

The transfer of high CCS traits from wild relatives to sugarcane using biochemical markers

SRDC Project Number CTA048

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Executive Summary

Issue

Over the last 40 years of sugarcane breeding in Australia there have been significant improvements in cane yield but little to no improvement in commercial cane sugar (CCS). It has been hypothesised that this lack of gain is due to the narrow genetic base of current breeding programs and has provided the impetus to examine new sources of germplasm that may provide desirable traits. Broadening the genetic base through the introgression of new *Saccharum* germplasm could allow the incorporation of 'new' genes for CCS into commercial sugarcane and result in increased CCS. However carefully targeted approaches are required to identify favourable genetic components of value from available new germplasm for introgression.

Project Outputs

- **The key finding that the enzyme sucrose-phosphate synthase (SPS) was significantly higher in activity in the upper internodes of high CCS clones as compared with low CCS clones in both an introgression and a backcross population segregating for CCS and sucrose concentration.** These findings are potentially very significant. The significant difference in SPS activity between high and low CCS clones measured in the uppermost internodes appears to be a robust biochemical marker having been observed in two populations representing two generations, segregating for CCS and sucrose content. The potential significance of a correlation between sucrose-phosphate synthase and high CCS has been communicated to the CRC for Sugarcane Innovation through Biotechnology (SIIB) researchers working in the marker area. They have developed a pilot project to turn this biochemical marker into a DNA marker through detection of SNPs (single nucleotide polymorphisms). These are currently being tested for an association between particular SNPs in the SPS genes and the high CCS and sucrose phenotype.
- **Derivation of a simple biochemical measure of the sucrose : hexose ratio to effectively group high or low sucrose accumulating or CCS clones.** The identification of an association between the sucrose : hexose ratio and high CCS is currently being further investigated as part of CTA047 and may lead to the development of a QTL for this marker.
- **A characterisation of potential new germplasm (184 clones of *Saccharum officinarum*), which may be introgressed into commercial sugarcane has been carried out.** A number of physical parameters, such as height and girth as well as CCS and sugar concentrations using the HPAE-PAD were performed. More detailed biochemical analysis was carried out upon a selection of five low CCS and ten

high CCS clones. These analyses contributed to the derivation of the sucrose : hexose ratio.

- **Detailed investigation of the measurement of CCS and how it relates to sucrose and hexose concentrations.**

From laboratory experiments it was determined that there is a negative effect of biological levels of hexoses upon Pol and under certain conditions it may be sufficient to affect CCS. The potential for a hexose induced effect upon Pol is greatest in actively growing cane when the hexose concentrations are high and the levels of glucose and fructose are similar. This possibility of 'Pol bias' is primarily of importance when examining a diverse source of germplasm such as the *S. officinarum* collection, which is likely to vary greatly in sucrose and hexose concentrations or when investigating the genetic and biochemical processes underpinning sucrose accumulation. When pursuing such investigations it is advisable to measure individual sugars rather than relying upon CCS measurements alone.

- **Rejuvenation of the sugarcane biochemical research area and development of a new approach to the measurement of a suite of key enzymes involved in sucrose metabolism.**

A strong sugarcane biochemistry research program had been a cornerstone of the CSR David North Laboratory. This research program was dismantled in the early 70's and the skills and knowledge dissipated.

R & D Methodology

As part of a strategy to introgress novel germplasm into commercial sugarcane, with the ultimate aim of improving CCS levels, a number of crosses have been made between novel *S. officinarum* parents and elite commercial cultivars. A two pronged approach was taken on these populations whereby both DNA (CTA047) and biochemical markers (CTA048) were sought which can be tested for linkages to desirable agronomic traits, in particular high CCS.

