Rough Translation

Scientific Cooperation Project

Brazil – Japan

Development of soybean plants

tolerant to drought and heat

Scientific cooperation – Brazil and Japan

Section 1 – Project identification

1.1 Title

Development of molecular breeding technology of crops with stress tolerance against degradation of global environment.

1.2. Predictable period

60 months

1.3. External resource

JST – *Japan Science and Technology Agency.*
JICA – *Japan International Cooperation Agency.*

Counterpart in Japan: JIRCAS - *Japan International Research Center for Agricultural Sciences*, under the coordination of the research Dra. Kazuko Yamaguchi – Shinozaki.

1.4. Estimated costs (In USA dollars)

External resources demand:

Brazil - US$ 3.000.000.00 / 5 years

US$ 600.000.00 / year

Japan – US$ 1.500.000.00 / 5 years

US$ 300.000.00 / year

Counterpart estimated from Brazil:

- Researchers payment: ~US$ 3.000.00/research/month
  Total of 14 researchers from Embrapa (60 months): US$ 2.520.000.00

- Laboratories technician payment: Annalists (10): US$ 2.000.00/technician/ month
  Total of technician from Embrapa (60 months): US$ 1.200.000.00

- Scholarships from CNPq (already obtained):
  DTI (1) – US$ 1.000.00/ month
  Masters (1) – US$600.00/ month
  Post-Doctoral (1) – US$1.500.00/ month
  Total scholarships (60 months): US$186.000.00

Total Counterpart estimated (60 months): **US$ 3.906.000.00**

OBS.: It was considered only average payments from researches and technicians (from Embrapa) and scholarships in the project. Costs on infra-structure and maintenance (equipments, laboratories, services) were not considered.

1.5. Proponent part

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1.6. Collaborators in Brazil

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- Paraná Agronomical Institute –PR.

1.7. Project abstract

Drought events frequency has increased significantly in the last decade, probably associated with climatic changes, due to global warming. South states in Brazil, responsible for 40% of national soybean production lost more than 25% of crop yield in the last two seasons. If considered indirect losses related to all agribusiness and economy on producer regions, these losses certainly has great impact on society. Many strategies can be used to reduce losses caused by drought, from adequate soil and crop management to irrigation. Other possibility is the development of cultivars more tolerant to water deficit conditions, and in this focus, biotechnology became an important tool in developing cultivars more tolerant / resistant to different biotical and abiotical stress conditions. In the last years, Brazil has assumed the third position on genetically modified plants (GMPs) production in the world, with soybean as the main component in this production. Seeds price equilibrium in the market, in the next decades, and Brazilian soybean competitivety maintenance will depend from Brazilian public and private companies to develop individually or in partnership with other institutes, strategies that make possible the development of GM soybean cultivars or not, which will solve Brazilian producers and market necessity. Since 2004, Embrapa is developing, in partnership, genetically modified plants containing genetic constructions with DREB technology. Many GM lines were obtained and actually, these events are being characterized (molecular, physiological and agronomical) in greenhouse with promising results. Next characterization stages needs to be developed in real fields conditions. During

the work period with DREB technology in Brazil, new advances were obtained from Dra Yamaguchi – Shinozaki team, at abiotic stress laboratory, at *Japan International Research Center for Agricultural Sciences* (JIRCAS), which allowed the improvement of genetic constructions that confers drought tolerance and among that, superiors results were obtained with construction containing *DREB2A* synthetic gene (Patent n° 3178672 PCT/JP2004/01003), which also promote heat tolerance and also constructions containing AREB family genes (Patent in process). Thus, in November 2007, EMBRAPA and JIRCAS signed an agreement to develop GM soybean containing these constructions. This partnership between EMBRAPA and JIRCAS has more than 10 years and has being incentivized and applauded, especially in the proposing of generating genetically modified plants tolerant to drought. This can be observed by the correspondence exchanged between the ministers of agriculture of Brazil and Japan that followed attached. Genes DREB/AREB encodes for transcription factors that activates other genes, responsible for protect cellular structures during dehydration. Using these genetic constructions, Dra. Yamaguchi – Shinozaki's team, already developed rice, wheat, tobacco and *Arabidopsis thaliana* plants with high levels of drought tolerance. Embrapa is the only institutions to develop GM soybean containing DREB construction, engineered and patented by Jircas. And, the new events to be generated containing the DREB2 and AREB constructions that would be considered promising, will be tested by Embrapa's ecophysiology team, in green house, with evaluations on physiological and agronomical responses, under drought conditions. In field conditions, the same studies will be performed, but events are going to be evaluated, only after attainment of authorization by the National Biosafety Commission. The final objective is the development to GM soybean lines tolerant to drought which will to be used in breeding programs at Embrapa to generate new commercial cultivars.

Key words: *DREB2A*, soybean, drought, heat, genetic transformation, GMOs, Real time PCR, plant physiology

Section 2 – Justification and situation diagnosis

Soybean crop importance in Brazil and in the world

Brazil is the second world soybean producer, exporting 75% of production to European markets (Anbio, 2006). In 2006/07 season, this crop occupied an area of 20.687 million hectares, totalizing a production of 58.4 million tons. The USA, major soybean world producer, responded for a production of 86.77 million tons of soybean. Brazilian soybean average productivity is 2.823 kg per hectares, reaching approximately 3.000 kg/ha in Mato Grosso state, the biggest producer state. At Paraná state, second in production, productivity reached 2.995 kg/ha (Embrapa, 2008).

Season 2006/07 crop numbers showed an increase of 1.3 million/ton, superior than season 2005/06, which totalized 53.4 million/ton. This increment occurred in reason of the productivity recovery, which in season 2005/06 was reduced due to drought (Conab, 2007).

Between 2000 and 2007 period, Brazilian exportations of agribusiness products jumped from US$ 20.6 billion to US$ 58.4 billion, an increase of 183.4%. The balance of agribusiness, in this period, grew 235.8%, from US$ 14.8 billion, in 2000, to a historical number of US$ 49.7 billion, at 2007. The main destiny of these external sales from agricultural sectors was the European Union which, in 2007 bought 35.8% of agribusiness exportations, totalizing US$ 20.9 billion.

The soybean complex contributed to the good performance of agribusiness balance. In 2000, Brazil exported US$ 4.2 billion of soybean and sub products and in 2007, this number jumped to US$ 11.4 billion, an increase on 171.3% (Cultivar, 2008).

Soybean sub products sometimes do not received the deserved importance; Brazil is the third major exporter of swine meat and the first in poultry exportation. For example, Japan imports from Brazil 75% of the poultry consumed in that country. And also, all supplied food in swine and poultry industry has soybean as principal component.

Climatic changes and the impact in soybean Brazilian production

In report about agricultural security emitted by the Planning Ministry, drought is appointed to be the main negative environment event for Brazilian agriculture, followed by excessive rain, hail and frozen. Thus, abiotic stresses can reduce crops yield and restrict latitudes and soils where important commercial species are cultivated affecting all society and not only the producers. Specially, in the reproductive phase, losses are highly significant to soybean crop. It would be important to develop plants more tolerant to drought, reducing losses in productivity once, with more time, which can mean days in field condition, the chances of a rainfall would increase.

In general, drought events frequency has increased significantly in the last decade, probably associated with climatic changes, due to global warming. (Stokstad, 2004; Schiermeier, 2006). What would happen with agriculture production if projections for the temperature elevation, due to global warming, were confirmed in the next years? According to some studies, areas adapted to cultivate coffee, rice, common beans, corn, soybean will be reduced practically to half, as soon as Earth temperature reach 5.8°C degrees above actual. This raise might occur in a 50 to 100 years, following predictions from the Intergovernmental Panel for Climatic Changes. For soybean crop, raises of 1°C degree and 3°C degrees in the temperature will reduce sowed area to 2.7 and 2.1 km², respectively. In the worst scenario, with a warming of 5.8°C degrees, adapted area for soybean would be 1.2 million km² (CT Agency, 2005).

For soybean culture, data shows that yield losses due to drought are constant. In 2003/04 season, a reduction of 120.1 million tons of (2.5%) occurred when compared to 2002/03 season, when no climatic problem happened. In the south states, rainfall scarcity caused losses of 1.9 million tons at Paraná and 4.1

million tons at Rio Grande do Sul state, a reduction of 47.8% on productivity (Conab, 2004).

Season 2004/05, was assaulted for severe climatic conditions. South states in Brazil, responsible for 40% of national soybean production lost more than 25% of crop yield. Direct losses were estimated in US$ 2.32 billion. In Rio Grande do Sul, losses were above 70% in soybean crop (Farias et al., 2005) and other states also lost due to water deficit periods (IBGE, 2005). This season was historical; 12.4 million ton grains were not harvest due to drought that assaulted the South and Center Brazilian regions. Soybean crop was again the product more affected, with losses estimated in US$ 2.7 billion.

Social and economic impacts resulted from losses in productivity

Other point that should be analyzed is the influence of drought events in agrarian questions, especially in Brazilian South producer regions. In Rio Grande do Sul and Paraná states; farmers work in smaller than 100 ha areas, representing however almost 50% of the soybean cultivated area. Producers with areas between 100 ha and 1.000 ha represents 40% of total area cultivate in Rio Grande do Sul and 44% at Paraná state (Balbinotti and Roessing, 2006). If for consecutive seasons these small soybean farmers suffer considerable losses and if together with this fact, grain price falls and cambial differences occurs, land sales or confiscation by financial agencies are unavoidable. Thus, these producers migrates to areas where land costs are smaller, such as North and Central Brazilian regions, increasing problems like native areas deforestation. These producers that losses their lands and have financial difficulties make agrarian problems bigger in the country.

Among the alternatives to minimize losses due to water deficit is the use irrigation. However economical factors and water availability are serious problems in this strategy. Other possibility is the development of plants more tolerant to drought. However, some difficulties occur to the breeding researcher, such as the quantification of the stress effect, neither for a lack of methodology nor for the instability and intensity of the stressing factor (Beever, 2000).

In fact, as many losses reduction mechanisms used smaller the producer's chances to have big damages. In this scenario, Biotechnology became an important tool in the development of cultivars more adapted to different biotical and abiotical stress conditions. Research groups in Brazil and in the world are directing efforts in developing molecular strategies that aims to obtain Genetically Modified Plants (GMPs) able to support longer periods of water deficiency. There's no doubt that this tendency in improving and searching for new technologies will continues, once the use of commercial GM crops is expanding radically, in the last decade, with the introduction of interesting characteristics that make possible solve agronomical problems and add value to the final product.

A recent report published in 2009 reveals that, in 2008, the number of countries planting biotech crops increased to 25, comprising 15 developing countries and 10 industrial countries. The top eight countries each grew more than 1 million hectares; in decreasing order of hectare they were; USA (662.55

million hectares), Argentina (221.00), Brazil (115.88), India (77.6), Canada (77.6), China (33.8), Paraguay (22.7), and South Africa (11.8 million hectares). The remaining 17 countries which grew biotech crops in 2008 in decreasing order of hectare were: Uruguay, Bolivia, Philippines, Australia, Mexico, Spain, Chile, Colombia, Honduras, Burkina Faso, Czech Republic, Romania, Portugal, Germany, Poland, Slovakia and Egypt. Biotech soybean continued to be the principal biotech crop in 2008, occupying 65.88 million hectares or 53% of global biotech area, followed by biotech maize (337.33 million hectares at 30%),, biotech cotton (115.55 million hectares at 12%) and biotech canola (55.9 million hectares at 5% of the global biotech crop area) (James, 2008).

In this same document, data shows that in 2008, the number of farmers benefiting from biotech crops globally in 25 countries reached 13.33 million, an increase of 1.3 million over 2007.Of the global total of 13.33 million beneficiary biotech farmers in 2008, (up from 12 million in 2007), remarkably over 90% or 12.33 million (up from 11 million in 2007) were small and resource-poor farmers from developing countries; the balance of 1 million were large farmers from both industrial countries such as the USA and Canada and developing countries such as Argentina and Brazil (James, 2008). These farmers are being benefited by biotechnological tools, once GM crops shows consisted performances with significant social and economical benefits for small and big producers, in developed and non developed countries, showing that molecular biology implies in humanitarian questions yet (James, 2008).

Other point to be observed is that seeds price equilibrium in the market, in the next decades, and Brazilian soybean competitivety maintenance will depend from Brazilian public and private companies to develop individually or in partnership with other institutes, strategies that make possible the development of GM or not soybean cultivars, which will attend Brazilian producers and market necessity. These strategies should enclose since the discovery, development and intellectual protection of molecular process aiming to solve agronomical problems and to aggregate value to the crop, until the search for international partners that might have commercial interests with Brazil and also technologies that improves Brazilian soybean crop.

Specifically, Japan has commercial interests in agricultural area, once imports more that 90% of consumed soybean, being a developed country that has being researching and developing from two decades, plant genetic engineering strategies, aiming to increase drought tolerance. Thus, Jircas, a public Japanese institution, it was developed and patented the DREB technology (Patents DREB1A n^{o} P3183458 and DREB2A n^{o} P3178672 PCT/JP2004/010003) and AREB (Patent in process) which evolves genetic constructions that activates other genes evolved in cellular defenses against desiccation, promoting drought tolerance in the plants that these construction are introduced.

It is important to stand out that Embrapa Soybean, in partnership with Jircas, is the only institution, in the world, that develop work with genetic construction containing *DREB1A* genes (Patent n° P3183458), *DREB2A* gene (Patent n° 3178672 PCT/JP2004/01003), and AREB gene in soybean.

Section 03 – Available scientific literature – a review

DREB technology

This technology already showed many results in different plant species and basically is based on recent molecular studies on gene regulation in plants submitted to dehydration. The DREB (***D**ehydration **R**esponsive **E**lement **B**inding*) transcription factors family also called CBF (***C**-repeat **b**inding **f**actors*) controls the expression of genes in response to environmental stress such as drought, salinity and low/high temperature.

Using the yeast hybridization assay, cDNAs clones were isolated from *DREB1A* gene and its homologues *DREB1B* and *DREB1C* and also, clones from *DREB2A* and its homologue *DREB2B.* Two clones homologues from CBF1 gene were also identified in homologues in *Arabidopsis*, *CBF2* and *CBF3* which corresponds to *DREB1C* and *DREB1A*, respectively (Liu et al,. 1998; Stockinger et al., 1997; Gilmour et al., 1998). Other genes homologues to this family were indentified, *OsDREB1A*, *OsDREB1B*, *OsDREB1C*, *OsDREB1D*, and *OsDREB2A* isolated from rice (Dubouzet et al., 2003), *GmDREBa*, *GmDREBb* and *GmDREBc* (Li et al., 2005) and *GmDREB2A* (Chen et al., 2007) in soybean and, *ZmDREB1A* (Qin et al., 2004) and *ZmDREB2A* (Qin et al., 2007) in corn.

