Drought Tolerance & Associated DNA markers in *Fragaria* sp.

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Abstract

Adaptive mechanisms of drought-tolerant plants are mainly determined by genetic and metabolism characteristics. Responses to drought in plants are extremely different according to their genetic background; inter- and intra-species variation in drought tolerance is described. In present study, to evaluate the degree of drought tolerance in *Fragaria* genotypes, a standard method for measurement of dehydration tolerance was established, so, a short-term water deficit experiment performed and determined leaf relative water content (RWC) and leaf water losing rate (WLR) as a quick screening method for monitoring of *Fragaria* genotypes in response to dehydration. The *Fragaria* genotypes were characterized in their response to drought stress by measurement of two eco-physiological parameters associated to the leaf water status (leaf WLR and RWC) and DNA fingerprints was performed for selected *Fragaria* genotypes using AFLP and candidate gene-EST markers. Lastly, the correlation between specific DNA markers and leaf WLR and RWC and the possibility of using association mapping in a small set of *Fagaria* accessions to create a set of correlated markers to the physiological traits which can be involved in drought tolerance in *Fragaria* were tested.

Key words: Strawberry, Fragaria sp., Drought stress, DNA markers, Genetic control, Marker Assisted Selection (MAS)

Introduction

Wild germplasm and landraces that are better adapted to local ecological conditions can be valuable genetic resources for breeding towards drought tolerance. The potential of such material and the available variability in the gene pools for tolerance to drought must be properly characterized at the physiological, morphological and genetic level as was already reported for cowpea and wheat (Hegde and Mishra 2009; Rampino et al. 2006; Peleg et al. 2005). Drought stress responses in strawberry include a decrease in net photosynthesis and in the leaf water potential at cellular level and a reduction of leaf area and yield at crop level (Klamkowski and Treder 2008; Razavi et al. 2008). In addition, osmotic adaptation resulted in higher sucrose levels under drought stress in strawberry 'Elsanta' (Razavi et al. 2008). Variation in drought tolerance was already assessed within the genus *Fragaria*, indicating *Fragaria chiloensis* as a more drought tolerant species compared to *F. virginiana* (Zhang and Archbold 1993). In this study interspecific variation was detected: higher solute accumulation and osmotic adjustment was observed under water deficit in *F. chiloensis* compared to *F. virginiana*. Also, plant water relation parameters like leaf water potential and relative water content (RWC) were variable between *Fragaria* species under water deficit (Archbold and Zhang 1993). Other reports confirm that response to water deficit is species specific; each particular *Fragaria* species shows a unique adaptive response to drought condition (VanDerZanden and Cameron 1996). Intraspecific variation in drought tolerance within *Fragaria chiloensis* clones was also described in this study. Up to now, only a small set of genes known to be of interest for abiotic stress tolerance have been characterized in *Fragaria* (Schwab et al. 2009), and

specially the information about the genetic control of drought tolerance in *Fragaria* is missing. An improved understanding of the genome structure in *Fragaria* enables to dissect the structural and functional basis of adaptive traits like drought tolerance (Folta and Davis 2006). In this view, a study of the genetic background of different *Fragaria* genotypes can be informative and this information ultimately might result in the detection of some DNA markers correlated to drought tolerance in *Fragaria*. In tea plants (*Camellia sinensis*), a DNA marker association study for drought stress could provide a useful alternative for QTL mapping in a limited set of genotypes (Mishra and Sen-Mandi 2004). A few studies in *Fragaria* used EST-derived SSRs to determine linkage with candidate genes (Bassil et al. 2006; Gil-Ariza et al. 2006; Sargent et al. 2004). EST markers developed in the coding region of functional genes have already proven to be transferable through genotypes and are powerful markers for fingerprinting in some crops (Scariot et al. 2007). However, these type of markers linked with drought tolerance are lacking in *Fragaria*. The main objectives of this research were to come to a fast screening method for drought tolerance of *Fragaria* genotypes and to correlate the genetic structure of different *Fragaria* genotypes, assessing by Expressed Sequence Tag (EST) and Amplified Fragment Length Polymorphism (AFLP) markers, and plant responses to drought stress.

