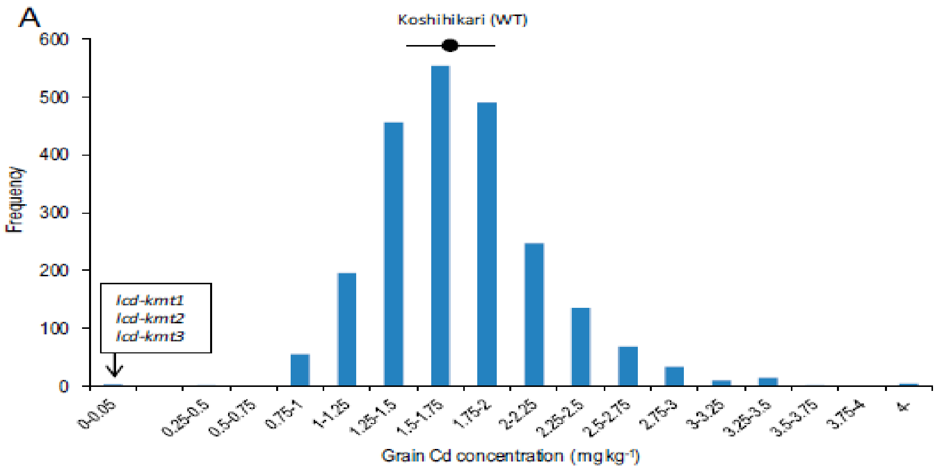
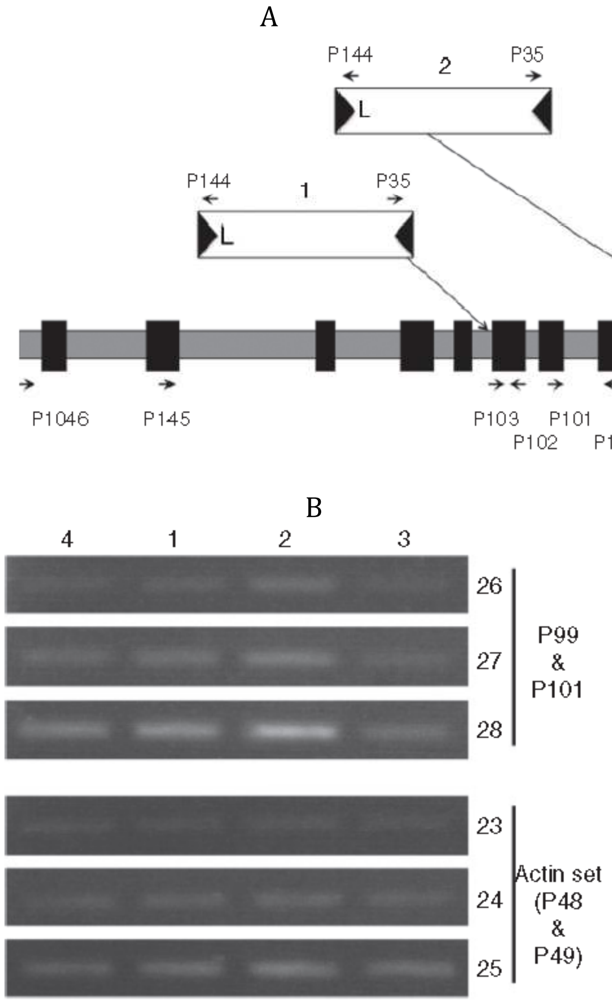


Fig.11: Cd concentration in grain of different rice genotypes

Recent research has revealed that metal-transporting transmembrane proteins have crucial roles in the uptake and translocation of heavy metals (Colangelo and Guerinot, 2006). Some groups of transporters act to transport heavy metals into the cytoplasm from intracellular compartments or from outside of the cell. Examples of such proteins include the natural resistance-associated macrophage protein, the zinc regulated transporter (ZRT), the IRT-related protein families and the Yellow Stripe 1-Like family (Curie *et al*., 2001). Another group of transporters pumps the heavy metals across the plasma membrane or organellar membrane from the cytoplasm. This class of transporters in the P1B-ATPase family, known as heavy metal ATPases, includes the copper/ silver (Cu/Ag) transporters and zinc/cadmium/cobalt/lead (Zn/Cd/Co/Pb) transporters. Cu/Ag transporters have been extensively characterized in *Arabidopsis thaliana* and include AtHMA5, AtHMA6/PAA1, AtHMA7/RAN1 and AtHMA8/ PAA2.