Yamaguchi - Shinozaki et al., (1992) realized the first studies on DREB transcription factors, identifying nine cDNAs named RD (***R**esponsive to **D**esiccation*) from *A. thaliana*. *Northern blot* analysis showed that these genes respond not only to water deficit but also to salt stress, low temperature and abscisic acid (ABA) treatment.

The *rd29 gene,* characterized by Yamaguchi - Shinozaki and Shinozaki (1993) encodes to a protein highly hydrophilic with a structure likewise LEA (late embryogenesis abundant) proteins (Baker et al., 1988; Yamaguchi - Shinozaki and Shinozaki, 1993). This gene has two cis - acting elements in promoter region evolved in a fast response to dehydration, inducing changes in the osmotic potential, which means an ABA independent pathway and an ABA dependent pathway, with a latter answer. This cis - acting element presents a conserved sequence of 9 bp, TACCGACAT, named DRE (***D**ehydration **R**esponsive **E**lement*), important in the *rd29A* gene expression regulation under drought conditions. Other response element evolved in *rd29*A induction is ABRE (***ABA R**esponsive **E**lement*) probably evolved in a latter activation of the *rd29*A promoter.

Promoter region from some genes evolved in protect cellular structures during stress, and that are part in the ABA independent metabolic pathway shows the DRE cis - acting element. These genes expression regulation is activated by DREB stress- inducible transcription factor (Kasuga et al., 2004). DREB proteins present a conserved domain of approximately 60 amino acids named domain ERF/AP2, initially indentified in an APETALA2 protein from *A. thaliana*, which recognize DRE region from these genes (Shinozaki and Yamaguchi - Shinozaki, 2000; Okamuro et al., 1997).

Many genes expressed in water deficit conditions activated by DREB1A protein were already identified and classified in functional categories (Kasuga et al., 1999; Seki et al.; 2001; Oono et al., 2003; Maruyama et al., 2004).

Thus, Kasuga et al., (1999) identified six gene induced by DREB1A protein, evolved in drought, salinity and cold tolerance; *rd29A*, *kin1*, *kin2/cor6.6*, *cor15a*, *rd17/cor47* and *erd10* genes. Seki et al., (2001) identified other six *FL3-5A3, FL5-2I22, FL5-94, FL5-77, FL3-27* and *erd4,* evolved in drought response and controlled by DREB1A.

Oono et al., (2003) identified yet 280 stress inducible genes, between dehydration and rehydration treatment in *A. thaliana*. Among them were identified DREB1A regulated gene such as *rd29A*, *cor15A*, *Kin1*, *Kin2*, *rd17*, *erd13*, *rd28*, *erd4*, *rd20*, *erd9*, *erd7* and *rd22.*

Also, Maruyama et al., (2004) using cDNA microarray assays identified 38 gene activated by DREB transcription factor in *A. thaliana* plants, containing *35S:DREB1A* genetic construction and submitted to cold. The identified downstream genes of DREB were classified into two groups. The first group includes proteins that probably function in stress tolerance such as LEA proteins, antifreeze proteins, hydrophilic proteins, RNA binding protein, an enzyme required for biosynthesis of sugars and protease inhibitors. The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably functions in response to stress. They are transcription factors and enzymes in phospholipid metabolism.

Genetically modified plants of *Arabidopsis thaliana*, tobacco and wheat containing DREB1A transcription factor were obtained, and under the control of the *rd29A* stress inducible promoter, results showed an increase on drought, salinity and cold tolerance in these species (Kasuga et al., 1999; Hsieh et al., 2002; Kasuga et al., 2004).

Tomato plants transformed with *35S:CBF1/DREB1B* genetic construction also presented grown reduction but ABA treatment reverted this retardation effect, indicating that *DREB* gene can acts in development process regulated by phyto hormones. Under water deficit conditions, transgenic tomato plants demonstrated higher drought tolerance when compared to control plants (Hsieh et al., 2002).

Transgenic wheat transformed with *rd29A:DREB1A* when treatment to showed water stress symptoms only after 15 days, while control plants began to show water stress symptoms after 10 days without water (loss of turgor and bleaching of the leaves) with evidencing severe symptoms (death of all leaf tissue) after 15 days without water. Tolerance to water stress was always associated with the presence of the transgene (Pellegrineschi et al., 2002; Oono et al., 2003; Bray et al., 2003).

Oh et al., (2005), also developed transgenic rice plants that constitutively expressed CBF3/DREB1A (CBF3) and ABF3, *Arabidopsis* genes that function in abscisic acid-independent and abscisic acid-dependent stress-response pathways, respectively. CBF3 in transgenic rice elevated tolerance to drought and high salinity, and produced relatively low levels of tolerance to low-temperature exposure. ABF3 in transgenic rice increased tolerance to drought stress alone.

After the drought treatments, control plants showed wilting and drought-induced rolling of young leaves with a concomitant loss of chlorophyll. In contrast to transgenic lines, plants exhibited leaf rolling within 2 days of the stress and exhibited considerably more visual symptoms of drought stress. After 4 days of

drought stress and sub-sequent watering for 5 days, the growth of transgenic lines was almost identical to non stressed control plants. In contrast, the growth of drought-stressed plants was severely inhibited, and these plants never recovered and finally died.

The target genes together with 13 and 27 additional genes are induced further upon exposure to drought stress, consequently making the transgenic plants more tolerant to stress conditions. Interestingly, transgenic plants exhibited neither growth inhibition nor visible phenotypic alterations despite constitutive expression of the CBF3 or ABF3, unlike the results previously obtained from *Arabidopsis* where transgenic plants were stunted.

In a recent study developed at Embrapa Soybean, *rd29A*:*DREB1A*, genetic construction containing *Arabidopsis DREB1A* gene fused to the *Arabidopsis* stress-inducible *RD29A* promoter (*RD29A:DREB1A*) was introduced into soybean, by biobalistics, aiming to obtain soybean plants more tolerant to drought. *AtDREB1A* gene expression under water deficit was confirmed in positive events and the transgene integration stability was checked in T1 generation (Beneventi, 2006).

In other study using the *rd29A*:*DREB1A* positive soybean events, morphological and anatomical traits indicated no large significant differences between GM soybean line named P58 and non-GM BR16 plants (control); no differences in distribution or quantities of xylem and phloem tissues were observed in transformed and non-transformed plants, or with different stress levels or different stages of development (Polizel, 2007). Also, *rd29A*:*DREB1A* insertion in soybean induced the expression of other genes evolved in the drought stress response mechanism when plants were submitted to water deficit treatment, including genes that are not DREB activated and even the expression of soybean endogenous DREB, suggesting indirect activation mechanisms (Polizel, 2007).

In general, the more favorable responses exhibited by transgenic soybean plants were related to the expression of genes evolved in dehydration response, such as, genes evolved in stomatal opening which confers higher stomatal conductance and photosynthetic and transpiration nets, presenting lower leaves temperature. Genes evolved in osmo protection, structural genes that encodes proteins which functions as water channel and guarantee higher water capitation and better water use efficiency and yet genes that among other functions, protects cellular structure and acts like molecular chaperones helping metabolic process pathway to develop, were also identified (Polizel, 2007).

Physiological and anatomical parameters analyzed in GM soybean plants containing *rd29A*:*DREB1A* genetic construction showed that transgenic plants presented higher stomatal conductance and consequently higher photosynthetic and transpiration nets, besides higher photosynthetic efficiency and higher chlorophyll content. Over expression of DREB1A did not induce xenomorphic characteristics in leaves, but probably caused thickness alterations. Although, GM soybean plants had presented physiological responses that suggests an increase to drought tolerance; they did not showed more productive agronomical characteristics (Polizel, 2007; Salinet et al., 2007). However, these parameters could have become limited, once experiments were performed in pots at green

house, avoiding transgenic lines to express all their potential, especially concerning root system. Thus new experiments, in field conditions are scheduled aiming to conduct a more detailed agronomical and physiological characterization on GM soybean lines. If an increased tolerance to drought was confirmed these plants will be transfer to specific drought tolerance breeding program generating cultivar that will reduce productivity losses in environment with water deficiency.

More recently, other transcription factor from DREB family is being used in studies aiming tolerance to drought and heat. DREB2A is important for the regulation of gene expression in response to heat stress as well as drought and salt stress. Although the DREB2A-regulated genes play important roles in drought-stress tolerance, they are not sufficient to withstand freezing stress.

DREB2A seems to function mainly in ABA-independent water stress–inducible gene expression, since expression of DREB2A is strongly induced by drought and high-salinity stresses but not by ABA treatment (Liu et al., 1998). Just like other proteins from DREB family, DREB2 also has a conserved ERF_AP2 DNA-binding domain and recognizes the DRE (***D****ehydration* ***R****esponsive* ***E****lement*) with core sequence, TACCGACAT (Sakuma et al., 2006b).

Over expression of DREB2A in transgenic plants neither caused growth retardation nor improved stress tolerance, suggesting that the DREB2A protein requires posttranslational modification, such as phosphorylation, for its activation (Liu et al., 1998), but the activation mechanism has not been elucidated yet.

DREB2A and *DREB2B* genes are activated, specifically, in response to drought and salinity and are located respectively, at chromosome five and three of *A. thaliana* (Nakashima et al., 2000). However, a study in the whole *Arabidopsis* genome revealed the presence of six homologues of DREB2, but *DREB2A* and *DREB2B* are the main responsible functional factor for these stresses responses (Sakuma et al., 2006b).

According to some studies, *DREB1A* and *DREB2A* genes present differences on DNA binding specificity, as *DREB1A* shows higher binding affinity form A/GCCGACNT sequence while *DREB2A* binds preferably ACCGAC sequence. Thus, based on the genes downstream DREB proteins were categorized into three groups (Sakuma et al., 2006b). The first group consists of downstream genes shared by DREB1A and DREB2A; most of these have ACCGACNT in their promoter regions. The second group consists of DREB1A-specific downstream genes; these genes have A/GCCGACNT in their promoters. The third group consists of DREB2A-specific downstream genes; we found ACCGACNA/G/C frequently in their promoter regions. These different downstream genes between the DREB1A and DREB2A proteins result in different stress tolerance to cold and drought in plants.

Working with *DREB2A* gene, Sakuma and co-workers identified two important domains, one in the center of the protein, between 136 and 165 amino acids which functions as negative regulatory domain and other with transcriptional activation, beginning at 254 position amino acid until the end of C-terminal region. The deletion of the negative regulatory domain transforms DREB2A into the constitutive active form (DREB2A CA) and microarray analysis in GM *Arabidopsis* transformed with *35S:DREB2A* genetic construction showed the induction not only of genes evolved in drought and salinity tolerance but also

in heat (Heat - shock) tolerance (Sakuma et al., 2006a). Among 21 up regulated genes in the *35S:DREB2A* CA plants, 14 genes (*rd29B*, *At1g52690*, *At1g69870*, *At3g53990*, *rd299A*, *rd17*, *Lea14*, *At2g23120*, *Cor15A*, *Kin1*, *Kin2*, *Cor15B, MT2A* and *At1g22985*) were up regulated under drought stress, and their promoter regions carry the DRE core motif(s). These results suggest that these 14 genes are candidates for direct targets of DREB2A (Sakuma et al., 2006b). Nine of these 14 genes encode LEA class proteins, which are thought to protect macromolecules, such as enzymes and lipids, from dehydration (Shinozaki and Yamaguchi-Shinozaki, 1999). Overproduction of these proteins probably improves drought stress tolerance in the transgenic plants.

In other study, also using DREB2A CA microarray analysis of transgenic *Arabidopsi*s over expressing *DREB2A CA* showed that the over expression of *DREB2A CA* induces not only drought- and salt-responsive genes but also heat-shock (HS) related genes such as heat shock factors (*AtHsfA3*), responsible specifically, for induction and regulation of heat shock responsive genes and molecular chaperones (*CPsHSP*, *At1g52560*, *At3g12580*, *At5g59720*) proteins that keep protein folding homeostasis and help metabolic maintenance and cellular structures during heat stress. DREB2A up-regulated genes were classified into three groups based on their expression patterns: genes induced by HS, genes induced by drought stress, and genes induced by both HS and drought stress. Thermotolerance was significantly increased in plants over expressing *DREB2A CA* and decreased in *DREB2A* knockout plants. Collectively, these results indicate that DREB2A functions in both water and HS-stress responses (Sakuma et al., 2006a; Schramm et al., 2008).

DREB2A gene is also gradually induced by H_2O_2 the level of its induction was lower than those by drought and salt stress. We then analyzed the effects of various plant hormone treatments. *DREB2A* was not induced by any plant hormones such as abscisic acid, cytokinin, ethephon, auxin, methyl jasmonate, and salicylic acid. Similarly, *DREB2B* is also induced by drought, salinity and H_2O_2 in *A. thaliana* (Sakuma et al., 2006a).

In maize, *DREB2A* homolog was isolated*, ZmDREB2A* whose transcripts were accumulated by cold, dehydration, salt and heat stresses in maize seedlings. Unlike *Arabidopsis DREB2A*, *ZmDREB2A* produced two forms of transcripts, and quantitative real-time PCR analyses demonstrated that only the functional transcription form of *ZmDREB2A* was significantly induced by stresses. Moreover, the *ZmDREB2A* protein exhibited considerably high transactivation activity compared with *DREB2A* in *Arabidopsis* protoplasts, suggesting that protein modification is not necessary for *ZmDREB2A* to be active. Constitutive or stress-inducible expression of *ZmDREB2A* resulted in an improved drought stress tolerance in plants.

Microarray analyses of transgenic plants over expressing *ZmDREB2A* revealed that in addition to genes encoding late embryogenesis abundant (LEA) proteins (*At3g53040*, *At1g52690*, *At2g36640*, *At3g15670*, *At5g06760*, *At1g01470*, *At2g35300* and *At1g32560*), some genes related to heat shock (*At1g52560*, *At5g03720* and *At3g12580*) metabolism (*At4g37990*, *At1g09350*, *At5g03860*, *At3g21720*, *At5g42800*, *At2g38530*, *At3g55940*, *At1g34580*, *At4g22870* and *At3g10450*) and detoxification (*At1g49570* e *At1g48130*) were also up regulated.

Furthermore, over expression of *ZmDREB2A* also enhanced thermotolerance in transgenic plants, implying that *ZmDREB2A* may play a dual functional role in mediating the expression of genes responsive to both water stress and heat stress.

DREB2 homolog, *GmDREB2A*, isolated from soybean was induced by drought, high salt and low temperature stresses and abscisic acid treatment. The *GmDREB2* bound specially to DRE element in vitro .Furthermore, the over expression of *GmDREB2* activated expression of downstream genes in transgenic *Arabidopsis*, resulting in enhanced tolerance to drought and high-salt stresses and did not cause growth retardation. Analysis of free proline contents in transgenic tobacco indicated that the over expression of *GmDREB2* accumulated higher level of free proline compared to the wild type plants under drought condition. The results from this study indicated that this novel soybean *GmDREB2* gene functions as an important transcriptional activator and may be useful in improving of plant tolerance to abiotic stresses in plants (Chen et al., 2007).