To create this material, a number of crosses were made in 1999 between novel *S. officinarum* parents and elite commercial cultivars. Detailed biochemical analyses were carried out on four high and four low CCS progeny clones from one segregating population derived from a cross between an *S. officinarum* clone (IJ76-514) and a commercial variety (Q165^A). A suite of key sucrose metabolising enzymes was measured. A novel technique to increase the sensitivity and throughput of enzyme assays has been developed using the HPAE-PAD system. Of particular significance was the finding that the activity of a key enzyme involved in sucrose synthesis, Sucrose-phosphate synthase (SPS), was significantly higher in the high CCS clones in the uppermost internodes, namely 1 to 3.

The measurement of the key enzyme SPS in four different positions down the stem profile has also been completed in four high and four low CCS progeny from a backcross population grown in a replicated field trial. These progeny were produced by crossing selected individual progeny, (KQ99-1410, measured to possess high CCS) from the first introgression population, with a different elite commercial cultivar (Mida). In the uppermost internodes (1 to 3), five-fold greater activity of SPS was observed in the high CCS clones as compared with the low CCS clones. Such a difference is similar to that observed in the first introgression population.

The precise measurement of sugars by application of the HPAE-PAD system, in addition to providing a novel means of enzyme measurement also led to the derivation of the biochemical measure of the sucrose : hexose ratio and clarified the relationship between CCS and sugar concentrations in developing cane.

The impact of project findings on the sugar industry and the Australian community

The successful achievement of the identification of a biochemical marker (sucrose : hexose ratio) and an enzymatic marker (SPS) provides potential tools to be used in the screening of populations for high CCS clones.

In addition to the direct application of these tools, if the use of SPS as the basis of SNP analysis to define genetic variation is successful, it will demonstrate the additional utility of the approach described herein. Both markers are undergoing further analysis in new projects which may lead to an impact upon the selection of high CCS varieties.

Background

Plant breeding has made substantial improvements in the performance of sugarcane over the last forty years. Empirical observations indicate that the principal contribution however has been through increased plant biomass (yield) whereas sucrose content (measured as CCS) has remained unchanged. From a whole of industry perspective, increases in CCS would make the production of raw sugar more efficient in that for the same sucrose yield, costs of harvesting, transport and milling would be reduced.

One factor that is believed to have contributed to the lack of progress in improving CCS is the narrow gene pool used in current commercial breeding programs. *Saccharum officinarum* is the principal contributor to high sucrose accumulation in modern commercial varieties and it is believed that some *Saccharum officinarum* genotypes have CCS levels that exceed those of commercial sugarcane hybrid varieties. Sugarcane breeders recognise that the introgression of genes associated with high CCS from *S. officinarum*, other *Saccharum* or related species offers significant potential for the improvement of CCS in commercial varieties. Currently the *S. officinarum* genes used in conventional plant breeding programs are derived from a small number of parents (19 *S. officinarum* clones), used in the original inter-specific hybridisations undertaken early in the century.

The principal aim of this project was to develop biochemical markers for high CCS, or more specifically high sucrose concentration, in wild clones of *S. officinarum* and to test whether such markers may have utility in introgression breeding programs to select for high sucrose concentration traits. The information gained during the identification of these biochemical markers may also improve our understanding of the underlying mechanisms that drive sucrose accumulation in sugarcane and its progenitors.

Objectives

To define the biochemical traits which determine the final level and rate of sucrose accumulation (maximal CCS and timing of CCS) in wild accessions of sugarcane relatives

No background information was available on the *Saccharum officinarum* collection of 184 clones which was to be the source of parents for introgression previously unused in breeding programs. This collection was characterised by measuring a number of physical parameters, CCS and sugar concentrations using the HPLC-PAD. A selection of five low CCS and ten high CCS clones were selected for more detailed biochemical analysis. The precise measurement of sugars enabled the derivation of a simple biochemical measure of the sucrose : hexose ratio to effectively group high or low sucrose accumulating or CCS clones. This marker is a clear indicator of maturity and unlike the CCS measurement is independent of moisture content and is not affected by fibre.