Widespread soil contamination with heavy metals has fostered the need for plant breeders to develop new crops that do not accumulate heavy metals. Metal-transporting transmembrane proteins that transport heavy metals across the plant plasma membrane are key targets for developing these new crops. *Oryza sativa* heavy metal ATPase 3 (OsHMA3) is known to be a useful gene for limiting cadmium (Cd) accumulation in rice. OsHMA2 is a close homolog of

OsHMA3, but the function of OsHMA2 is unknown. To gain insight into the function of OsHMA2, they analyzed three Tos17 insertion mutants. The translocation ratios of zinc (Zn) and Cd were clearly lower in all mutants than in the wild type, suggesting that OsHMA2 is a major transporter of Zn and Cd from roots to shoots (**Satoh-Nagasaw *et al.*, 2011**)



Fig' Schematic representation of the Tos17 insertion sites in OsHMA2 and RT–PCR detection of OsHMA2 transcripts in the transposon mutants. (A) Diagram of insertion positions of Tos17 in OsHMA2. OsHMA2 has nine exons (black boxes) and eight introns (gray boxes). Three mutants [oshma2-1 (1), oshma2-2 (2) and osham2-3 (3)] with Tos17 insertions (open bars with black rectangles) were used in this study. The letter 'L' in the open bars indicates the left border of Tos17. Arrows show the positions and orientations of the primers used for PCR to clone cDNAs and perform RT–PCR. The numbers under the arrows give the primer name available in (B) Semi-quantitative RT–PCR analysis of OsHMA2 transcripts in the roots of 2-month-old plants of the wild type (4), oshma2-1 (1), oshma2-2 (2) and osham2-3 (3). The number immediately to the right of the gel image is the number of PCR cycles performed. The primer sets are identified in the rightmost column. Bands from the actin primer sets are positive controls showing the total amount RNA

The accumulation of OsHMA2 transcripts in the mutant lines was determined by reverse transcription–PCR (RT–PCR) using the primers P99 and P101.This indicates that the OsHMA2 gene was normally transcribed with Tos17 insertion and was accumulated. All mutants had stop codons within the first 30 nucleotides that differ from the wild type and all aberrant versions of proteins of OsHMA2 are shorter than the normal OsHMA2 protein. To confirm the OsHMA2 they were grown under hydroponic conditions with Cd. The concentrations of Cd and Zn in the roots of osHMA2 mutants were not significantly different from those of the wild type but conc. in shoot is lower than wild type. These results indicate that OsHMA2 is not responsible for the uptake of heavy metals but is involved in the translocation of Cd and Zn from roots to shoots.

MARKER ASSISTED BREEDING

Marker-assisted (or molecular-assisted) breeding is the use molecular markers to track the genetic makeup of plants during the variety development process. It provides a dramatic improvement in the efficiency with which breeders can select plants with desirable combination of genes. A molecular marker is a genetic tag that identifies a particular location within a plant's DNA sequences. Markers can be used in transferring a single gene into a new cultivar or in testing plants for the inheritance for many genes at once. Markers can be based on either DNA or proteins. Recent advances in understanding molecular and physiological mechanisms of abiotic stress responses, along with breakthroughs in molecular marker technologies, have enabled the dissection of the complex traits underlying stress tolerance in crop plants. Quantitative trait loci (QTLs) controlling different abiotic stress traits form the basis for a precise marker-assisted backcrossing (MABC) strategy to rapidly transfer tolerance loci into high-yielding, but stress-sensitive varieties.

Hybrid chlorosis in F_2 generation has been reported only in rice (*Oryza sativa* L.) and interspecific crosses among *Melilotus* species .Sato and co-worker in 1984 incidentally found a case of hybrid chlorosis in the F_2 population from a cross between two Japanese native cultivars: J-147 and J-321. Its first symptom was discoloration of the second or third leaf. The yellowish part expanded gradually. Then the whole plant died within 20 days, yielding no seed. The phenomenon was caused by a set of mutually independent duplicated recessive genes, named *hca-1* and *hca-2.* Ichitani *and coworkers in* 2012 conducted a study to identify the location of these genes in chromosome. Linkage analysis with gene specific was done in F4 population derived fron the cross J-147 and IR24. In this study, linkage analysis of the genes causing hybrid chlorosis in F_2 generation in rice, *HCA1* and *HCA2*, was performed. *HCA1* and *HCA2* are located respectively on the distal regions of the short arms of chromosomes 12 and 11.