Many transgenic plants more tolerant to drought using DREB2A constitutively active form (DREB2A CA) were developed, using genetic construction with constitutive promoter (35S) and stress inducible promoter (rd29A) and DREB2A homologues genes from *Arabidopsis* (Sakuma et al., 2006a, 2006b; Schramm et al., 2008), maize (Qin et al., 2007) and soybean (Chen et al., 2007). All studies related that not only drought tolerance was increased but also high salinity and heat tolerances were increased in GM transgenic *Arabidopsis*, suggesting that *DREB2A* can induce downstream genes in response to these stresses, in different plants species.

Thus, *A. thaliana* was transformed with both *35S: DREB2A CA* and *rd29: DREB2A CA* genetic constructions and results under stress were compared between lines generated. For drought stress treatment, water was withheld for 2 weeks. They were then watered and grown under control conditions for 3 days. This treatment killed all wild-type plants, but; 60% of the *35S:DREB1A* plants survived. Like *35S:DREB1*A, the *35S:DREB2A CA* plants exhibited tolerance to drought, with survival rates of 62.8 to 83.3%. For the freezing stress treatment, the plants were exposed to a temperature of –6 C for 30 h and returned to 22 C for 5 days. All the wild-type plants died in this treatment, whereas; 40% of the *35S:DREB1A* plants survived. By contrast, only 5.0 to 11.7% of the *35S:DREB2A CA* plants survived. These results indicate that the genes downstream of *DREB2A* play an important role in drought stress tolerance but are not sufficient to withstand freezing stress. The tolerance of the *RD29A:DREB2A CA* plants to drought were compared with that of the *35S:DREB2A CA* and wild-type plants. Only 21.3% of the wild-type plants survived. However, the survival rates of *RD29A:DREB2A CA* plants were much higher, between 83.3% and 88.3% (Sakuma et al., 2006b).

In *Arabidopsis* transformed with *GmDREB2* gene, drought tolerance analysis indicated that after drought treatment and rewatering all the wild type *Arabidopsis* plants died (0/58), whereas 45.9%(28/61) and 21%(8/38) of the *35S:GmDREB2* and the *Rd29A:GmDREB2* transgenic plants survived, respectively, although their survival rate under normal growth condition was

100%. When submitted to saline stress (200 mM NaCl) the phenotypes of the wild type and the transgenic plants were different and the *35S:GmDREB2* plants displayed strong tolerance against high-salt stress with surviving rate higher than that of the wild type plants. These results indicated that the expression of *GmDREB2* gene improved drought tolerance of the transgenic plants. In this study, it was also observed that *35S:GmDREB2* transgenic tobaccos accumulated higher levels of free proline that function as osmolyte in the stress tolerance of plants, than the wild type plants under unstressed and drought stress conditions. These results suggest that over expression of *GmDREB2* gene activated the expression of some downstream genes involving free proline biosynthesis, which, in turn, enhanced tolerance to drought stresses in transgenic plants (Chen et al., 2007).

Thus, based in on results from tests performed in laboratories and green house, many analyses showed that the development of soybean lines containing *rd29A: DREB1A* or *rd29A: DREB2A* genetic constructions are proved promising strategies to obtain events with drought tolerance improved. Many studies demonstrated the importance of transcription factors in acquisition of different stress tolerance, contributing to agriculture and environment, once GM plants transformed with stress inducible allows over expression of many other genes associated to acquisition of stresses tolerance. Although some negative effects such as grown retardation of plants can occur, strategies like the use of stress-inducible promoters to control transcription factors can avoid or reduce these effects (Kasuga et al., 1999; Wang, 2003).

Section 04 – Project description

Expected situation at the end of the project

This project will allow the generation of GM soybean plants containing genetic construction with DREB and AREB family genes, expressing in high levels transcription factors that activate rapidly and also in high levels genes responsible for cellular defenses during water deficit and heat stresses in soybean.

The identification of the best soybean “elite” events, considerable genetically stable and presenting physiological and agronomical characteristics for drought tolerance and also for biosafety issues will be transfer to Embrapa Soybean breeding program, which will introduce the interested characteristic in commercial cultivars by conventional breeding.

With this strategy at cellular defense level we hope to increase cells activity time frame during water deficit and heat situations, fact that can reflect positively in the maintenance of important metabolic and physiological mechanisms that composes agronomical characteristics.

Thus, if it became possible, soybean plants would have more time for a rain fall to occur, reducing or eliminating water deficit. In case it occurs, it will be possible to develop GM soybean plants which will present lower productivity losses during drought periods.

4.1. Project working plan

The acquisition of soybean plants transformed with drought tolerance characteristics will allow minimizing problems due to water deficit. The chosen methodology to develop and realize this strategy is described below in activities.

Activity 1: Project administration

The leadership of the project will be Dr. Nepomuceno's responsibility. Activity 01 will be in charge of the project administration and will integrate research actions, trough exchange of information via email and meetings every six months between project members. At the end of each year, it's intended to join all members responsible for activities to perform presentations, discussions and make compatible all results.

At the end of the second year, it's intended to conduct a meeting between Embrapa and Jircas researchers in a local not defined yet. However, constant communication between both institutions will be considered priority to project coordination in Brazil. Besides, Dr. Kanamori research member from Dra. Y-Shinozaki laboratory is already in Brazil working at Embrapa Soybean set with biotechnology team to develop GM soybean tolerant to drought.

Activity 2: Biobalistics transformation of soybean embryos with DREB, AREB and others genetic constructions

This activity will be responsibility of Dr. Nepomuceno, although at the laboratory this process will be coordinated by Dr. Renata Fuganti (Pos-doc, PhD in Molecular Biology) and Ms Silvana Marin (Embrapa staff member with Bs in chemistry) with the support of Larissa Giroto (MSc in biotechnology) and two biology graduation students, Juliana Leite and Juliane Marinho who will work in developing the biobalistics and *Agrobacterium* methods.

The drought-sensitive Brazilian soybean cultivar BR16 (Oya et al. 2004), will be transformed by particle bombardment transformation, according to Aragão et al., (2000) and Rech et al., (2008).

After surface sterilization, seeds will be soaked in distilled water for 16 h to 18 h. Embryonic axes will be excised and the apical meristems will be exposed and then disposed in circle, in the bombardment medium [BM: MS basal salts supplemented with 3% sucrose and 0.8% phytagel at pH 5.7]. After bombardment, formation of multiple shoots will be induced by transferring and completely immersing the embryonic axes in culture medium [MS basal salts medium, supplemented with benzylaminopurine (5 mg/mL), 3% sucrose and 0.6% agar at pH 5.7], for 16 hours, in the dark, at 28°C degrees.

The explants will be transferred to selection medium (MS basal salts medium, 3% sucrose, 0.15 µM imazapyr and 0.8% agar, vitamin B_5, pH 5.7), at 28°C, with a 16-h photoperiod (50 µmols $m^{-2}s^{-1}$) and relative humidity > 80%, for 45 days. Embryonic axes with multiple shoots will be then individually transferred

to a sand:vermiculite (1:1) mixture moistened with nutrient solution, placed in an acclimation chamber for 28 days, then transferred to a greenhouse at 25°C ±2°C degrees, relative humidity of 80% ± 20% and a light flux density of 1.000 µmol m^{-2} s^{-1}.

To confirm GM soybean positive lines for *DREB* and *AREB* genetic constructs insertions; different sets of primers will be used in PCR reactions.

Activity 3: *Agrobacterium tumefaciens* transformation method

Soybean Brazilian cultivar BR 16 will be used in the experiments. Embryonic tip, cotyledonary node and hypocotyl segment will be used as explants for transformation.

For embryonic tip, mature, dry seeds will be sterilized with sodium hypochlorite and 70% ethanol. Seeds will be then washed three times with distilled water and then soaked for 16 hours in the dark. The cotyledons and primary leaves on the embryonic axes will be excised to expose the meristem.

For cotyledonary node, to prepare the explants, a longitudinal cut along the hilum will be made to separate the cotyledons, and the seed coat and the embryonic axis will be removed. Moreover the cotyledons will be cut horizontally.

For hypocotyl segment, sterilized soybean seeds will be germinated on filter paper wetted with distilled water for one week. Upper hypocotyl segments (about 1 cm long) will be excised from seedling.

All experiments utilized *A. tumefaciens* strain EHA105, which contains a bar gene and β-glucronidase (*gus*) gene. *A. tumefaciens* will be cultured in 100 mL of liquid YEP medium for 1 day. The bacterial culture will be then centrifuged at 5.000 rpm for 10 min, after which the pellet will be re-suspended with co-incubation medium, and the OD will be adjusted to 0.8.

For infection, explants will be incubated in the *Agrobacterium* suspension for 2 hours. Then, the explants will be blotted onto filter paper, and placed on 0.9 % agarose-solidified co-incubation medium (CIM) in the dark, at 20°C degrees, for 5 days.

Explants will be washed with distilled water containing 500 mg/L carbenicillin and 100 mg/L cefotaxime for three times. Then, the explants will be briefly blotted onto filter paper, placed on 0.9% agarose-solidified shoot elongation medium (SEM1) in the dark, at 28°C, under a 16 h / 8 h (light / dark) for 1 week, SEM2 for 1 week and SEM3 for 1 week.

Transgenic plants will be recovered 6-7 months after the beginning of the in vitro cultivation.

Activity 4: Analyses of DREB/AREB constructions in GM soybean plants T_0 and in the following generations

DREB and *AREB* integrity in host genome in the T_0 plants generation and segregation in next generations T_1, T_2,...T_n will be perform using PCR technique and primers sets specifically for inserted constructions.

Activity 5 - Molecular characterization

All these activities will be conducted under the supervision of Dr. Alexandre Lima Nepomuceno and Dra. Francismar C. Marcelino, researchers from Embrapa's staff. They will be done by one PhD Student (Ms Amanda Paiva) and one MSc Student (Ms Cibelle Angels) as part of their thesis practical work.

Activity 5.1. Real time PCR for relative expression of DREB/AREB transgene quantification under drought treatment – a hydroponics experiment

In all GM soybean positive events obtained, DREB/AREB transcription factors expression level will be evaluated (Sakuma et al., 2006a, 2006b).

A hydroponics experiment will be done to induce drought condition. This methodology will allow not only collecting leaves samples, but also roots samples to be analyze concerning DREB/AREB transcription factors expression.

Thus, GM soybean lines and control plants will be put in a hydroponics system. Plants will be grown in plastic containers (30 L) an aerated pH 6.6 balanced nutrient solution (Hewitt, 1963). Seeds will be pre-germinated on moist filter paper in the dark at 25° C ±1°C and 65% ±5% of temperature and relative humidity, respectively. Then, plantlets will be placed in expanded polystyrene trays in such a way that the roots of the seedlings will be completely immersed in the solution. Each tray containing 15 seedlings and was maintained in a greenhouse at 25°C ±2°C degrees and 60% ±5% of temperature and relative humidity, respectively, under natural daylight [photosynthetic photon flux density (PPFD) = 1.5 x 10^3 moles m^{-2} s^{-1} , equivalent to 8.93 x 10^4 lux] and a 12 hours day length (Martins et al., 2008).

After 15 days seedlings will be submitted to different drought induction treatments in which plants will be removed from the hydroponic solution and kept in a tray in the dark without nutrient solution or water for 0 min (control), 30 min, 60 min and 90 min, 15 seedlings being used per treatment.

To gene expression analysis using RT-qPCR, samples from leaves and roots from all treatments will be collected in separate, immediately frozen in liquid nitrogen, and stored at -86°C degrees, until total RNA extraction. Samples will be collected at moderate stress and severe stress of water deficit treatment. Total RNA will be extracted using Trizol (Invitrogen) reagent according to the manufacturer's instructions. The total RNA was transcribed into cDNA first strand using the Reverse Transcriptase MML (Invitrogen) as described by Panchuk et al., (2002) using an oligo-dT primer.

All primers to amplify the chosen genes will be designed using Primer Express v. 3.0 (Applied Biosystems) software, next to 3' gene region, taking in account criteria such as product length (amplicons from 75 to 180 bps), optimal PCR annealing temperature and the likelihood of primers self-annealing.

As a reference gene for normalization, lectin gene (Accession No.: K00821.1) will be used. The PCR reactions will be carried out in technical triplicates using 500 nM for each forward and reverse primers, 12.5 μL of Platinum SYBR Green qPCR SuperMix UDG Kit (Invitrogen) and 5 μL of a 1:10

(v/v) dilution of cDNA in a total volume of 25 μL. PCR parameters will be 50ºC for 2 min, 95ºC for 2 min, followed by 40 cycles of 95°C for 15 sec, 62°C for 30 sec and 72°C for 1 min.

An amplification efficiency curve will be made to test each gene, using four different cDNA dilutions (10, 10^{-1}, 10^{-2} and 10^{-3}). To verify nonspecific PCR products and primer dimmers formation, a melting curve will be performed, immediately after amplification. All analysis will be performed according to $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001) to estimate the relative gene expression.

Statistical analysis of the data obtained will be performed using the software REST 2008 version 2.0.7 (Pfaffl et al., 2002) which allows the hypothesis test *P* value to be calculated for each sample group and determined 95% confidence intervals. *P* represents the probability that the difference between sample and control group is due only to chance and is calculated by performing 2000 random reallocations of the data obtained.

To develop this study, besides Real Time PCR relative quantification, RPA (*Ribonuclease Protection Assay* (RPA) and *Northern Blot* according to Ausubel et al., (1995) will be performed using GM and control samples submitted to drought.

To develop this study, besides Real Time PCR relative quantification, two other techniques will be performed to evaluate gene expression in GM and control samples submitted to drought: RPA (*Ribonuclease Protection Assay* (RPA) following manufacturer's instructions and *Northern Blot* according to Ausubel et al., (1995).

Activity 5.2. Real time PCR for relative expression of genes under drought treatment- induced and not induced by DREB/AREB transcription factors

Some gene known to be DREB/AREB proteins induced and genes known to be drought induced but not by these transcription factors will be also evaluated using the same protocols described above concerning sample collecting and Real Time PCR relative quantification.

Thus, after a search in the scientific available literature for genes that respond to drought thought modulation of gene expression levels, and that presented an up-regulated gene expression in *Arabidopsis thaliana* GM plants over expressing for example DREB during water deficit events, some candidates gene will be selected, such as the stress-inducible target genes of DREB, gene *Gm*LEA14 (*Late Embryogenesis Abundant*) to contribute to osmotic stress protection in both embryonic and vegetative tissues (Accession No.: CA784216), gene *Gm*GR-RBP, a RNA binding protein rich in glycine (Accession No.: AF169205), gene *Gm*PI-PLC, a phospholipase C (Accession No.: U41474), and gene *Gm*STP, a sorbitol transporter protein (Accession No.: AJ563367) among others.