*To test the utility of assays for these biochemical/physiological processes as markers in progeny populations derived from wild crosses between sugarcane varieties and *Saccharum officinarum*.*

High throughput assays for the key enzymes involved in sucrose metabolism, namely sucrose synthase, three isoforms of invertase (neutral, acid and cell wall) and sucrose phosphate synthase (SPS) were successfully developed and used upon an introgression (IJ76-514 x Q165^A) population.

The enzyme measurements performed upon a backcross population (KQ99-1410 x Mida) were restricted to sucrose-phosphate synthase, vacuolar acid and neutral invertase. From these measurements, the enzyme SPS measured in the uppermost internodes (1-3), consistently exhibited increased activity in the high CCS as compared with the low CCS clones, thereby demonstrating the robustness of this biochemical marker.

Although the objectives for this project were ambitious, the identification of a robust biochemical marker (sucrose :hexose ratio) as well as an enzymatic marker have clearly demonstrated their achievement.

Methodology and Outputs

As part of a strategy to introgress novel germplasm into commercial sugarcane, with the ultimate aim of improving CCS levels, a number of crosses were made between novel *S. officinarum* parents and elite commercial cultivars.

A two-pronged approach was taken on these populations whereby both DNA (CTA047) and biochemical markers (CTA048) were sought which can be linked to desirable agronomic traits, in particular high CCS. As no background information was available on the 184 *S. officinarum* clones, the potential parents for the introgression approach, a screen to measure physical parameters and stalk sugar content was carried out. From the initial screen, a subset of ten high and five low sucrose accumulating clones were selected for more detailed analysis. Internode samples were taken at various positions down the stem profile and sugar content (sucrose and hexoses) determined for three stalks of the selected clones. A summary of this data and a description of the introgression strategy is described in the manuscript published in the Proceedings of the 24th ISSCT Conference entitled ***Introgression of S.officinarum: A biochemical and molecular marker approach to improve CCS*** and attached as Appendix 1.

To create the introgression populations, a number of crosses were made in 1999 between novel *S. officinarum* parents and elite commercial cultivars. Detailed biochemical analyses were carried out on four high and four low CCS clones from one progeny population (IJ76-514 x Q165^A) to measure a suite of key sucrose metabolising enzymes. Of particular significance was the finding that the activity of a key enzyme involved in sucrose synthesis, SPS, was significantly higher in the high CCS clones in the uppermost internodes, 1 to 3.

A novel technique to increase the sensitivity and throughput of enzyme assays has been developed using the HPAE-PAD system.

This methodology is described in the manuscript attached as Appendix 2, entitled ***Application of high pH anion exchange-pulsed amperometric detection to measure enzyme activities in sugarcane and its wider application to other biologically important enzymes*** which is to be published in Journal of Chromatography B.

The measurement of the key enzyme SPS in four different positions down the stem profile has also been completed in four high and four low CCS progeny from a backcross population grown in a replicated field trial. These progeny were produced by crossing select progeny (measured to possess high CCS) from the

first introgression population with a different elite commercial cultivar (KQ99-1410 x Mida).

Corroborative evidence that this enzyme represents an appropriate biochemical marker and possible early screening tool for high CCS has been gathered (Appendix 3).

We have thereby demonstrated that biochemical markers can be successfully applied to field grown material and their use is not restricted to material grown under controlled environmental conditions.

The use of the HPAE-PAD system also facilitated high sensitivity measurement of sugars in the *S. officinarum* collection as well as in the introgression populations, although less sensitive techniques would also be satisfactory for such measurements. However, the HPAE-PAD also enables the identification of sugars other than sucrose, glucose and fructose, which may be present in unknown samples. A more detailed sugar measurement of clones, other than CCS, enabled the calculation of a sucrose to hexose ratio, a biochemical indicator of maturity, that unlike the CCS measurement is independent of moisture content and is not affected by fibre. The direct measurement of sugars at an internode level will provide a better understanding of the sucrose accumulation process and may provide greater scope to identify traits associated with sucrose accumulation. These ideas were presented by Peter Albertson at the SRDC sponsored Sugarcane Physiology Workshop held in Brisbane in September 2003 and are described in greater detail in the manuscript attached as Appendix 4, entitled ***Measurement of sucrose at internode level rather than whole stalk CCS alone can increase our understanding of sucrose accumulation in sugarcane.***

The direct measurement of sugars in conjunction with CCS has highlighted some of the deficiencies associated with the CCS measurement.