Linkage map showing the respective locations of *HCA1* and *HCA2* on rice chromosomes 12 and 11. (a) Linkage map of *HCA1* on rice chromosome 12 constructed from an F_4 line derived from the cross between J-147 and IR24 (b) RFLP framework maps of rice chromosomes 12 and 11. (c) Linkage map of *HCA2* on rice chromosome 11constructed from another F_4 line derived from the cross between J-147 and IR24

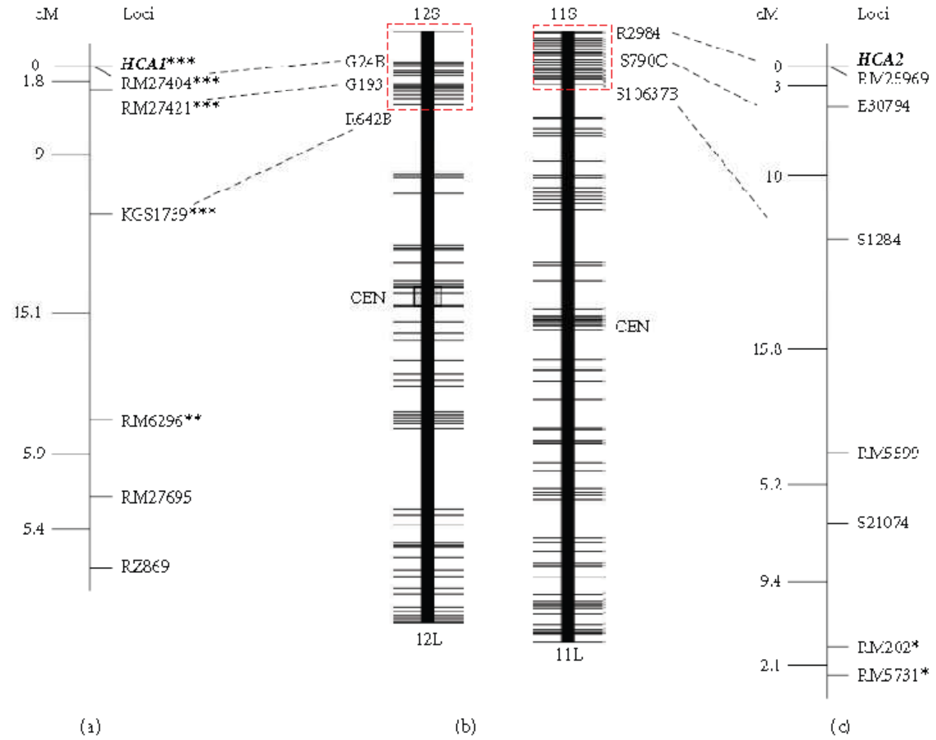


Fig.16: Linkage map of *HCA1* and *HCA2 gene in chromosome 11 and 12.*

In this study, linkage analysis of the genes causing hybrid chlorosis in F2 generation in rice, *HCA1* and *HCA2*, was performed. *HCA1* and *HCA2* are located respectively on the distal regions of the short arms of chromosomes 12 and 11. These regions are known to be highly conserved as a duplicated chromosomal segment. The molecular mechanism causing F_2 chlorosis deduced from the location of the two genes was discussed. The possibility of the introgression of the chromosomal segments encompassing *HCA1* and/or *HCA2* was also discussed from the viewpoint of Indica-Japonica differentiation.

Rice (*Oryza sativa* L.) grain is a major dietary source of cadmium (Cd), which is toxic to humans, but no practical technique exists to substantially reduce Cd contamination. Carbon ion-beam irradiation produced three rice mutants with <0.05 mg Cd·kg^{-1} in the grain compared with a mean of 1.73mgCd·kg^{-1} in the parent, Koshihikari.Sequence analysis revealed that the mutants have different mutations of the same gene (OsNRAMP5), which encodes a natural resistance- associated macrophage protein. Functional analysis revealed that the defective transporter protein encoded by the mutant osNRAMP5 greatly decreases Cd uptake by roots, resulting in decreased Cd in the straw and grain. In addition they developed DNA markers to facilitate marker-assisted selection of cultivars carrying osNRAMP5 (**Ishikawa *et al.,* 2012**).

PHYTOREMEDIATION

Phytoremediation is the direct use of living green plants for in situ, or in place, removal, degradation, or containment of contaminants in soils, sludges, sediments, surface water and groundwater. To remove pollutants from soil, sediment and/or water, plants can break down, or degrade, organic pollutants or contain and stabilise metal contaminants by acting as filters or traps. The uptake of contaminants in plants occurs primarily through the root system, in which the principal mechanisms for preventing contaminant toxicity are found. The root system provides an enormous surface area that absorbs and accumulates the water and nutrients essential for growth, as well as other non-essential contaminants. Researchers are finding that the use of trees (rather than smaller plants) is effective in treating deeper contamination because tree roots penetrate more deeply into the ground. In addition, deep-lying contaminated ground water can be treated by pumping the water out of the ground and using plants to treat the contamination.