Gene expression of other genes known to be drought inducible in soybean such as *GmP5CS* (Genbank accession No.: 32345693), which belongs to the gene induction *DREB1A* cascade and encodes the galactinol synthase enzyme (GolS, UDP-galactose:myo-inositol galactosyltransferase, EC 2.4.1.123) with key function in raffinose biosynthesis, an important substance to protect seed from

desiccation; and *GmPip1* (Genbank accession No.: U27347), which encodes a plasma-membrane intrinsic protein (PIP), part of a channel responsible for controlling water movement between cells, will be evaluated, among many others.

Activity 5.3. Real time PCR for transgene copy number quantification

The determination of number of transgene copies will be perform using $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001; Ingham et al., 2001) using the amplification of lectin gene as endogenous to all samples and as calibrator, sample from cultivar BR16 non transgenic. This strategy was possible once lectin gene presents only one copy in soybean genome. The number of copies form target gene, normalized by the endogenous reference is compared to the calibrator.

PCR quantitative reactions were conducted in ABI PRISM® 7300 Sequence Detection System equipment (Applied Biosystems, 2003). To amplify the target fragment, multiplex reactions were done using *TaqMan*® methodology. Ideal primers and probe concentrations were initially standardized, considering efficiency amplification for each system. Efficiency was calculated using $E=10^{-1/slope}-1$ formula (Livak et al., 2001).

Activity 5.4. Southern blot method for transgene copy number quantification

The number of transgene copy number will be also determined using Southern Blot technique. To perform the analysis, leaves from GM soybean lines will be collect. After DNA extraction digestion reaction will be done using restriction enzymes. To probe preparation a specific sequence from *DREB/AREB* or other genes will be used.

After digestion, DNA fragments will be separate in agarose gel and then, samples will be transfer to a nylon membrane. Probe will be labeled with ^{32}P – dCTP and hybridized overnight, in room temperature. After that membranes will be washed and exposed in X-ray film.

Activity 6 – Biochemical analysis of multiple enzymes evolved in drought response in DREB plants

Increased production of activated oxygen species such as superoxide radicals and H_20_2 is likely to occur during drought stress. Drought-related physiological changes, such as decrease in leaf water content and stomatal closure, result in limited $C0_2$ availability and the channeling of reducing equivalents to the production of active oxygen species rather than to C02 fixation. In addition, production of H_20_2 also results from the increased catalytic activity of glycolate oxidase in peroxisomes that coincides with elevated photorespiratory activity during drought.

Some enzymes evolved in drought response pathways will be analyzed, in GM and non GM soybean lines, such as ascorbate peroxidades (APX), superoxide dismutase (SOD) and catalase (CAT).

Enzymes activities will determined by spectro photometric methods. Thus, APX activity will be evaluated according to Amako et al., (1994), while SOD and CAT activities will be evaluated by Gupta et al., (1993) and Costa et al., (2005), respectively.

Activity 7 – Microarray analysis of DREB/AREB plants under water deficit and control conditions

To perform an initial gene expression screening, a microarray analyze will be performed using GM and non GM soybean plants aiming to identify genes differential expressed up or down regulated under water deficit and control conditions.

Thus, an experiment will be performed in green house using GM lines generated by transformation and BR16 (original variety) as control. The plants will be divided into two groups: a control group at 15% of gravimetric humidity (GH) (near field capacity) and a stressed group at 5% of GH (moderate stress) (Casagrande et al., 2001).

Each group will comprised twelve plants of each genotype, sown in 10-L pots with sand and nutrient solution under greenhouse conditions (day $30^{o}C \pm 2^{o}C$ degrees; night $22 \pm 2^{o}C$; RH 40%±5%) in a complete randomized block design.

All plants will be allowed to develop for 45 days in normal conditions (15% GH). Drought stress treatment will be initiated by withholding irrigation, until sand humidity reached 5% GH in the flowering stage (R_2); the control group will be kept at 15% GH until the conclusion of the experiment.

Activity 8 – Real time PCR for relative expression of genes up/down regulated in drought conditions identified in microarray studies

After microarray analyses, aiming to obtain a high-degree accuracy of genes differentially expressed under drought conditions in the GM soybean lines, some genes will be chosen to perform a real time PCR relative quantification. Protocols that will be used were already described above in other activities.

Activity 9 – Full transcriptome of DREB/AREB plants using pyro sequencing

Full transcriptome from soybean GM lines containing DREB/AREB genes obtained in this project will be done using Pyrosequencing process. **This** technology was licensed to 454 Life Sciences. The 454 system was developed an array-based pyrosequencing technology which has emerged as a platform for large-scale DNA sequencing.

Activity 10 - Experimental procedure in greenhouse conditions for water deficit induction treatment – pots experiment

All experiments at greenhouse and field conditions will be in the responsibility of Dr. José Renato Farias (PhD in Ecophysiology) and Dr. Norman Neumaier (PhD in Plant Physiology), both Embrapa staff scientists.

To induction and analyze of *DREB/AREB* gene expression experiments will be performed initially in green house conditions. Thus, soybean GM plants and control plants will be cultivated in pots containing sandy soil, at 15% gravimetric humidity (GH) for 31 days post-sowing, until reproductive stage, R_1 (Ferh & Caviness,1979).

After this development period, the irrigation will be withheld from the drought-stress treatments pots until the GH values decreased to 5% (moderate stress). Twenty nine days after, the irrigation will be again withheld to 2.5% GH (severe stress) until harvesting, approximately 30 days. Control plants will be remained with 15% of GH throughout the experiment. To keep the pots in the desired GH values, they will be weight twice a day and water will be added as needed (Casagrande et al., 2001).

The experiment will be performed in randomized blocks design (RDB) with a 2 x 2 factorial arrangement of the treatments involved two gravimetric humidity (drought stress and normal conditions plants) and two genotypes (GM and no GM plants), with four blocks and three biological repetitions per treatment inside each block. The temperature and air-humidity in greenhouse will be monitored and maintained between 17ºC and 40ºC degrees, and between 25% and 90%, respectively. All pots will be irrigated twice a week with 50 mL of pH 6.6 balanced nutrition solution (Hewitt, 1963).

Activity 11 - Experimental procedure in green house conditions for water deficit induction treatment – rewatering experiment

Other experiment in greenhouse conditions just like the one described above will be performed with the difference that after the application of severe stress, a rewatering procedure will be done aiming to analyze *DREB/AREB* gene expression after rehydration.

Activity 12 - Physiological characterization

All experiments and analyses concerning physiological characterization will be in the responsibility of Dr. José Renato Farias (PhD in Ecophysiology) and Dr. Norman Neumaier (PhD in Plant Physiology), both Embrapa staff scientists, who will be in charge of plant physiology and agronomy studies at green house and field conditions.

Physiological parameters such as net photosynthesis, stomatal conductance, chlorophyll content and transpiration rate will be sampled and analyzed in all treatments (control stressed, control no-stressed, GM line stressed and GM line no-stressed) at 06, 12, 20, 27, (moderate stress) 34, 38, 41, 43, 48, 50, 54 and 57 days (severe stress) of water deficit treatment.

Also, foliar area, relative grown net, foliar temperature, intracellular CO_2 concentration, carbon discrimination ($^{13}C/^{12}C$) and macro and micro nutrients in leaves will be also evaluated.

Activity 13 - Metabolic characterization

All experiments and analyses concerning metabolic characterization of GM soybean lines obtained as a result from this project will be coordinated by Dra. Clara Beatriz Hoffman (PhD in Entomology) an Embrapa staff scientist.

Thus, GM soybean samples submitted to the water deficit treatments described above will be evaluated concerning their chemical composition and will be compared to non GM plants.

To identify gliceolines (soybean fitoalexinas), extract from leaves will be analyzed in High Performance Liquid Chromatography (HPLC) equipment while other signal molecules to plant resistance to biotical and abiotical stresses will be evaluated using gas chromatography.

Proline free content in GM plants stressed and no-stressed treatments will also be quantified as proline is an osmoprotect very important in drought conditions.

Activity 14 - Anatomical characterization

Anatomical analyses will be performed in leaves with cuts of $1cm^2$, using a photonic microscopy and a scanning electron microscopy. GM and no-GM genotypes stressed and non-stressed will be collected in two distinct time-points: at R_2 plant development phase, at 20 days post treatment during moderate stress (5% GH), and at R_4 plant development phase, at 34 days post treatment during severe stress (2.5% GH).

For photonic microscopy analysis, sections of leaves will be submerged in 50% FAA (formol 0.5mL, acetic acid 0.5mL and alcohol 50%, 9mL) fixative reagent. The samples will be dehydrated in an alcoholic series and diafanized in xylene. Infiltration and blocking will be done in paraffin and materials will be sectioned in a rotary microtome, in a 10µm thickness before affixing on glass microscope slides for several hours at 40ºC. Deparafination and rehydration will be carried out by soaking the slides in a series of jars with xylene for 40 min, ethanol-xylene (1:1) for 1 min and five changes with descending ethanol concentration for 2 min each. Sections will be stained with astra blue for 5 min and excess stain will be removed by washing the slides with running water for a few seconds, before stained with basic fuchsine for 15 min and washing again.

The slides will be dehydrated in a series of five changes with ascending ethanol concentration for 2 min each and washed with ethanol-xylene (1:1) for 2 min and xylene for 5 min. Sections mounted in Canada balsam will be covered with glass cover slips before microscopic analysis (Jonhasen, 1940). Histometric analysis will be done using Motic Images 2000 1.3 software. An ANOVA analysis and Tukey test will be performed using SAS software (Sass, 1951).

For the scanning electron microscopy, the samples will be previously fixed in glutaraldehyde/paraformaldehyde (Karnovsky modified – glutaraldehyde 2.5%, paraformaldehyde 2.5% in 0.1M phosphate buffer and distilled water), washed in 0.1M phosphate buffer, fixed in 1% OsO_4, washed again in phosphate buffer, dehydrated in gradient ethanol solutions (50-100% ethanol) and critical point

dried with CO_2, in Bal-Tec/CPD-030 (Critical Point Dryer) equipment. Dried samples will be mounted onto aluminum stubs, fixed with carbon tape, coated with gold powder, in Bal-Tec/SCD-050 (Sputter Coater) equipment. Anatomical evaluations and registers will be carried out in a scanning electron microscopy (PhilipsFEI – Quanta 200).

Activity 15 – Biosafety analysis ???

Activity 16 – Crosses between GM DREB/AREB soybean lines X commercial varieties – breeding program

After full characterized, the most promising GM soybean lines containing DREB/AREB genes will be chosen to perform crosses between these lines and commercial varieties aiming to obtain cultivars with drought tolerance increased. Dr. Carlos Arrabal Arias and Dr. Antonio Pipolo are the project breeders (EMBRAPA staff scientists) responsible for this activity together with the whole Embrapa Soybean breeding program team.

Activity 17 - Experimental procedure for water deficit induction in field conditions –Planned liberation on environment

The project leader Dr. Alexandre Nepomuceno had already submitted to National Biosafety Commission (CTNBio) the request for permission to sow and grow GM soybean in the fields of Embrapa Soybean experimental farm.

The experiment will be performed in randomized blocks, containing GM lines containing DREB/AREB genes and non GM lines, sub divided in the treatments: irrigated (I), non irrigated (NI) and under rain out shelters (RS) which will be programmed to close every rain fall, in a period of 30 days between the more sensible stages for water deficit in soybean, stages R_3 to R_6.

Soybean crop management during the season will be conducted as technical recommendations of Embrapa's soybean manual for this region.

During filed experiment, climatic conditions such as water balance, rain fall precipitation, water content in the soil, solar radiation, temperature, air humidity among other parameters will be monitor, as well as physiological and agronomical evaluations will be done by Embrapa's Ecophysiology staff.

4.2. Institutional board

EMBRAPA Soybean has already a structure for development of Genetically Modified soybean lines caring traits of interest for the soybean crop production in South America. The leadership in the project is responsibility of Dr. Alexandre Lima Nepomuceno. Dr. Kanamori (from Jircas) should be the counterpart from the Japanese side in Brazil. In Japan is Dr. Yamaguchi-Shinozaki.

Dr. José Renato Farias (PhD in Ecophysiology) and Dr. Norman Neumaier (PhD in Plant Physiology), both EMBRAPA staff scientists, are in charge of plant physiology and agronomy activities at green house and field conditions. Their main objective is to prove the concept *in vivo*, at actual conditions. Under Dr.

Farias and Dr. Normam supervision two technicians (Mr Nelson Delattre and Mr. Claudinei Toledo) and three field workers; Mr Roseli Cardoso, Mr Cleber Oliveirada Silva and Mr Everaldo Carmo will develop the work needed.

For the transformation process, the coordination will be done by Dr. Renata Fuganti (Pos-doc, PhD in Molecular Biology) and Ms Silvana Marin (Embrapa staff member with Bs in chemistry). As a support team in the transformation work two technicians (Mr. Cesar Silveira, Embrapa staff member with Bs in chemistry; Ms. Larissa Giroto, MSc in biotechnology) develop the activities. One PhD Student (Ms Amanda Paiva), one MSc Student (Ms Cibelle Angels) and two biology graduation students, Juliana Leite and Juliane Marinho also work in developing the biobalistics and *Agrobacterium* methods.

Dra. Francismar C. Marcelino is coordinating the stewardship program that is responsible for organizing the information in terms of biosafety and pre-breeding. Dr Francismar also participates in the molecular characterization experiments with Dr. Ricardo Abdelnoor.

All statistical support and analyses will be performed by Dra. Maria Cristina Neves de Oliveira (PhD in statistics), a member of Embrapa's cientific staff.

Dr. Carlos Arrabal Arias and Dr. Antonio Pipolo are the project breeders (EMBRAPA staff scientists). They will be helping the project coordinator to choose the best events for possible use in the breeding program, and they will do the crosses necessary for the introgression of GM lines project.

Section 5 – Objective and results

5.1. Development objective

Introduce in soybean, by biobalistics and *Agrobacterium tumefaciens* methodologies, *DREB/AREB* genes aiming to obtain GM soybean plants with drought and heat tolerances increased and identify and characterize molecular, physiological and agronomical responses of soybean GM plants to be used at Embrapa breeding program.

5.2. Immediate objective

- Characterize concerning genetic stability, transgene copy number and gene expression levels GM soybean lines containing DREB/AREB constructions;
- Evaluate the induction and gene expression levels of cellular defense native genes in GM soybean lines, containing DREB/AREB constructions when compared to non GM lines, submitted to drought and heat stresses;
- Characterize physiological and agronomical responses of GM soybean lines, containing DREB/AREB constructions in greenhouse and in field conditions.

5.3. Expected results

As a result from all activities implemented in this project developed in partnership with Jircas, the objective is to accomplish every specific activity and objective. Thus, it will be generated many GM soybean lines that will be fully

characterized aiming to identity “elite” events to be transfer to breeders from Embrapa program and partners institutions.

In this focus, after the end of this project, with the success of acquisition soybean positive events containing *DREB/AREB* constructions, new options to develop GM soybean cultivars will be available for breeding programs.