Materials and methods

Some strawberry cultivars (*Fragaria* × *ananassa* Duch.) (20) from different breeding programmes and two ecotypes of the European diploid species *F. vesca* and one American octaploid species *F. chiloensis* were included in this study. All plants were grown for 3 months in a greenhouse of Ghent University (51.3 N, 3.4 E)

according to good horticultural practices for runner production. Daughter plants were cut from the stolons, and after a production cycle of 18 months plants were transferred to a growth chamber two weeks prior to the start of the experiment for preconditioning. They were well-watered and grown under constant temperature (22°C) and relative humidity (60%). An 16 hour photoperiod (from 6h00 till 22h00) was given and 100 µmol m^{-2} s^{-1} PPFD was supplied during the light period (Philips, MASTER
.20HPI-T Plus, 400 Watt). As a fast screening of the *Fragaria* genotypes, the leaf water losing rate (WLR) and relative water content (RWC) of detached leaves was assessed as described by Suprunova et al. (2004) and Verslues et al. (2006). For DNA extraction of the leaf material, the Qiagen DNeasy Plant Mini Kit was used. A modified AFLP protocol (Vos et al. 1995) was followed according to De Riek et al. (1999). Selective amplification was carried out using four fluorescent 6-FAM or HEX labeled *Eco*RI/*Mse*I primers combinations with six selective bases: *Eco*RI-ACT/*Mse*I-CGA (PC_1), *Eco*RI-AGG/*Mse*I-CAA (PC_2), *Eco*RI-ACC/*Mse*I-CAT (PC_3), *Eco*RI-ACC/*Mse*I-CTA (PC_4). Of the final PCR product, 1µl was mixed with 13.5 µl Hi-Di™ Formamide (Applied Biosystems) and 0.5 µl of the GeneScan™-500 Rox® Size Standard (Applied Biosystems). Products were denatured by heating for 3 minutes at 90°C. Capillary electrophoresis and fragment detection were performed on an ABI Prism 3130*xl* Genetic Analyzer (Applied Biosystems). Polymorphic bands were scored as present or absent (1/0) using the GeneMapper® 4.0 software (Applied Biosystems). Thresholds for marker frequency ($0.15<f<0.85$) and average marker peak height (h>100) were set according to De Riek et al. (1999). Candidate genes for the development of EST markers were initially selected based on their putative function in plant drought tolerance and The EST marker development was carried out as described in Razavi et al. (2012). EST marker analysis was

performed as described in Razavi et al. (2012). Polymorphic alleles of EST markers were scored as present or absent (1/0). Quantity One software (Version 4.5.1) from BioRad (Hercules, CA, USA) was used for scoring and sizing of all bands. In the case of ESTs, we calculated the number of polymorphic alleles per locus (n_p) and also the average number of alleles per locus n_{av}= number of polymorphic alleles (n_p)/ number of loci (L) (Scariot et al. 2007; Belaj et al. 2003). RWC and WLR were analyzed with one way ANOVA and cultivars were grouped using Tukey's test (P=0.01). Calculation of genetic similarities (Jaccard similarity coefficient), canonical discriminant analysis, principle coordinate analysis (PCO), Kruskal-Wallis analysis ($P \leq 0.05$), correlation analysis (Pearson coefficient), hierarchical cluster analysis (UPGMA), chi-square (X^2) test ($P \leq 0.05$) and biplot (scatter dot graph) were performed with SPSS 11.01 for Windows (SPSS Inc., Chicago IL). To evaluate the reproducibility, bootstrapping (1000 permutations) was done using the Tree Con package for Windows (version 1.3b). Mantel analysis (Mantel 1967; Mantel Nonparametric Test Calculator for Windows, Version 2.00, 1999, by Adam Liedloff) was employed for testing of correspondence between matrices. The significance of the statistics was evaluated by permutations (1000×) and expressed as a probability (Smouse et al. 1986).