Sugars other than sucrose may confound estimates of CCS when spectrophotometric methods such as polarimetry and NIR are used. However, in highly selected commercial varieties they are unlikely to have a significant impact on CCS measured at the time of harvest. The potential for hexose induced negative bias of Pol and CCS is greatest in actively growing cane and for research directed at mechanisms underpinning the sucrose accumulation process, the direct measurement of sugars is highly recommended. This work entitled ***The effect of hexose upon Pol, Brix and calculated CCS in sugarcane: a potential for negative Pol bias in juice from actively growing cane*** is appended as Appendix 5.

The potential for using sucrose, glucose, fructose and sucrose : hexose ratio directly to identify QTLs has been tested in a preliminary way on the IJ76-514 x Q165^A population by the CRC for Sugarcane Innovation through Biotechnology. A sample of juice from the August sampling time in 2001 was taken for each individual from each replicate. The juice was analysed for sucrose, glucose and fructose using enzyme linked assays (the HPAE-PAD system was fully booked

making the enzyme assay measurements reported above), Using the sugar measurements and the markers scored across the population (CTA047) single factor analysis was used to identify putative QTLs. A pair of figures highlighting the points made below appear as Appendix 6. As not a part of this project, a fuller treatment of this data will be presented elsewhere. However, the results illustrate the benefits of the combined biochemical and DNA based approaches and that the project CTA048 has provided valuable insights that are being followed up.

- A QTL for sucrose was identified on linkage group 17 (Q165^A) where no association was identified for CCS.
- A QTL for both fructose and glucose was identified at the bottom of linkage group 81 (Q165^A). There was no QTL identified for sucrose but CCS did identify a QTL in this region. This kind of analysis is starting to explain which components of the several variables that make up CCS is contributing to which QTL.
- There are also several QTLs identified by CCS that are not associated with the sugar measurements (one is at the top of linkage group 81). CCS is affected by water and fibre independently of sugar content on a dry weight basis and composition.
- On the IJ76-524 map, which contains fewer markers, fructose and glucose co-located with a different QTL for CCS than sucrose. Glucose and fructose contributed to a QTL not associated with CCS.
- In both maps the sucrose : hexose ratio also identified QTLs. They always co-located with QTLs found with hexoses, sucrose or both.

Although the sugar measurements have only been made for one year the preliminary data shows that there is additional information to be gained from the sugar measurements. The size of the QTLs were similar to those for CCS, accounting for 3-9% of the variation. These measurements will be made again in the backcross populations to be sampled in June/July 2004. This will test the robustness of the QTL found and further test whether these measurements and the ratio derived from them will be useful in aiding sugarcane improvement.

Intellectual Property

The possible use of SPS and other key enzymes involved in sucrose metabolism as SNP markers may represent intellectual property.

Expected Outcomes

This project was aimed at developing a strategic research platform to underpin the new breeding initiatives being developed in the sugar industry. It has achieved its objectives and provided a number of useful tools, which can be applied to drive a targeted approach to sugarcane improvement.

The enzyme sucrose-phosphate synthase has been demonstrated to be a robust enzymatic marker of high sucrose and CCS in two introgression populations. A pilot project to detect SNPs (single nucleotide polymorphisms) in SPS has been established to determine the possible application of this biochemical marker to detect genetic variance in sugarcane. If successful, this work will be extended to a wider range of enzymes involved in sucrose metabolism.