It’s important to stand out that genetic engineering strategies that are being developed confer not only drought tolerance but also heat tolerance, according to results published in the scientific literature obtained from other plant species as already discussed in this project. Extreme temperatures commonly occur together with water deficit periods, so, more tolerant plants will be available to producers, specially favoring the ones that cultivate in regions more badly assaulted by these environmental stresses. The economical and social benefits can be significant from small-medium to big producers, in Brazil and in developing countries showing that genetic engineering is an important tool to be used to solve or mitigate serious environmental and humanity questions.

Section 6 – Requested external cooperation

6.1. External resource choice - justification

Brazil and Japan have already a strong connection, not only culturally, but also scientifically. Specifically with Embrapa, our partnership with Jircas has been of prominent importance for the Brazilian agriculture and livestock in the last years. Jircas, through its visiting researchers, in conjunction with the Brazilian researchers, has been contributing to expressive achievements regarding crop-livestock integration and techniques for sustainable management of agroecosystems. The results achieved in Brazil have also been of help for the development of the agriculture and livestock in neighbor countries in South America, such as Argentina, Paraguay and Bolivia. Besides the technical contribution, the exchange of Japanese and Brazilian researchers, promoted by JIRCAS and EMBRAPA, has also collaborated for the strengthening of the cultural relations between the two countries.

Thus, in November 2003, a MOU (*Memorandum of Understanding*) was signed between Jircas and Embrapa to develop transgenic soybean tolerant to abiotic stress, with *DREB1A* gene construction. Since then, works with Dra. Yamaguchi – Shinozaki’s team, from Jircas abiotic stresses laboratory, allowed the improvement of genetic constructions that confers drought and heat tolerances, significant problems to soybean crop. Once again in 2008, these two institutes signed a second agreement to develop soybean line containing *DREB2A/AREB* genetic constructions, in a complement to the fist agreement, signed for *DREB1A* gene.

It’s important to stand out that Embrapa is the only institute that works on GM soybean containing the DREB constructs, developed by and patent by Jircas IRCAS (*DREB1A* - Patent n° P3183458 and *DREB2A* - Patent n° 3178672 PCT/JP2004/01003).

6.2 Trainings

- Training on DNA microarray analyzes – Tsukuba, Japan. Two months.
- Training on genetic construction methods – Tsukuba, Japan. Two months.

Also the EMBRAPA-JICA project on the development of drought tolerant soybean is programming to give training, not only to EMBRAPA Scientists, but also to many graduation, master and doctorate students since EMBRAPA has a great connection with Brazilian Universities.

6.3. Permanent material

Since 1997, Embrapa Soybean headquarter has structure for work in many areas of molecular biology, such as rooms for radioisotopes manipulation, tissue culture, plant transformation, for cleaning and preparation of material, electrophoresis, among others.

Also, Plant Biotechnology laboratory has a Bioinformatics structure with a Dell Power Edge 2950 server, what allows data bank accesses as well as access to bioinformatics tools developed at the laboratory and/or from public domains, to researchers and partner institutions.

Embrapa Soybean headquarter has yet laboratories specialized in soil and plant nutrition, plant physiology, biochemistry, biological control, entomology, phytopathology, seeds analyses, microbiology, agro meteorology and soil biotechnology. They are all modern and well equipped laboratories, which offer necessary conditions to execute all activities proposed in this project. Besides laboratorial structure, Embrapa Soybean headquarter also has basic structure on greenhouses and field.

6.4. Buildings and installations

Embrapa's biotechnology laboratory is being amplified and will allow at the end of july 2010, a bigger area to students and technicians to work.

Section 7 – Risks and difficulties

There was initially the risk concerning on the recognition from soybean of *A. thaliana* genetic elements. However, previous studies revealed that *At*DREB1A construction eliminated this possibility (Beneventi, 2006). Each event is independent and according to the locus insertion, number of inserted copies or interaction with other chromossomical regions it can occur alteration or inhibition of transgene expression. Thus, events to be analyzed not necessarily will express the DREB/AREB protein introduced.

So, based in previous results with *At*DREB1A genetic construction, it's believed that these constructions will be recognized by soybean.

Also, it's possible that even if DREB and AREB proteins be expressed, they won't activate soybean cellular defenses native genes. Or even if activating these soybean defense genes, they won't reach enough levels to increase drought and heat tolerance.

Section 8 – Social – environmental impact

Drought is one of the most major factors limiting crop production worldwide. In Brazil, the southern states of Brazil, which are responsible for 40% of national production, lost more than 25% of their production due to water deficits in the

2003/04 and 2004/05 soybean crop seasons. In dollars, more that 4 billions were lost due to drought.

Also, abiotic stress, such as water deficit periods not only reduces production yield, but also restrict latitudes and soils were important commercial crops were sown, affecting producers and all society.

Some studies predict that, in a period of 50 to 100 years, Earth temperature will increase between 1 to 5.8 Celsius degrees. This temperature raise will change rain seasons, water evaporation and plant transpiration. Thus, areas that were used to crop production won't be adequate to cultivate anymore. Following global warming, drought episodes will probably increase, thus, it's urgent the necessity of more tolerant cultivars that supports more days in the field without water, increasing the chances of rainfall.

With cultivars more tolerant to abiotic stresses, such as drought and heat, losses can be mitigating. Besides, number recuperation in soybean production, small and medium producers, sometimes assaulted by drought for many crops, will be able to recover their economy, resulting in social and financial improvement.

Section 9 – Personal, biological and environmental security

Embrapa Soybean unit follows the Biosafety Brazilian law (Law number 11.105 from March 24, 2005) in accord to all discard determinations and risks reduction settle by the National Technical Biosafety Commission (CTNBio) and Internal Biosafety Commissions (CIBio) to work with Genetically Modified Organisms (GMOs).

Also Embrapa Soybean has the Biosafety Quality Certification (CQB – number 02/97) and can conduct experiments and works with organisms classified as Risk Class 1 (project situation) following Normative Regulations n^{o}1 and n^{o}2 from CTNBio.

Any alteration on the project schedule, execution difficulties or accident with the GMOs will be immediately communicated to CTNBio. Reports will be made regularly and send to CIBio to compound the Annual Report from the Soybean headquarter to be send to CTNBio.

Section 10 – Embrapa's Soybean staff

- **Researcher's staff**

	Name	Institution	Major	Working Groups (WG)				Remarks
				1. gene transfer	2. molecular analysis	3. physiological analysis	4. field test	
1	Dr. Alexandre Nepomuceno	EMBRAPA	Project Manager/ Molecular Biology / Plant Physiology	○	○	○	○	◎
2	Dr. Norihito Kanamori	JIRCAS	Plant Molecular Biology	○	○			
3	Dr. Norman Neumaier	EMBRAPA	Plant Physiology			○	○	
4	Dr. José Renato B. Farias	EMBRAPA	Agrometheorology			○	○	
5	Dr. Carlos Arrabal	EMBRAPA	Breeding				○	
6	Dr. Antonio Pipolo	EMBRAPA	Breeding				○	
7	Dr. Renata Fuganti	EMBRAPA	Transformation	○	○	○	○	
8	Dr Ricardo Abdelnoor	EMBRAPA	Molecular Biology		○			
9	Dra Franscismar C. Marcelino	EMBRAPA	Molecular Biology	○	○			
10	Dra Maria Cristina Neves	EMBRAPA	Statistics			○	○	
11	Dra Clara Beatriz Hoffman	EMBRAPA	Metabolomics		○	○		
12	Dr Júlio Franchini	EMBRAPA	Crop Management				○	
13	Dr Henrique Debiasi	EMBRAPA	Crop Management				○	
14	Dr. Fabiana Rodrigues	EMBRAPA	Molecular biology	○	○	○	○	
15	Dr. Elibio Rech	CENARGEN	Molecular biology	○				
16	Dr. Celso Marur	IAPAR	Plant Physiology			○	○	
17	Dr. Gedi Sfredo	EMBRAPA	Plant Nutrition			○	○	
18	Dra. Maria Fatima Grossi	CENARGEN	Molecular Biology	○	○			

- **Student's staff**

Amanda A P Rolla – PhD student
Cibelle Engles – Master student
Larissa Girotto – Master
Juliana P Leite – Biology graduation student
Juliane P Marinho – Biology graduation student

- **Technician's staff**

Silvana R R Marin – Biotechnology laboratory
Cesar A Silveira - Biotechnology laboratory
Marcia

- **Green house and field support staff**

Roseli Cardoso
Cleber Oliveira da Silva
Everaldo Carmo

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Section 12 – Annexes

MINUTES OF MEETINGS
BETWEEN
THE JAPANESE DETAILED PLANNING SURVEY TEAM
AND
BRAZILIAN AGRICULTURAL RESEARCH CORPORATION
- EMBRAPA SOYBEAN
ON JAPANESE TECHNICAL COOPERATION
FOR THE RESEARCH ON
"DEVELOPMENT OF SOYBEAN WITH TOLERANCE
TO DROUGHT AND HEAT"

In response to the request made by Brazil for the research project on "Development of soybean with tolerence to drought and heat" (hereinafter referred to as "the Project"), Japan International Cooperation Agency (hereinafter referred to as "JICA") dispatched to Brazil a detailed planning survey team (hereinafter referred to as "the Team") headed by Mr. Narihide Nagayo from August 23 to September 5, 2009. During its stay in Brazil, the Team and Brazilian Agricultural Research Corporation-National Soybean Research Center (hereinafter referred to as "Embrapa Soybean") had a series of meetings and exchange of opinions about the content of the Project and necessary adequate measures by both parties for the smooth implementation of the Project.

As a result of the survey, both parties have reached common understandings concerned with the matters in the documents attached hereto.

These texts were done in both English and Portuguese, each texts being equally authentic. In case of any divergence of interpretation, the English text shall prevail.

Londrina, August 31, 2009

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1

ATTACHED DOCUMENT

1. Background of the Project

Gradual warming of the earth, firstly caused by increasing greenhouse gases with rapid population growth and industrialization, subsequently raises global problems such as aridification of cropland, reduction of crop yield and security of food and feedstuff. Although conventional crop breeders challenged to produce crop plants tolerant to drought, they have not reached outstanding results so far. On the other hand, recent technical progress in genetic engineering based on plant genome research attracts attention to develop crops having improved tolerance to drought by gene transfer. Under such situations research for elucidation of genes involved in drought tolerance in crops utilizing the outcome of genomic research and development of genetic engineering technology utilizing these genes has become important .

Such technology of genetic engineering has built a big success in soybean, maize or cotton with herbicide- and/or insect-resistance, which leads their dominance in global trade. Development of drought-tolerant soybean and maize is now considered as the most important target of such technology because they are grown on a large scale in areas of relatively low rainfall. Japan is a big importer of these crops and needs to ensure a stable food supply from the world. Based on such conditions stated above, the government of Brazil submitted a proposal on "Development of Soybean with Tolerance to Drought and Heat" to Japan.

This proposal intends to develop the genetic engineering technology of soybean adapted to tolerate drought and heat aiming that the soybean production is stabilized in Brazil, in cooperation between Japan International Research Center for Agricultural Sciences (hereinafter referred to as "JIRCAS") and Embrapa Soybean. The research group firstly takes steps in isolating useful genes related to environmental stress tolerance and stress-inducible promoters in soybean on the basis of research outcome of genes involved in environmental stress tolerance and rapidly evolving soybean genome research. It selects candidate combinations of such genes and promoters, and then introduces them to soybean plant. It further evaluates environmental stress tolerance of transgenic soybean plants in greenhouse and field conditions, feedback the results to improve the combination of useful genes and promoters, and select elite transgenic lines with improved tolerance to environmental stresses.

2. Objectives of the detailed planning survey

Based on the proposal by the government of Brazil in September 2008, JICA conducts a detailed planning survey. It includes discussions with Embrapa Soybean, on-site investigation, and verification of relevance in operating the proposal as planned. Common understandings as a result of the survey will be documented as Minutes of Meetings and signed by both parties in Brazil and Japan.

Concrete procedures are as follows;

1) To collect fundamental information for the project; global warming impact on food and feedstuff production in Brazil, current status of crop varieties with drought tolerance in breeding and their demands.
2) To get a picture of current situation of participating institutes and related institutes to clarify the implementation structures available for the Project.
3) To confirm what research and development should be implemented in this project, then consider and discuss necessary inputs for it.
4) To evaluate the project from five criteria and prepare Project Design Matrix (PDM) and Plan of Operation (PO).
5) To discuss necessary steps to start the project.

3. Results of the survey

3-1 Brief overview of the survey

The Team visited Embrapa Soybean in Londrina, Brazil, from August 23 to September 5, 2009, and examined the current status of facilities and research structures of the institute. It also made a series of discussions on the framework, implementation methods of the Project, necessary inputs for it, and other matters involved.

3-2 Framework of the Project

3-2-1 Framework for the Science and Technology Research Partnership for Sustainable Development (Annex 1)

Both sides have confirmed that the Project is implemented under the Science and Technology Research Partnership for Sustainable Development (hereinafter referred to as "SATREPS*") promoted by JICA and Japan Science and Technology Agency (hereinafter referred to as "JST") in collaboration.

JICA will take measures for the technical cooperation such as dispatch of Japanese researchers, provision of equipment and training of counterpart researchers, and other supports related to the Project in Brazil. JST will support the Japanese research institutes/researchers for the project activities in Japan.

*SATREPS aims to develop new technology and its applications for tackling global issues, and also aims at the human resources development and capacity-building of researchers and research institutes in both countries.

3-2-2 Project Title

The Project was originally named as "Development of soybean with tolerance to drought and heat" in proposal, however, it shall be changed to the one that shows a clear design of the Project.

The Project is now named as "Development of genetic engineering technology of crops with stress tolerance against degradation of global environment". It may be modified and finalized over the course of discussions prior to the official signing of the document titled Record of Discussions (hereinafter referred to as "R/D").

3-2-3 Project Design Matrix and Plan of Operation

A basic framework of the Project is as shown in Project Design Matrix (hereinafter referred to as "PDM") in Annex 2. The Plan of Operation (hereinafter referred to as "PO") as shown in Annex 3.

PDM describes the clear details of the Project, its purpose, expected outputs and activities. It is used to manage, implement and monitor the Project, and referred to its evaluation.

However PDM and PO may be modified in the Reseach Group Unit (hereinafter referred to as "RGU"*), if necessary, within the framework approved in the R/D. The RGU must inform the modification to the Joint Coordination Committee (hereinafter referred to as "JCC"*).

* The function of JCC and RGU is mentioned in 3-2-8 8) and 9) respectively.

3-2-4 Site of the Project

Londrina City, the State of Parana, Brazil

3-2-5 Duration of the Project

Duration of the Project is for five(5) years from Jan. 2010 to Dec. 2014 tentatively.

3-2-6 Summary of the Project

1) Overall Goal (that is expected to be achieved after five years at the end of the Project)

Soybeans adapted to environmental stresses are developed, which contributes to the stabilization of the soybean production in Brazil.

2) Project Purpose

Genetic engineering technology of soybean with environmental stress tolerance is developed.