Results and discussion

Significant genotype variations were observed for both the RWC $_{\text{at harvest}}$ and the RWC $_{\text{after 4h}}$. The rate of decline of RWC (ΔRWC) showed also significant differences between the genotypes (data not shown). Moreover the rate of water loss as assessed by WLR varied significantly between the genotypes (P=0.01). A canonical discriminant analysis was employed to maximize separation of the genotypes and two main classes were grouped: Class *a* was defined

as drought sensitive; and class *b* defined as the drought tolerant (Fig 1).

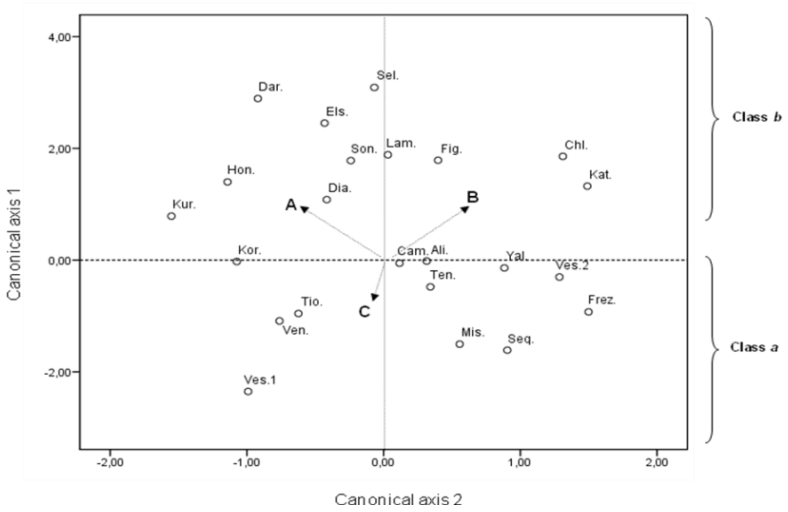

Fig. 1: Grouping of *Fragaria* genotypes based on discriminant factors of measured ecophysiological traits (Class a <0.00 and class b >0.7 on the reference line of canonical axis 1) (arrow A shows RWC at harvest, arrow B shows RWC after 4h and arrow C shows WLR, based on standardized canonical discriminiant function coefficients), Class a: Genotypes defined as drought sensitive showing a high WLR and low RWC after 4h, Class b: Genotypes defined as drought tolerant showing a low WLR and high RWC after 4h

General genetic relationship between *Fragaria* genotypes was revealed by AFLP and EST markers. Using the selection settings for frequency and average marker peak height, total number of polymorphic AFLP markers included in the analysis were 369. The polymorphism rate was similar between primer combinations (PC1: 98; PC3: 103; PC4: 104), except for PC2 with only 64 polymorphic markers. Twenty candidate genes were selected for EST marker development and in the end, 24 EST-based markers were available for genetic characterization. In 23 *Fragaria* genotypes, 121 alleles could be disclosed, 99 of them were polymorphic, on average 78% of these markers were polymorphic in the dataset. The number of polymorphic bands per genotype was variable between different ESTs and genotypes. The average number of alleles per locus (nav) for an EST was four. For both marker types, the pairwise genetic similarities between individual plants and hierarchical cluster analysis revealed the general relationship of the genotypes. The diploid species *Fragaria vesca* was clearly separated from the other genotypes both with AFLP and EST. The octaploid species *Fragaria chiloensis* clustered with the other octaploids but was clearly distant from the strawberry cultivars. AFLP confirmed the genetic similarity between some related genotypes (data not shown). By hierarchical cluster analysis, EST markers confirmed the genetic similarity within some studied strawberry cultivars (data not shown). ESTs appears to be better to group cultivars adapted to semi-arid conditions. Overall, clustering as generated from the AFLP and EST data were generally in good agreement with the taxonomic classification of *Fragaria* genotypes. The Mantel test showed significant (P=0.001) correlation among Jaccard matrices calculated from both marker techniques (R=0.81) (data not shown). Genetic relationships were also revealed and quantified by using PCO.