Several factors influence the value of calculated CCS, including the level of soluble compounds other than sucrose in juice, stalk fibre and stalk moisture. The direct and sensitive measurement of sugars (in this project performed using the HPLC-PAD) enabled the calculation of the sucrose to hexose ratio, a biochemical marker independent of moisture and not affected by fibre. Furthermore, direct sucrose measurement may facilitate the development of QTL markers associated with high sucrose accumulating clones as the contribution of additional factors to the CCS calculation which may obfuscate the correlation are removed.

An effective extraction and assay procedure has been used for the suite of key enzymes during the course of this project. This is particularly relevant as the enzyme sucrose-phosphate synthase, the robust enzymatic marker is highly labile. These procedures have been passed on to groups within the CRC who are attempting to manipulate metabolic processes in sugarcane.

Recommendations and Future Research Needs

The key outputs from this project have provided a platform from which to launch other projects aimed at developing high sugar genotypes. Major recommendations are as follows:

1. The potential of SPS as a molecular marker for high sucrose be assessed. This is currently underway as part of a CRC SIIB project.
2. The biochemical marker sucrose : hexose ratio be assessed in the backcross population to be phenotyped in 2004. Samples are proposed to be collected to test the utility of this marker in June/July 2004 in a CRC SIIB project.
3. Pursue QTL analysis using the sugars sucrose, glucose and fructose to determine if QTLs can be identified which are independent of those associated with CCS. This may facilitate the dissection of genetic components driving sucrose accumulation and hexose to sucrose conversion.
4. Further research projects looking at CCS should be encouraged to look at individual sugars where possible. Such an approach may explain observed phenomena and illuminate details of the sucrose accumulation process.

Publications

Grof CPL (2001). Molecular manipulation of sucrose metabolism. *Proceedings 24th International Sugar Cane Technologists* (Brisbane, 17-21 September, 2001) p 586.

Albertson PL, Aitken KS, Grof CPL, Jackson P, McIntyre L, Piperidis G and Berding N (2001). Introgression of *S.officinarum*. A biochemical and molecular marker approach to improve CCS. *Proceedings 24th International Sugar Cane Technologists* (Brisbane, 17-21 September, 2001) p 567-572.

Albertson PL and Grof CPL (2002). Introgression of *S. officinarum* to improve CCS via the identification of potential biochemical markers. *Proceedings of the Australian Society of Sugar Cane Technologists* 24, 481.

Albertson PL and Grof CPL (2003). The effect of hexose upon Pol, Brix and calculated CCS in sugarcane: A potential for negative Pol bias in juice from actively growing cane. *25th Conference of the Australian Society of Sugar Cane Technologists*, Townsville, Qld.

Albertson PL and Grof CPL (2004). Application of high pH anion exchange-pulsed amperometric detection to measure enzyme activities in sugarcane and its wider application to other biologically important enzymes. *Journal of Chromatography B*. (in press).

Albertson PL and Grof CPL (2004). The effect of hexose upon Pol, Brix and calculated CCS in sugarcane: A potential for negative Pol bias in juice from actively growing cane. *J of the American Society of Sugarcane Technologists* (accepted with minor changes).

Albertson PL and Grof CPL (2004). Measurement of sucrose at an internode level rather than whole stalk CCS alone can increase our understanding of sucrose accumulation in sugarcane. Submitted to *Field Crops Research*.

The data presented in Appendix 3 is currently being prepared for publication.

Acknowledgements

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The data in Appendix 6 was generated by Donna Glassop, Karen Aitken and Graham Bonnett within the CRCSIIB.

Appendix 1

Introgression of S. officinarum: A biochemical and molecular marker approach to improve CCS.

Appendix 3

Results of enzyme measurements from introgression population (IJ76-514 x Q165) and backcross population (KQ99-1410 x Mida).