3) Outputs

1. Useful genes related to environmental stress tolerance are identified.

2. Stress-responsive promoters are isolated and combinations with useful genes are optimized.

3. Transgenic soybean lines containing constructs of promoters and useful genes are produced.

4. Transgenic soybean lines with environmental stress tolerance are selected.

4) Activity

1-1. Genes involved in regulation of stress tolerance are identified in plants such as soybean.

1-2. Genes involved in stress perception are identified in plants such as soybean.

1-3. Genes involved in regulation of stress response are identified in plants such as soybean.

2-1. Stress-responsive genes are searched in soybean.

2-2. Stress-responsive promoters are identified in soybean.

2-3. Constructs of useful genes and promoters are optimized.

3-1. Genetic engineering technology is established in soybean.

3-2. Constructs of useful genes and promoters are introduced in soybean.

3-3. T1 seeds of transgenic lines are collected.

4-1. Drought-inducible genes are identified and transgenic lines are selected based on gene analysis.

4-2. Heat-inducible genes are identified and transgenic lines are selected based on gene analysis.

4-3. Gene expression of transgenic plants is analyzed.

4-4. Evaluation methods of stress tolerance of soybean are established.

4-5. Stress tolerance of transgenic soybean lines is evaluated in greenhouse.

4-6. Stress tolerance of transgenic soybean lines is evaluated in field.

3-2-7 Measures to be taken

1) Measures to be taken by the Japanese side

1. Dispatch of Japanese researchers and the Project Coordinator to Brazil

JICA will dispatch the Japanese researchers and the Project Coordinator as listed in Annex 4.

2. Provisions of machinery and equipments

JICA will provide machinery and equipments (hereinafter referred as "the Equipment") necessary for the implementation of the Project as Listed in Annex 5-1 within the budget allocated for the Project.

3. Budget allocation

The items and budget listed in Annex 6 will be allocated by the JICA to maintain effective implementation of the Project.

4. Receiving/sending of Brazilian researchers in Japan or to third countries

According to necessity, JICA will receive/send the Brazilian researchers and other personnel related to the Project in Japan or to third countries.

2) Measures to be taken by the Brazilian side

1. Assignment of personnel

The Brazilian side will assign Brazilian researchers and related personnel as listed in Annex 4.

2. Preparation of facilities and Provision of the Equipment

Office space, facilities, experimental field and the Equipment listed in Annex 5-2 for the Project will be prepared and secured by Embrapa Soybean.

3. Budget allocation

The items and budget listed in Annex 6 will be allocated by the Brazilian side to maintain effective implementation of the Project.

3-2-8 Implementation structure of the Project

For effective implementation of the Project, the Project will organize the management structure written in Annex 7.

1) Managing institute will be Embrapa Headquarter, and implementing institute in Brazilian side will be Embrapa Soybean.
2) Managing and implementing institute in Japanese side will be JIRCAS, and the collaborative research institutes will be RIKEN and The University of Tokyo.
3) The Brazilian side will be requested to assign the Project Director and the Project Leader in Brazil. The Japanese side will assign the Project Leader and the person in charge of the collaborative research institutes respectively. And both parties will assign the responsible person for each research group.
4) The Head General of Embrapa Soybean, as the Project Director, takes full responsibility for the management and implementation of the Project in Brazil.
5) The Project Leader in Brazil will bear responsibilities regarding the implementation of the Project in Brazil. He/She is also responsible for discussing and coordinating the Project with the Project Leader.
6) The Project Leader will bear all responsibilities for the implementation of the Project. He/She is also responsible for discussing and coordinating the Project with the Project Leader in Brazil. In addition he/she coordinates with the collaborative research institutes in Japanese side.
7) Japanese researchers dispatched from Japanese institutes will take a role of doing activities in cooperation with Brazilian researchers.
8) For the effective and successful implementation of the Project, JCC will be established and its functions and composition are described in Annex 8.
9) RGU will be established and its functions and composition are described in Annex 9.
10) Mid-Annual reports (every six months), Mid-Term report and Final report will be made by RGU and submitted to JCC. The evaluation of the Project will be conducted jointly by Japanese and Brazilian

authorities concerned, at the middle and during the last six months of the Project term in order to examine the level of achievements and to recommend direction adjustments of the Project activities, if necessary.

4. Justification of the Project from five criteria

4-1 Predictions of Relevance

Since Brazil, as the world's leading producer, contributes the balance of demand and supply of soybean transaction in the world, the Project is expected to reduce uncertainty of soybean production by drought and heat. This expectation ensures the validity of the Project. Soybean is one of the strategically important crops for Brazil while it is a pressing issue for Japan to ensure food security, and therefore the Project is directly in accordance with the national interests of both countries. In addition, the Project contributes to the diplomatic policy of Japan and Brazil that considers a stronger partnership between both countries in science and technology as an important issue.

4-2 Predictions of Effectiveness

The Project is considered to have enough effectiveness with setting up adequate methods and goals for developing genetic engineering technology of crops with stress tolerance against degradation of global environment. Therefore, the effectiveness is high.

4-3 Predictions of Efficiency

Embrapa and JIRCAS have conducted a collaborative research since 1995. It is expected for both parties to enhance the strongest areas and compensate for the weaknesses of each other through cooperative research of the Project. Embrapa has a long-term cooperation with Japan to be commended by Minister of Foreign Affairs of Japan previously, and is a major counterpart of the Japan-Brazil Partnership Program (JBPP) as a trilateral cooperative partner of high capability. Such accumulated experience and high capability of Embrapa ensure efficient implementation of the Project.

4-4 Predictions of Impact

Development of soybean crop with tolerance to drought and heat helps to address climate instability that causes considerable damage to soybean production in Brazil. It means that the Project could directly contribute to stable production of soybean in Brazil and in the world.

4-5 Predictions of Sustainability

Useful genes identified and introduced in the Project are potentially applicable to other agriculturally important crops, and continued research on site is expected after the Project is over. This research theme gains growing importance under the increasing uncertainty in global environment and the increasing food demand in the world.

5. Implementation of Record of Discussion (R/D)

An official document titled Record of Discussion, which defines the Project, will separately be signed and exchanged by both parties before the commencement of the Project. It will be finalized based on a preliminary draft of R/D shown in Annex 10 and Minutes of Meetings (M/M) signed here.

6. Supplementary Note

1) Memorandum of Understanding (MOU)

JIRCAS and Embrapa, for implementing a collaborative research, will sign the Memorandum of Understanding (MOU) which includes the following clauses.

a. Objective and plan

b. Implementation

c. Confidentiality and intellectual property rights

d. Access to genetic resources

e. Publication of research outcome

f. Dispute resolution

g. Duration of agreements

h. Compliance with laws and regulations

* The above clauses may be modified in accordance with research contents.

7. Points to be confirmed

1) Expenses of contract with post-doctoral researchers and technicians

Expenses of contract with two(2) post-doctoral researchers (or one post-doctoral and one post-master researcher) and two(2) technicians (one is a student of the master course, and the other of the doctoral course) to improve the implementation structure of the Project is covered by JICA until two(2) years and half after the Project begins.

The cost for remaining period of the Project, however, will be covered by Brazilian side, considering that self-sustaining development of the Project in Brazil is required.

8. Points to be considered for implementation of the Project

1) Biological experimental materials may be provided under Material Transfer Agreements for the Project implementation when necessary. Each party shall make necessary measures for providing/receiving the materials smoothly following each country's legislation.

2) Considering the total budget available, it could be possible to substitute equipments listed in Annex 5-1 for other equipments or reagents and other materials according to necessity.

9. Procedures to take before the Project starts

1) Both the Brazilian and the Japanese sides secure a sufficient budget for the Project.

2) Both sides assign the researchers and personnel concerned to the Project.

3) Both sides sign and exchange Record of Discussion.

4) The Project starts.

ANNEXES

Annex 1 Project Implementation Scheme

Annex 2 Project Design Matrix (PDM)

Annex 3 Plan of Operation (PO)

Annex 4 List of Researchers

Annex 5 List of the Equipment

Annex 6 List of Undertakings

Annex 7 Organization Chart

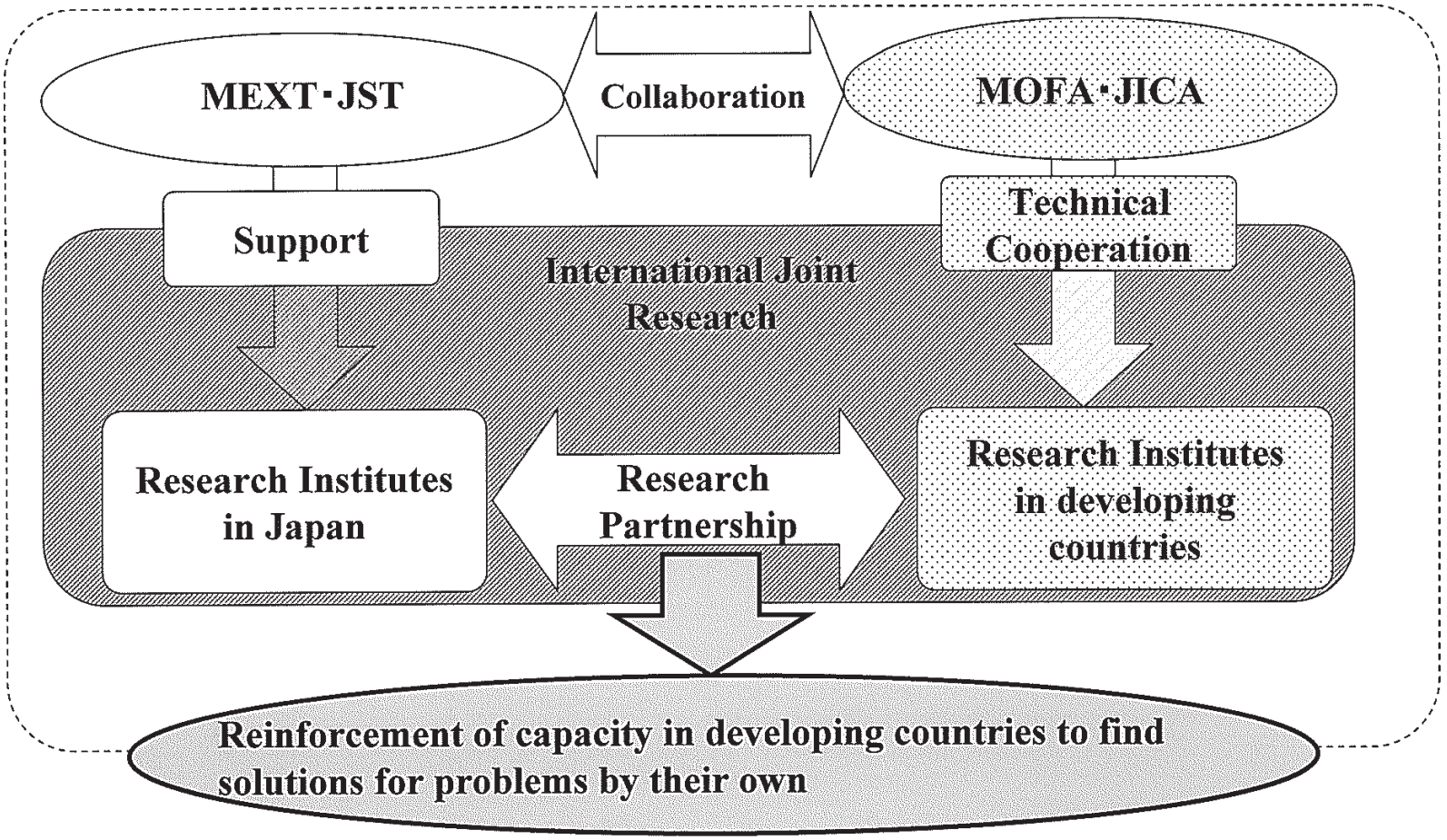
Annex 8 Joint Coordination Committee

Annex 9 Research Group Unit

Annex 10 Preliminary Draft of Record of Discussion

Annex 1: Project Implementation Scheme

Science and Technology Research Partnership for Sustainable Development (SATREPS)



MEXT: Ministry of Education, Culture, Sports, Science and Technology
JST: Japan Science and Technology Agency

MOFA: Ministry of Foreign Affairs
JICA: Japan International Cooperation Agency

ver. 1

Annex 2

PDM "Development of soybean with tolerance to dry and heat"(SATREPS in Brazil)

Site of the Project: Londrina City, the State of Parana, Brazil

Direct beneficiary: Embrapa Soybean

Period of coolaboration: From Jan 2010 to Dec 2014

Brief of project	Index	Index acquisition method	Outside condition
【Overall goal】 Soybeans adapted to environmental stresses are developed, which contributes to the stabilization of the soybean production in Brazil.	Soybeans adapted to environmental stresses are developed before 2019.		
【Project Purpose】 Genetic engineering technology of soybean with environmental stress tolerance is developed.	1. At least 10 useful genes related to environmental stress tolerance are identified in plants such as soybean. 2. At least five(5) stress-responsive promoters are identified and combinations with useful genes are optimized. 3. At least five(5) constructs of usefull genes and promoters are introduced in soybean and at least three transgenic lines are produced for each construct. 4. At least one(1) stress-tolerant line with environmental stress toralance is sellected.		
【Outputs】 1. Useful genes related to environmental stress tolerance are identified. 2. Stress-responsive promoters are isolated and combinations with useful genes are optimized. 3. Transgenic soybean lines containing constructs of promoters and useful genes are produced. 4. Transgenic soybean lines with environmental stress tolerance are selected.	1-1 At least five(5) genes involved in regulation of stress tolerance are identified in plants such as soybean. 1-2 At least two(2) genes for membrane proteins involved in stress perception are identified in plants such as soybean. 1-3 At least three(3) genes involved in regulation of stress response are identified in plants such as soybean. 2-1 At least 100 stress-responsive genes are identified in soybean. 2-2 At least three(3) stress-responsive promoters are identified in soybean. 2-3 At least five(5) constructs of useful genes and promoters are optimized. 3-1 Genetic engineering technology with more than 2 % of transformation effeciency is established in soybean. 3-2 At least five(5) constructs of useful genes and promoters are introduced in 3-3 T1 seeds of at least three(3) lines are collected. 4-1 At least two(2) drought-inducible genes are identified and at least two(2) transgenic lines are selected for each construct based on gene analysis. 4-2 At least two(2) heat-inducible genes are identified and at least two(2) transgenic lines are selected for each construct are selected based on gene analysis. 4-3 Gene expression of at least two(2) independent lines derived from at least two(2) constructs is analyzed. 4-4 Evaluation methods of stress tolerance of soybean in greenhouse and field are established. 4-5 Stress tolerance of at least two(2) independent lines derived from at least two(2) constructs is evaluated in greenhouse. 4-6 Stress tolerance of at least two(2) independent lines derived from at least two(2) constructs is evaluated in field..		
【Activity】 1-1 Genes involved in regulation of stress tolerance are identified in plants such as soybean. 1-2 Genes involved in stress perception are identified in plants such as soybean. 1-3 Genes involved in regulation of stress response are identified in plants such as soybean. 2-1 Stress-responsive genes are searched in soybean. 2-2 Stress-responsive promoters are identified in soybean. 2-3 Constructs of useful genes and promoters are optimized. 3-1 Genetic engineering technology is established in soybean. 3-2 Constructs of useful genes and promoters are introduced in soybean. 3-3 T1 seeds of transgenic lines are collected. 4-1 Drought-inducible genes are identified and transgenic lines are selected based on gene analysis. 4-2 Heat-inducible genes are identified and transgenic lines are selected based on gene analysis. 4-3 Gene expression of transgenic plants is analyzed. 4-4 Evaluation methods of stress tolerance of soybean are established. 4-5 Stress tolerance of transgenic soybean lines is evaluated in greenhouse. 4-6 Stress tolerance of transgenic soybean lines is evaluated in field.	【Inputs】 【Japanese side】 ・the Project Coordinator ・Short-term researchers ・Invitation of Brazilian researchers to Japan ・the Equipment ・Partial contribution for expenses of contract with two post-doctoral researchers (or one post-doctoral and one post-master researcher) and two technicians. ・Cost for project and others	【Brazilian side】 ・Arrangement of researchers and technicians ・Partial contribution for expenses of contract with two post-doctoral researchers (or one post-doctoral and one post-master researcher) and two technicians. ・Offer of facilities and project office ・the Equipment and running cost ・Securement of cost for project	Conditions precedent