Jaccard similarity matrix of 23 genotypes was calculated based on combined AFLP and EST data and used as input for PCO analysis (Fig 2).

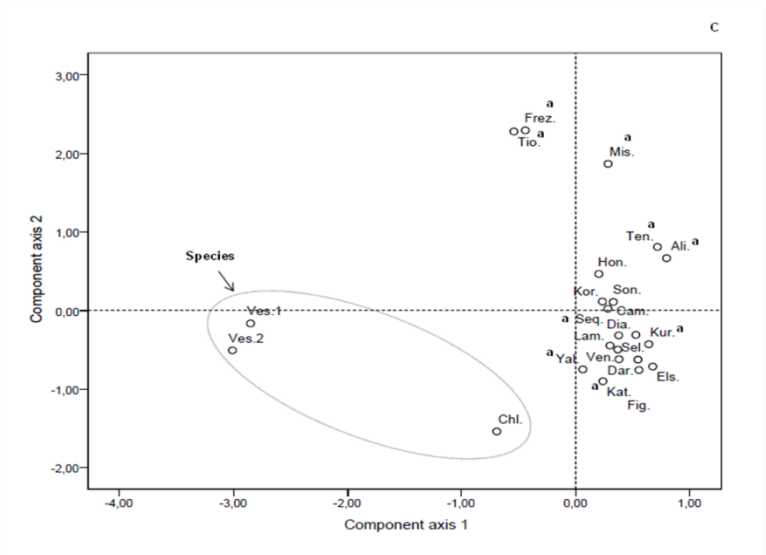

Fig. 2: Biplot of principal coordinate analysis (PCO) using combined AFLP and EST data ([a] Cultivars are adapted to Iranian climate condition; in dendrograms: Jaccard similarity coefficient, UPGMA clustering, bootstrap values from 1000 re-sampling cycles)

Association testing of DNA markers with physiological traits was performed as well. Kruskal-Wallis analysis ($P \leq 0.05$) was applied to determine AFLP and EST markers linked to the individual measured physiological traits as well as to the canonical discriminant factors. In AFLP, most of the markers correlated to the individual physiological traits are also linked to the discriminant factors of these traits, although there remain still some differences (data not shown). In ESTs, 10 markers linked to the measured physiological traits are also correlated to their derived discriminant factors, while 9 ESTs are only linked to the traits and not to their discriminant factors (data not shown). Hierarchical clustering of *Fragaria* genotypes according to AFLP/EST markers linked to the WLR, $RWC_{after\ 4h}$ at one side and discriminant factors of all measured physiological traits at the other side was made; the correspondence between the obtained ordinations with two main physiological classes of genotypes (tolerant/sensitive) was studied (Fig 3).