Materials and Methods

Plant Material

A subset of eight clones from one population (IJ76-514 x Q165) representing the extremes of the CCS spectrum were selected for detailed biochemical analysis. Three stalks of each clone were randomly selected from each of three plots, totalling nine stalks per clone. Each clone was separated into internodal sections and the pith of the internodes 1-3, 5-6, 9-10, middle (internode midway between internode 10 and the bottom of the stalk) was cored from each section using a cork borer. The tissue was finely chopped and internodal material from stalks taken from the same plot was pooled. Two sub-samples, one of 5 g for enzyme analyses and a second of 1 g for sugar determinations, were taken from this pool and rapidly cooled in liquid nitrogen. The plant material was transferred to dry ice and subsequently stored at -80°C.

A backcross population was produced by crossing one individual from the first introgression population, measured to possess high CCS, with a different elite commercial cultivar (KQ99-1410 x Mida). The progeny from this backcross were sampled as single stools in September 2002 in order to establish replicated plots of a smaller number of select clones representing the extremes of the spectrum with regard to sucrose content in the overall backcross population. CCS measurements were carried out at Kalamia Mill and juice samples were transferred to Brisbane to measure sucrose concentration from 288 clones using the HPAE-PAD. Twenty high and 20 low CCS clones were selected and planted in replicated plots in a random block design. Four high and four low CCS clones were sampled during July 2003 in an identical manner to the first introgression population, for detailed biochemical analysis.

Enzyme assays and protein determination

Enzymes were extracted from 5 g tissue in 50 mM Hepes pH 7.5, containing 10 mM MgCl₂, 5 mM DTT, 5mM α -aminocaproic acid, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM Benzamidine, 1 mM Benzamide, 2 μ M Leupeptin, 2 μ M Antipain and 0.1% Triton-X 100 as previously described (Albertson et al. 2001). The crude extract was concentrated by a two-step precipitation and resuspension procedure utilising 15% PEG (polyethylene glycol 8000 MWt.). A second PEG precipitation was required to remove residual sugars from the extract. The final pellet was resuspended in 2-3 mL and desalted on a Sephadex G25 column (50 mm x 10 mm) and stored on ice until required.

Enzyme assays and protein determination

Assays were subsequently conducted with 50 mM Hepes, pH 7.3, except for acid invertase, which was assayed at pH 4.5 in 50 mM McIlvaines buffer (citrate/phosphate buffer). Substrates for the enzyme assays were well above reported K_m values thereby facilitating maximum enzyme activity (V_{max}).

Sucrose cleaving enzymes

Invertase and SS(r) assays were conducted in the presence of 125 mM sucrose, the SS(r) assay also included 15 mM UDP.

Sucrose synthesising enzymes

The SS(f) assay was conducted in the presence of 30 mM UDP-glucose and 15 mM fructose.

The SPS assay was conducted in the presence of 30 mM UDP-glucose, 13.8 mM glucose-6-phosphate, and 4 mM fructose-6-phosphate.

All assays were conducted for 30 min at 37 °C except for the SPS assay, which was allowed to proceed for 60 min. The enzyme assay was initiated by adding a 100 µL aliquot of crude extract to 200 µL of 2x assay buffer plus 100 µL of enzyme substrates. Aliquots at time zero (t_0) and 30 min (t_{30}) or 60 min (t_{60}) for all enzyme assays except for acid invertase, were stopped initially by freezing in liquid nitrogen and once all assays were completed were heated to 80°C for 5 min. The acid invertase assay tubes were then neutralised with Imadazol prior to heating. All tubes were then stored at -20 °C until the reaction products were analysed by HPAE-PAD. The difference in product present in the two samples ($t_{30} - t_0$) or ($t_{60} - t_0$) was then used to calculate enzyme activity.

Aliquots of partially purified extracts were frozen for subsequent protein determinations using the Bradford reagent and BSA as the standard.

Results

The measurements of CCS, brix and pol in four high and four low CCS clones from the introgression (IJ76-514 x Q165) and backcross population (KQ99-1410 x Mida) are presented in Tables 1 and 2 respectively. The measurements of the introgression population, which are evidently low, were performed when the plants were approximately 8 months old and therefore had not yet reached maturity. Nonetheless a clear difference is discernable between high and low CCS clones.