PO "Development of soybean with tolerance to dry and heat"(SATREPS in Brazil)

			2009 FY	2010 FY				2011 FY				2012 FY				2013 FY				2014 FY		
			Ⅳ	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅰ	Ⅱ	Ⅲ
1. Useful genes related to environmental stress tolerance are identified.	1-1 Genes involved in regulation of stress tolerance are identified in plants such as soybean.	Search of genes involved in regulation of stress tolerance in model plants.																				
		Search of genes involved in regulation of stress tolerance in soybean.																				
		Functional analysis of genes involved in regulation of stress tolerance using protoplasts.																				
		Functional analysis of genes involved in regulation of stress tolerance using transgenic plants.																				
	1-2 Genes involved in stress perception are identified in plants such as soybean.	Search of genes involved in stress perception in model plants.																				
		Search of genes involved in stress perception in soybean.																				
		Functional analysis of genes involved in stress perception using transgenic plants.																				
	1-3 Genes involved in regulation of stress response are identified in plants such as soybean.	Search of genes involved in regulation of stress response in model plants.																				
		Search of genes involved in regulation of stress response in soybean.																				
		Functional analysis of genes involved in regulation of stress response using transgenic plants.																				
2. Stress-responsive promoters are isolated and combinations with useful genes are optimized.	2-1 Stress-responsive genes are searched in soybean.	Making of oligo-array of soybean.																				
		Search and idenfication of stress-responsive genes using oligo-array.																				
	2-2 Stress-responsive promoters are identified in soybean.	Identification of stress-responsive genes using cDNA array of soybean.																				
		Isolation of stress-responsive promoters of soybean.																				
	2-3 Constructs of useful genes and promoters are optimized.	Collection of soybean full-length cDNA and identification of promoter sequences.																				
		Optimization of combinations of promoters and useful genes.																				
3. Transgenic soybean lines containing constructs of promoters and useful genes are produced.	3-1 Genetic engineering technology is established in soybean.	Establishment of high-efficient gene transfer technology for soybean.																				
	3-2 Constructs of useful genes and promoters are introduced in soybean.	Introduction of constructs containing useful genes.																				
		Check of existance of transgenes.																				
	3-3 T1 seeds of transgenic lines are collected.	Collection of next generation seeds.																				
4. Transgenic soybean lines with environmental stress tolerance are selected.	4-1 Drought-inducible genes are identified and transgenic lines are selected based on gene analysis.	Creation of gene expression database of soybean.																				
		Selection of transgenic soybean lines using gene analysis.																				
	4-2 Heat-inducible genes are identified and transgenic lines are selected based on gene analysis.	Creation of gene expression database of soybean.																				
		Selection of transgenic soybean lines using gene analysis.																				
	4-3 Gene expression of transgenic plants is analyzed.	Gene expression analysis of transgenic soybean plants using oligo-array.																				
	4-4 Evaluation methods of stress tolerance of soybean are established.	Establishment of evaluation methods of stress tolerance of soybean.																				
		Selection of transgenic soybean using PCR.																				
	4-5 Stress tolerance of transgenic soybean lines is evaluated in greenhouse.	Stress tolerance test of transgenic soybean plants in greenhouse.																				
	4-6 Stress tolerance of transgenic soybean lines is evaluated in field.	Stress tolerance test of transgenic soybean plants in field.																				

FY : Fiscal Year in Japan (Ⅰ :Apr.to Jun. Ⅱ :Jul.to Sep. Ⅲ :Oct.to Dec. Ⅳ :Jan. to Mar.)

Annex 4

LIST OF RESEARCHERS AND COORDINATOR

Both Brazilian and Japanese side organize the research team below, managed by Dr. Nepomuceno (Brazilian side) and Dr. Yamaguchi-Shinozaki (Japanese side).

It may be modified and finalized over the course of discussions prior to the official signing of the document titled Record of Discussions.

Japanese side

	Name	Institution	Major	Research Group Unit (RGU) in Japanese side				Remarks
				1. Identification of useful genes	2. Identification of promoters	3. Construct selection	4. Analysis of transgenic plants	
1	Dr. Kazuko Yamaguchi-Shinozaki	JIRCAS/ The University of Tokyo	Project Leader/ Plant Molecular Biology	○	○	○	○	◎
2	Dr. Kazuo Nakashima	JIRCAS	Plant Molecular Biology	○	○			
3	Dr. Yasunari Fujita	JIRCAS	Plant Molecular Biology	○		○		
4	Dr. Kyonoshin Maruyama	JIRCAS	Bioinfomatics				○	
5	Researcher A	JIRCAS	Plant Molecular Biology	○	○	○	○	
6	Dr. Yuriko Osakabe	The University of Tokyo	Plant Molecular Biology	○		○		
7	Researcher B	The University of Tokyo	Bioinfomatics	○	○		○	
8	Dr. Kazuo Shinozaki	RIKEN	Plant Molecular Biology	○	○	○	○	◎
9	Dr. Taishi Umezawa	RIKEN	Plant Molecular Biology	○		○		
10	Researcher C	RIKEN	Plant Molecular Biology	○	○	○	○	
11	Dr. Tetsuya Sakurai	RIKEN	Bioinfomatics	○	○			
	Name	Institution	Major	Research Group Unit (RGU) in Brazilian side				
				1.gene transfer	2. molecular analysis	3. physiological analysis	4. field test	
12	Dr. Norihito Kanamori	JIRCAS	Plant Molecular Biology	○	○			
13	Dr. Hiroshi Kudo	JICA	The Project Coordinator					

Brazilian side

	Name	Institution	Major	Research Group Unit (RGU) in Brazilian side				Remarks
				1. gene transfer	2. molecular analysis	3. physiological analysis	4. field test	
1	Dr. Alexandre Lima Nepomuceno	Embrapa Soybean	Project Leader in Brazil/ Molecular Biology / Plant Physiology	○	○	○	○	◎
2	Dr. Norman Neumaier	Embrapa Soybean	Plant Physiology			○	○	
3	Dr. José Renato B. Farias	Embrapa Soybean	Agrometeorology			○	○	
4	Dr. Carlos Alberto Arrabal Arias	Embrapa Soybean	Breeding				○	
5	Dr. Antonio Eduardo Pipolo	Embrapa Soybean	Breeding				○	
6	Dr. Renata Fuganti	Embrapa Soybean	Transformation	○	○	○	○	
7	Dr Ricardo Vilela Abdelnoor	Embrapa Soybean	Molecular Biology		○			
8	Dr Franscismar Correia. Marcelino	Embrapa Soybean	Molecular Biology	○	○			
9	Dr Maria Cristina Neves de Oliveira	Embrapa Soybean	Statistics			○	○	
10	Dr Clara Beatriz Hoffmann-Campo	Embrapa Soybean	Metabolomics		○	○		
11	Dr Júlio Franchini dos Santos	Embrapa Soybean	Crop Management				○	
12	Dr Henrique Debiasi	Embrapa Soybean	Crop Management				○	
13	Dr. Fabiana Rodrigues	Embrapa Soybean	Molecular biology	○	○	○	○	
14	Dr. Elibio Rech	Embrapa CENARGEN	Molecular biology	○				
15	Dr. Gedi Jorge Sfredo	Embrapa Soybean	Plant Nutrition			○	○	
16	Dr. Maria Fatima Grossi de Sa	Embrapa CENARGEN	Molecular Biology	○	○			
17	Researcher A	Embrapa Soybean	Molecular Biology	○	○			

Annex 5-1

LIST OF THE EQUIPMENT

This is Tentative List of the Equipment by Japanese side so that it may be modified and finalized over the course of discussions prior to the official signing of the document titled Record of Discussions.

	Item	Quantity
1	Air conditioner system	4
2	Biometric system to control access	4
3	BOD Incubator	1
4	Centrifugal refrigerated with rotor plate	2
5	Centrifuges	2
6	Fluorescence Scanner	1
7	Freezer	3
8	Horizontal autoclaves	1
9	Horizontal electrophoresis system	2
10	Ice machine	1
11	Incubator with agitation	2
12	Laminar flow	2
13	Magnetic stirrer with heating	3
14	Microcomputer	5
15	NanoDROP	1
16	Oven	1
17	Print laser	2
18	Refrigerated centrifuge for microtube	3
19	Refrigerator	3
20	Rocking shaker	2
21	RT-PCR (7500 Real time PCR system)	1
22	Sample concentrator mod. 5310 centrifugal vacuum concentrator	1
23	Shake master	1
24	Stereomicroscopy with camera	2

25	Thermocycler 96 well gradient Veritti	3
26	Ultrafreezer	1
27	Ultrasonic washing machines(L)	1
28	Ultrasonic washing machines(S)	1
29	Vertical electrophoresis system for 6 plates	2
30	4 wheel Jeep	1
31	Air conditioner system	2
32	Computers (10 pcs + 2 printers)	1
33	Photosynthetic apparatuses (not portable)	2
34	Photosynthetic apparatuses (portable)	1
35	Cold chamber for seed storage	1
36	Monitoring system in the field	1
37	Oximeters	3
38	Psycrometers (100) + Dataloger set (water potential)	1
39	Mini digital thermometers	20
40	Meteorological stations sensors + dataloger	2
41	GPS equipment	2
42	Analytical balance	2
43	Rain Out Shelter	4
44	Seed moisture reader	1
45	Phytotron(Screening Greenhouse/LemnaTec)	1
46	High-Frequence Soil Water Content Data Logger and sensors	2
47	Leaf Porometer	2
48	Water Potential Reader WP4	2
49	Neutrons Probe - Soil Experiments	1
50	GreenSeeker	1
51	Leaf area reader	1
52	Microscope	1
53	Compressor for pressure plate extractor	1
54	pH meter/conductivity meter/multimeter	2
55	Balance to weigh vessels (25kg)	2
56	Palmtops	2

57	Guelph Permeameter	2
58	Hydroponics equipment	5
59	Laptops	2

Note:

The Equipment will become the property of Embrapa on being delivered C. I. F. (cost, insurance and freight) to the Brazilian authorities concerned at the posts and/or airports of disembarkation.

Annex 5-2

LIST OF THE EQUIPMENT

This is Tentative List of the Equipment by Brazilian side so that it may be modified and finalized over the course of discussions prior to the official signing of the document titled Record of Discussions.

	Item	Quantity	US$
1	Sap flow meter system	1	12,000
2	Bioanalyser	1	35,000
3	Automated DNA extractor	1	220,000
4	Automated PCR preparation system	1	including in No.3
5	Horizontal electrophoresis system	1	7,000
6	Incubator with agitation	1	5,500
7	Laminar flow	1	5,000
8	Microcomputer	1	1,500
9	Print laser	1	800
10	Refrigerator	1	1,000
11	Shake master	1	5,000
12	Stereomicroscopy with camera	1	20,000
13	Thermocycler 96 well gradient Veritti	1	15,000
14	Ultrafreezer	1	30,000
15	Air condition air system	1	3,000
16	Centrifuge	1	20,000
17	Freezer	1	1,300
18	Analytical balance	1	3,500
19	Microscope	1	10,000
20	Hydroponics equipment	1	2,500
21	Laptops	1	1,500

Annex 6

LIST OF UNDERTAKINGS

1. Both Brazilian and Japanese side will utilize the existing equipment, materials, furniture and facilities.
2. All financial matters for the Project will need to be complied with the regulations respectively by both sides.

The table below shows the equipment and others necessary to implement the Project effectively. Both sides are responsible to cover costs in procuring and/or financing the items as follows.