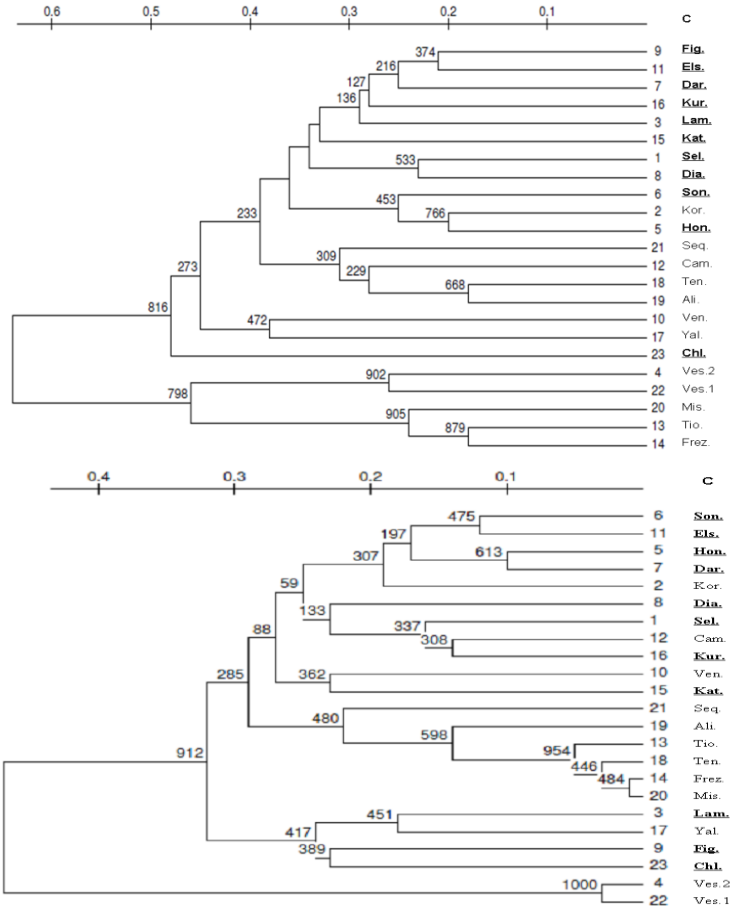

Fig 3: Dendrograms of 23 *Fragaria* genotypes obtained using: 114 AFLP markers correlated to the canonical discriminant functions derived of measured physiological traits by canonical discriminant analysis (Above) and 10 EST markers correlated to the canonical discriminant functions derived of measured physiological traits by canonical discriminant analysis (Below) (Jaccard similarity coefficient, UPGMA clustering, bootstrap values from 1000 re-sampling cycles; class *b* is marked as underlined bold names)

In conclusion, in a limited set of *Fragaria* genotypes, it was possible to come to an integrated method, combining fast screening tools for plant leaf dehydration and associated markers from random AFLP or candidate gene ESTs. Phenotypic classes of plants grouped to their drought response better corresponded to groupings made on correlated markers. Through this study, also some potential candidate genes involved in *Fragaria*

drought tolerance were identified but need further characterization. As plant responses to drought stress are a complex quantitative phenomenon, higher drought tolerance could be attributed to the combination of different factors that cannot be genetically analyzed as a monogenetic character. Therefore, mapping of them as QTLs is needed to better identify the regions involved in the regulation of this trait (Suprunova et al. 2004; Teulat et al. 2003). The resulted correlated markers in our study still need to be further evaluated for their association to the plant function in drought condition and LD-mapping in a larger set of unrelated genotypes. A more powerful approach would be to analyze these markers and check their trait-association and neutrality on genetic structure. This is probably not so easy for the highly bred and narrow gene pool of *Fragaria* × *ananassa*, but the linked markers identified in this study still are a good starting point for using this approach. Eventually these markers might be applied in germplasm screening for drought tolerance in *Fragaria* sp. Further investigation is necessary to characterize the sequences which were used for development of correlated EST markers to the plant responses to drought stress in this work. The expression analysis of the genes that were evaluated in the present study will give more information about the genetic control of drought response in *Fragaria* sp.

Reference

Bassil N.V., Gunn M., Folta K., Lewers K. (2006). Microsatellite markers for *Fragaria* from 'Strawberry Festival' expressed sequence tags. Mol Ecol Notes, 6: 473-476.

Belaj A., Satovic Z., Cipariani G., Baldoni L., Testolin R., Rallo L., Trujillo I. (2003). Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and of their effectiveness in establishing genetic relationships in olive. Theor Appl Genet, 107: 736-744.

Folta K.M., Davis T.M. (2006). Strawberry genes and genomics. Crit Rev Plant Sci, 25: 399-415.

De Riek J., Dendauw J., Mertens M., De Loose M., Heursel J., Van Bockstaele E. (1999). Validation of criteria for the selection of AFLP markers to assess the genetic variation of a breeders' collection of evergreen azaleas. Theor Appl Genet, 99: 1155-1165.

Gil-Ariza D.J., Amaya I., Botella M.A., Munoz Blanco J., Caballero J.L., Lopez-Aranda J.M., Valpuesta V., Sanchez-Sevilla J.F. (2006). EST-derived polymorphic microsatellites from cultivated strawberry (*Fragaria* × *ananassa*) are useful for diversity studies and varietal identification among *Fragaria* species. Mol Ecol Notes, 6: 1195-1197.