Main steps in phytoextraction:

1. A metal fraction is absorbed at root surface
2. Bioavailable metal moves across cellular membrane into root cells
3. A fraction of the metal absorbed into roots is immobilized in the vacuole
4. Intracellular mobile metal crosses cellular membranes into root vascular tissue (xylem)
5. Metal is translocated from the root to aerial tissues (stems and leaves)

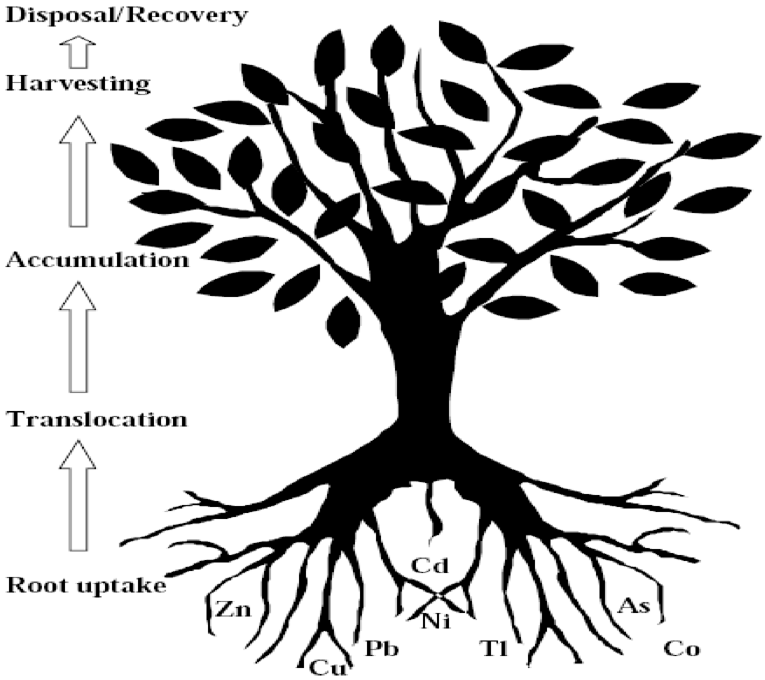


Figure 1. Phytoextraction of metals.

So one way of improvement of tolerance is to developed tolerant cultivars, which has got all the above mentioned tolerance mechanism. Phytoremediation is another indirect way to improve tolerance to heavy metals by reducing toxicity of heavy metals from the soil. Certain plant has got enormous tolerance mechanism due to which they can absorb more heavy metals from soil and can clean soil. Phytoextraction will be more economically feasible if, in addition to metal removal, plants also produce biomass with an added value (Vassilev *et al*., 2004). For example, energy crops (oilseed and willow), fibres and fragrance producing plants could be used to recover these valuable products (Schwitzguébel *et al*., 2005). Sunflower (*Helianthus annuus* L.) is a promising plant to remove heavy metals like zinc or copper (Lin *et al*., 2003), and several radionuclides from contaminated environment (Dushenkov *et al*., 1997). A negligible concentration of toxic metals in sunflower seeds and oil should also limit the risk of food chain contamination and allow the use of oil for technical purpose (Madejón *et al*., 2003).

IMPROVEMENT OF PHYTOREMEDIATION

Nehnevajoba, 2001 has reported that when mutagenised M_2 seeds along with control plant grown in hoagland media, some of the sunflower mutants accumulated a higher amount of metals in roots than the control, but metal accumulation in the shoots was very low. A mechanism, responsible for metal immobilisation in the roots and subsequent inhibition of ion translocation to the shoots, is metal binding to the cell wall. Metals can also be complexed and sequestered in cellular structure (e.g., vacuole), becoming unavailable for translocation to the shoots (Lasat *et al*., 1998). Mutants showed an enhanced uptake of all three metals Cd, Zn and Pb than the control one.

Plants regenerated from metal tolerant tissue cultures were presumed to carry heavy metal tolerance. Hydroponic experiments led to the testing of 30 individual *B. juncea* variants originated from Cd or Pb selection lines. A 2-3 times higher metal accumulation in shoots and even a 6 times enhanced Cd shoot extraction compared to the control plants indicated success of the *in vitro* selection. From the 30 individual variants, 7 regenerants showed a significantly higher shoot metal extraction than the control plants (**Nehnevajoba, 2001**). These findings are in good agreement with Guadagnini, 2000 who used the *in vitro* breeding for selection of tobacco variants with enhanced metal uptake. He has observed 15 % of the tested variants with significantly higher shoot metal accumulation, as compared to the control.