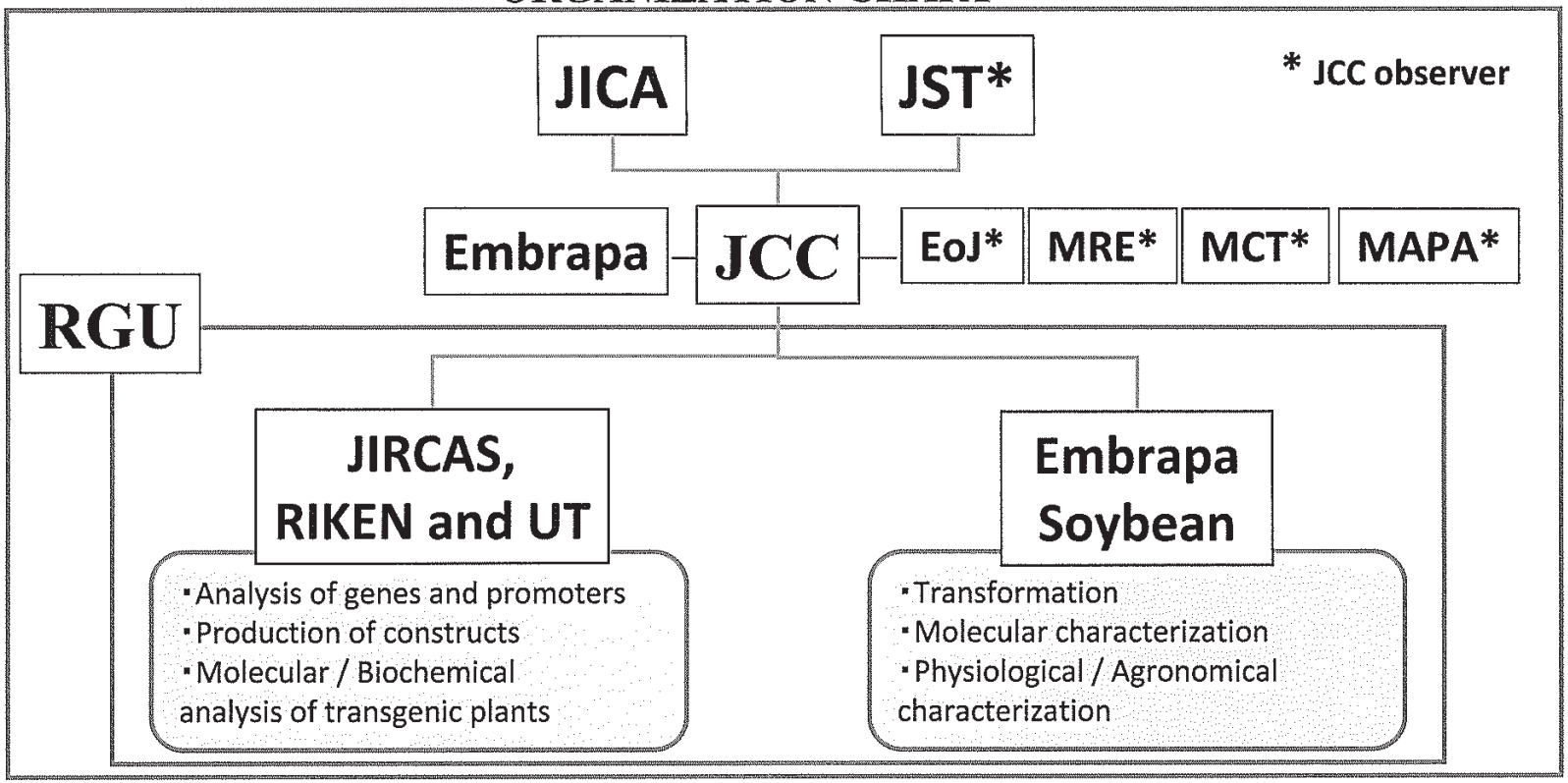
It may be modified and finalized over the course of discussions prior to the official signing of the document titled Record of Discussions.

Items	Prepared by	
	Brazilian side	Japanese side
Office equipment for the project offices		
PC	○	○
PC software	○	○
Printer	○	○
Air conditioner	○	○
Internet connection	○	
Utilities	○	
Others	To be discussed and agreed by both sides	
Expenses on activities		
Expenses of contract for two post-doctoral (or one post-doctoral and one post-master) researchers for five years for Embrapa	○	○
Expenses of contract for a technician (doctoral course) for five years for Embrapa	○	○
Expenses of contract for a technician (master course) for five years for Embrapa	○	○

Expenses for consumption articles for project activities such as chemical reagent, experimental equipment/tool for five years	○	○
Others	To be discussed and agreed by both sides	
Vehicles		
4-wheel Jeep		○
Maintenance, spare parts, insurance, gasoline and other running cost for vehicles	○	
Maintenance, spare parts and running cost of the Equipment		
Expenses for maintenance and spare parts of the Equipment	○	
Seminar, workshops, conference, reception related to the Project held in Brazil		
Fees for registration	○	
Fee for helpers	○	
Venue	○	
Refreshment	○	
Lunch	○	
Stationery	○	
Handout, textbooks, brochures, photocopying	○	
Conference, seminar held outside Brazil		
Fees for registration		○
Expenses for transportation and accommodation		○
Others		
Biotechnology building (2 stories, 300m^2)	○	

Annex 7

ORGANIZATION CHART



Abbreviations

MRE	Ministry of External Relations
MCT	Ministry of Science and Technology
MAPA	Ministry of Agriculture, Livestock and Food Supply
EoJ	Embassy of Japan
Embrapa	Brazilian Agricultural Research Corporation
JIRCAS	Japan International Research Center for Agricultural Sciences
RIKEN	RIKEN
UT	The University of Tokyo
JST	Science and Technology Agency
JICA	Japan International Cooperation Agency
JCC	Joint Coordination Committee
RGU	Research Group Unit

Annex 8

Joint Coordination Committee

1. Functions

The Joint Coordination Committee(JCC) will meet at least once a year and whenever the necessity arises, and its functions are as follows;

(1) To discussthe annual research plan based on PDM and PO reported from RGU
(2) To evaluate the overall goal progress of the project activities as well as the achievements of the annual plan
(3) To discuss any other issues pertinent to the smooth implementation of the Project

2. Composition

(1) Chairperson: Executive Director, Embrapa

(2) Members

1) Brazilian side

a. The Head of International Relations Department, Embrapa
b. The Head General, Embrapa Soybean (Project Director)
c. Project Leader in Brazil, Embrapa Soybean

2) Japanese side

a. Resident Representative, JICA Brazil Office
b. Project Leader
c. Representative of JIRCAS, The University of Tokyo and RIKEN
d. The Consultation Team from JICA (if necessary)

(3) Observer

1) Representative of Embassy of Japan
2) Representative of MRE
3) Representative of MCT
4) Representative of MAPA
5) Others (Researchers from both sides、Project Coordinator)
6) Representative of JST
7) Personnel recommended by the Chairperson

Annex 9

Research Group Unit (RGU)

1. Functions

The Research Group Unit (RGU) will meet at least once in half a year and whenever the necessity arises, and its functions are as follows;

(1) To examine and report the project activities of both Brazilian and Japanese research institutes

(2) To discuss about the modification and future plan of the Project

(3) To submit Mid-Annual reports (every six months), Mid-Term report and Final report to JCC members, JICA and JST

(4) To examine proposed working and financial plans under the conditions stated in R/D

(5) To examine the modification of the PDM and PO and inform to JCC if necessary

(6) To discuss any other issues ensuring the smooth implementation of the Project

2. Composition

(1) Chairperson

The Project Leader in Brazil and the Project Leader jointly

(2) Members

- Representative of researchers in Embrapa Soybean
- Representative of researchers in JIRCAS, The University of Tokyo and RIKEN
- Related researchers from both sides
- Project Coordinator

RECORD OF DISCUSSIONS
BETWEEN
JAPAN INTERNATIONAL COOPERATION AGENCY,
THE MINISTRY OF EXTERNAL RELATIONS
AND
AUTHORITIES CONCERNED OF THE FEDERATIVE REPUBLIC OF BRAZIL
ON JAPANESE TECHNICAL COOPERATION
FOR
"DEVELOPMENT OF GENETIC ENGINEERING TECHNOLOGY OF CROPS WITH STRESS TOLERANCE AGAINST DEGRADATION OF GLOBAL ENVIRONMENT"

Japan International Cooperation Agency (hereinafter referred to as "JICA") through Coordinator for Technical Cooperation of Japan in Brazil, exchanged views and had a series of discussions with the Brazilian concerned authorities with respect to desirable measures to be taken by both Japanese and Brazilian Governments for successful implementation of "Development of genetic engineering technology of crops with stress tolerance against degradation of global environment" (hereinafter referred to as "the Project").

As a result of the discussions, the Coordinator for the Technical Cooperation of Japan in Brazil and the Brazilian authorities concerned agreed upon the matters referred to in the document attached hereto, in accordance with the provision of the Agreement on Technical Cooperation between the Government of Japan and the Government of the Federative Republic of Brazil signed in Brasilia, Brazil on September 22nd, 1970 (hereinafter referred to as "the Agreement").

The project was referred to in the Second Meeting of the Joint Committee on Japanese-Brazilian Cooperation in Science and Technology held on May 14th, 2009 in accordance with the Article III of "the Agreement between the Government of Japan and the Government of the Federative Republic of Brazil on Co-operation in the Field of Scientific and Technology" signed on May 25, 1984.

These texts were done in both English and Portuguese, each texts being equally authentic. In case of any divergence of interpretation, the English text shall prevail.

"date of signing", Brasilia, Brazil

Mr. Katsuhiko Haga
Resident Representative
JICA
Japan

Dr. Pedro Antonio Arraes Pereira
President Director
Embrapa
Federative Republic of Brazil

Dr. Ademar Seabra da Cruz Junior
Chief of Division of Science and Technology
Ministry of External Relations (MRE)
Federative Republic of Brazil

THE ATTACHED DOCUMENT

I. COOPERATION BETWEEN JICA AND THE GOVERNMENT OF THE FEDERATIVE REPUBLIC OF BRAZIL

1. The Government of the Federative Republic of Brazil will implement the Project in cooperation with JICA.
2. The Project will be implemented in accordance with the Master Plan and the Plan of Operation which is given in Annex 1 and 2 respectively.

II. MEASURES TO BE TAKEN BY JICA

In accordance with the laws and regulations in force in Japan and the provisions of Article III of the Agreement, JICA, as the executing agency for technical cooperation by the Government of JAPAN, will take, at its own expense, the following measures according to the normal procedures of its technical cooperation scheme.

1. DISPATCH OF JAPANESE EXPERTS

JICA will provide the services of the Japanese experts as listed in Annex 3. The provision of Article IV- (1) of the Agreement will be applied to the above-mentioned experts.

2. PROVISION OF MACHINERY AND EQUIPMENT

JICA will provide such machinery, equipment and other materials (hereinafter referred to as "the Equipment") necessary for the implementation of the Project as listed in Annex 4. The provision of Article IX of the Agreement will be applied to the Equipment.

3. TRAINING OF BRAZILIAN PERSONNEL IN JAPAN OR THIRD COUNTRIES

JICA will receive the Brazilian personnel connected with the Project in Japan or neighboring countries. The provision of Article III-(1) will be applied to the training

4. BUDGET ALLOCATION

The part of items and budget listed in Annex 5 will be allocated by the Japanese side to maintain effective implementation of the Project

III. MEASURES TO BE TAKEN BY THE GOVERNMENT OF FEDERATEIVE REPUBLIC OF BRAZIL

1. The Government of the Federative Republic of Brazil will take necessary measures to ensure that the self-reliant operation of the Project will be sustained during and after the period of Japanese technical cooperation, through full and active involvement in the Project by all related authorities, beneficiary groups

and institutions.

2. In accordance with Article IV of the Agreement, the Government of the Federative Republic of Brazil will ensure that the technologies and knowledge acquired by the Brazilian nationals as a result of the Japanese technical cooperation will contribute to the economic and social development of the Federative Republic of Brazil.

3. In accordance with the provisions of Articles V, VI and VIII of the Agreement, the Government of the Federative Republic of Brazil will grant in Brazilian privileges, exemptions and benefits to the Japanese experts referred to in II-1 above and their families during their stay in the Federative Republic of Brazil.

4. In accordance with the provisions of Article IX of the Agreement, the Government of the Federative Republic of Brazil will take the measures necessary to receive and use the Equipment provided by JICA under II-2 above and equipment, machinery and materials carried in by the Japanese experts referred to in II-1 above.

5. The Government the Federative Republic of Brazil will take necessary measures to ensure that the knowledge and experience acquired by the Brazilian personnel from technical training in Japan will be utilized effectively in the implementation of the Project.

6. In accordance with the provision of Article V-(1)-(i) of the Agreement, the Government of the Federative Republic of Brazil will provide the services of Brazilian counterpart personnel and administrative personnel as listed in Annex 3.

7. In accordance with the provision of Article V-(1)-(ii) of the Agreement, the Government of the Federative Republic of Brazil will provide the buildings and facilities as listed in Annex 4 and 5.

8. In accordance with the laws and regulations in force in the Federative Republic of Brazil, the Government of the Federative Republic of Brazil will take necessary measures to supply or replace at its own expense machinery, equipment, instruments, vehicles, tools, spare parts and any other materials necessary for the implementation of the Project other than the Equipment provided by JICA under II-2 above.

9. In accordance with the laws and regulations in force in the Federative Republic of Brazil, the Government of the Federative Republic of Brazil will take necessary measures to meet the running expenses necessary for the implementation of the Project.

IV. IMPLEMENTATION STRUCTURE OF THE PROJECT

For effective implementation of the Project, the Project will organize the Management structure as follows;.

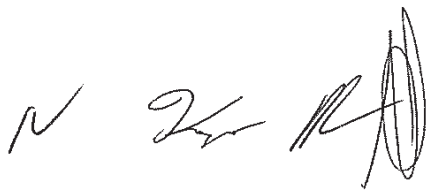
1. Managing institute will be Embrapa Headquarter, and implementing institute in Brazilian side will be Embrapa Soybean.
2. Managing and implementing institute in Japanese side will be JIRCAS, and the collaborative research institutes will be RIKEN and The University of Tokyo.
3. The Brazilian side will be requested to assign the Project Director and the Project Leader in Brazil. The Japanese side will assign the Project Leader and the person in charge of the collaborative research institutes respectively. And both parties will assign the responsible person for each research group.
4. The Head General of Embrapa Soybean, as the Project Director, takes full responsibility for the management and implementation of the Project in Brazil.
5. The Project Leader in Brazil will bear responsibilities regarding the implementation of the Project in Brazil. He/She is also responsible for discussing and coordinating the Project with the Project Leader.
6. The Project Leader will bear all responsibilities for the implementation of the Project. He/She is also responsible for discussing and coordinating the Project with the Project Leader in Brazil. In addition he/she coordinates with the collaborative research institutes in Japanese side.
7. Japanese researchers dispatched from Japanese institutes will take a role of doing activities in cooperation with Brazilian researchers.
8. For the effective and successful implementation of the Project, JCC will be established and its functions and composition are described in Annex 6.
9. RGU will be established and its functions and composition are described in Annex 7.

V. JOINT EVALUATION

Mid-Annual reports (every six months), Mid-Term report and Final report will be made by RGU and submitted to JCC. Japanese and Brazilian sides will jointly conduct a mid-term evaluation at the middle of the cooperation term and a final evaluation during the last six months of the cooperation term in order to examine the level of achievement and to recommend direction adjustments of the Project activities, if necessary.

VI. CLAIMS AGAINST JAPANESE EXPERTS

In accordance with the provision of Article VII of the Agreement, the Government of the Federative Republic of Brazil undertakes to bear claims, if any arises, against the Japanese experts engaged in technical cooperation for the Project resulting from, occurring in the course of, or otherwise connected with the discharge of their official functions in the Federative Republic of Brazil except for those arising from the willful misconduct or gross negligence of the Japanese experts.



VII. MUTUAL CONSULTATION

There will be mutual consultation between JICA and the Government of the Federative Republic of Brazil on any major issues arising from, or in connection with this Attached Document.

VIII. MEASURES TO PROMOTE UNDERSTANDING OF AND SUPPORT FOR THE PROJECT

For the purpose of promoting support for the Project among the Brazilian people, the Government of the Federative Republic of Brazil will take appropriate measures to make the Project widely known to Brazilian people.

IX. TERM OF COOPERATION

The duration of the technical cooperation for the Project under this Attached Document will be five (5) years from the departure date of the first JICA expert dispatched for the Project.

Aneex 1 Master Plan
Annex 2 PO
Annex 3 List of Researchers
Annex 4 List of the Equipment
Annex 5 List of Undertakings
Annex 6 Joint Coordination Committee
Annex 7 Research Group Unit