Hegde V.S., Mishra S.K. (2009). Landraces of cowpea, *Vigna unguiculata* (L.) Walp., as potential sources of genes for unique characters in breeding. Genet Resour Crop, 56: 615-627.

Klamkowski K., Treder W. (2008). Response to drought stress of three strawberry cultivars grown under greenhouse conditions. J Fruit Ornam Plant Res, 16: 179-188.

Peleg Z., Fahima T., Abbo S., Krugman T., Nevo E., Yakir D., Saranga Y. (2005). Genetic diversity for drought resistance in wild emmer wheat and its ecogeographical associations. Plant Cell Environ, 28: 176–191.

Rampino P., Pataleo S., Gerardi C., Mita G., Perrota C. (2006). Drought stress response in wheat: physiological and molecular analysis of tolerant and sensitive genotypes. Plant Cell Environ, 29: 2143-2152.

Razavi F., Pollet B., Steppe K., Van Labeke M.C. (2008). Study of chlorophyll fluorescence as a tool for evaluation of drought stress in strawberry. Photosynthetica, 46: 631-633.

Razavi F. (2012). Molecular and physiological responses to drought stress in *Fragaria sp.* PhD thesis dissertation, Ghent Uniuversity.

Mantel N. (1967). The detection of disease clustering and generalized regression approach. Cancer Res 27: 209-220.

Mishra R.K., Sen-Mandi S. (2004). Molecular profiling and development of DNA marker associated with drought tolerance in tea clones growing in Darjeeling. Curr Sci, 87: 60-66.

Sargent D.J., Davis T.M., Tobutt K.R., Wilkinson M.J., Battey N.H., Simpson D.W. (2004). A genetic linkage map of microsatellite, gene-

specific and morphological markers in diploid *Fragaria*. Theor Appl Genet, 109: 1385-1391.

Scariot V., De Keyser E., Handa T., De Riek J. (2007). Comparative study of the discriminating capacity and effectiveness of AFLP, STMS and EST markers in assessing genetic relationship among evergreen azaleas. Plant Breed, 126: 207-212.

Schwab W., Schaart J.G., Rosati C. (2009). Functional molecular biology research in *Fragaria*. In: Folta K.M., Gardiner S.E. (eds.) Genetic and Genomics of *Rosaceae*. Springer, New York, pp 457-486.

Suprunova T., Krugman T., Fahima T., Chen G., Shams I., Korol A., Nevo E. (2004). Differential expression of dehydrin genes in wild barley, *Hordeum spontaneum*, associated with resistance to water deficit. Plant Cell Environ, 27: 1297- 1308.

Smouse P.E., Long J.C., Sokal R.R. (1986). Multiple regression and correlation extension of the Mantel test of Matrix correspondence. SystZool, 35: 627-632.

Teulat B., Zoumarou-Wallis N., Rotter B., Ben Salem M., Bahri H., This D. (2003). QTL for relative water content in field-grown barley and their stability across Mediterranean environments. Theor Appl Genet, 108: 181-188.

VanDerZanden A.M., Cameron S.J. (1996). Effect of water deficit stress on 11 native *Fragaria chiloensis* clones selected as ornamental groundcovers. Sci Hort, 66: 241-253.

Verslues P.E., Agarwal M., Katiyar-Agarwal S., Zhu J., Zhu J.K. (2006). Methods and concept in quantifying resistance to drought, salt and freezing, abiotic stressed that affect plant water status. The Plant J, 45: 523-539.

Vos P., Hogers R., Bleeker M., Reijans M., Van de Lee T., Hornes M., Fijters A., Pot J., Peleman J., Kuiper M., Zabeau M. (1995). AFLP: a new technique for DNA fingerprinting. Nucl Acids Res, 21: 4407-4414.

Zhang B., Archbold D.D. (1993). Solute accumulation in leaves of a *Fragaria chiloensis* and a *F. virginiana* selection responds to water deficit stress. J Amer Soc Hort Sci, 118: 280-285.