GENETIC ENGINEERING FOR IMPROVEMENT OF PHYTOREMEDIATION

Introduction of DNA encoding enzymes or other proteins from other living organisms. Modification of primary and secondary metabolism and by adding new phenotypic and genotypic characters to plants with the aim of understanding and improving their phytoremediation properties (Davison, 2005). Many reports have supported the increase of valuable natural products through the over expression of biosynthetic genes with a strong promoter and a suitable Signal sequence to control the preferred subcellular localization (Ohara *et al*., 2004).

Genes to change the oxidation state of heavy metals

Introduction of bacterial merA and merB gene encoding mercuric oxide reductase which convert Hg^{2+} to low toxic volatile mercuric oxide (Rugh *et al*., 1996). Hg (o) later release as a volatile compound. The resulting form of the metal is volatile, so that one can create plant capable of metal remediation by Phytovolatilization. But Regulatory concerns restrict the use of plants modified with *merA and merB.*So to avoid this problem Plants engineered to express MerBpe (an organomercurial lyase under the control of a plant promoter) may be used to degrade methyl-mercury and subsequently remove ionic mercury via extraction.

Phytochelations for metal sequestering

Genes controlling the synthesis of peptides that sequester metals, like phytochelatins e.g., the Arabidopsis *cad1* gene (Howden *et al.,* 1995) can be engineered to improve metal sequestration. Cadmium sensitive mutants of *Arabidopsis thaliana, were* sensitive to cadmium to different extents and were deficient in their ability to form cadmium-peptide complexes. Mutants had wild-type levels of glutathione, *in vitro* assays demonstrated that each of the mutants was deficient in PC synthase activity. These results demonstrate conclusively the importance of PCs for cadmium tolerance in plants.

Plant metal transporters

These are generally proteins that are found in the cell membrane, which have an affinity for metal ions. Some of the transporters identified so far include *Arabidopsis IRT1* gene that encodes a protein that regulates the uptake of iron and other metals (Eide *et al*., 1996).

Proteins for metal accumulation

A protein, NtCBP4 that can modulate plant tolerance to heavy metals found in tobacco. (Arazi *et al*., 1999). Transgenic lines having higher levels of NtCBP4, exhibiting improved tolerance to Ni and hypersensitivity to Pb. First plant protein that modulates plant tolerance and accumulation of Pb. This gene could be useful for improving phytoremediation strategies (Alkorta *et al.,* 2004).

CONCLUSSION

The numerous deleterious health effects upon exposure to toxic HM's in the environment is a matter of serious concern and a global issue. So it is necessary to tackle with the toxicity of the heavy metal to plant as well as human health. Molecular and cellular adaptation of plant cells in response to HM stress appears to be necessary to improve plant HM tolerance. The increased availability of gene deletion mutation or of plant over or under expressing certain key gene will provide valuable information in relation to the tolerance mechanism. Huge variation for heavy metal tolerance which is present in nature may be used to select and breed heavy metal tolerant crop plant.

Year	Zinc	Copper	Cadmium	Lead	Nickel	Chromium	Total
2011	1,818.4	1,625.1	10.1	1,106.9	180.1	623.7	5,364
2012	1,894.2	1,692.9	10.5	1,153.0	187.6	649.7	5,588
2013	1,973.1	1,763.4	11.0	1,201.0	195.4	676.8	5,821
2014	2,055.3	1,836.9	11.4	1,251.1	203.5	705.0	6,063

2015	2,141.0	1,913.4	11.9	1,303.2	212.0	734.3	6,316
2016	2,230.2	1,993.2	12.4	1,357.5	220.9	764.9	6,579
2017	2,323.1	2,076.2	12.9	1,414.1	230.1	796.8	6,853
2018	2,420.0	2,162.8	13.4	1,473.0	239.7	830.0	7,139
2019	2,520.8	2,252.9	14.0	1,534.4	249.6	864.6	7,436
2020	2,625.8	2,346.8	14.6	1,598.3	260.0	900.7	7,746
2021	2,735.3	2,444.6	15.2	1,664.9	270.9	938.2	8,069
Total	24,737	22,108	137	15,057	2,450	8,485	72,975

Table.1: Total amount of heavy metal generate in year in India (in tons)

Crosses		particulars			
	Anthers inoculated	Callus formation	S/R	Green plants	Albino plants
Parbhavati x B 370	560	7 (12%)	45	22	23
Parbhavati x IET 8573	450	Callus could not be obtained			
Parbhavati x Karnal Local	530	Callus could not be obtained			

Table.2: Callus formation in different hybrids

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