The sucrose concentrations in four different internodes down the stem profile of selected clones from the first introgression population (IJ76-514 x Q165) are shown in Fig 1. The difference between high and low CCS clones appears to be reduced when sucrose is expressed on a dry weight basis highlighting the significance of moisture content upon the calculation of CCS.

The activities of the key enzymes, sucrose- phosphate synthase and soluble invertases were measured in four high and four low CCS clones from the IJ76-514 x Q165 cross by the novel application of the HPAE-PAD system and are shown in Fig. 2. Panels a) to c) show enzyme activities in four internodes down the stem of the high CCS clones, KQ99-1317 and 1362 and the low CCS clones KQ99-1292 and 1397. The activity of SPS and neutral and soluble acid invertase in an additional 2 high (KQ99-1446 and 1501) and 2 low (KQ99-1318 and 1355) CCS clones from the same population measured 18 months later are shown in panels d) to f).

The activities of the key enzymes, sucrose- phosphate synthase and soluble invertases were also measured in four high and four low CCS clones from the KQ99-1410 x Mida cross. The KQ99-1410 parent was one of the progeny from the IJ76-514 x Q165 cross. These measurements are shown in Figure 3.

Figure 4 shows sucrose synthase activity measured in the forward (a) and reverse (b) directions down the stem profile of four high and four low CCS clones from the backcross population KQ99-1410 x Mida.

Albertson PL, Peters KF and Grof CPL (2001). An improved method for the measurement of cell wall invertase activity in sugarcane tissue. *Aust. J. of Plant Physiol.* **28**, 323-328.

Table 1. Measurements of CCS, brix and pol in four high and four low CCS clones sampled in July 2001 from introgression population IJ76-514 x Q165.

Clone	Brix (Mean \pm SE)	Pol (Mean \pm SE)	CCS (Mean \pm SE)
1317	17.15 \pm 0.93	54.6 \pm 4.40	7.13 \pm 0.86
1362	15.55 \pm 0.31	45.7 \pm 1.51	5.23 \pm 0.34
1446	14.85 \pm 0.78	43.8 \pm 4.51	4.95 \pm 1.0
1501	16.38 \pm 0.38	52.4 \pm 2.55	6.85 \pm 0.58
1292	12.05 \pm 0.33	30.8 \pm 2.67	2.33 \pm 0.71
1318	12.9 \pm 0.75	33.2 \pm 3.97	2.68 \pm 0.92
1355	13.85 \pm 0.82	38.2 \pm 3.64	3.75 \pm 0.75
1387	13.7 \pm 0.71	36.3 \pm 4.08	3.25 \pm 0.90

Table 2. Measurements of CCS, brix and pol in four high and four low CCS clones sampled in July 2003 from backcross population KQ99-1410 x Mida.

Clone	Brix (Mean \pm SE)	Pol (Mean \pm SE)	CCS (Mean \pm SE)
3177	23.80 \pm 0.20	95.73 \pm 1.33	15.90 \pm 0.30
3115	22.57 \pm 0.38	87.47 \pm 2.08	14.13 \pm 0.43
3105	22.27 \pm 0.62	86.93 \pm 3.37	14.13 \pm 0.69
3060	21.33 \pm 1.32	81.73 \pm 7.87	13.10 \pm 1.65
3225	21.10 \pm 0.45	79.87 \pm 2.40	12.67 \pm 0.50
3051	20.17 \pm 0.38	75.60 \pm 1.40	11.87 \pm 0.22
3020	19.13 \pm 0.24	69.73 \pm 1.35	10.63 \pm 0.30
3264	18.77 \pm 0.69	64.67 \pm 4.74	9.33 \pm 1.09

Fig. 1. Sucrose concentrations in selected internodes on a fresh weight (a) and dry weight basis (b) for four high (KQ99-1501, 1317, 1362, 1446) and four low (KQ99-1292, 1387, 1318, 1355) CCS clones from the introgression population (IJ76-514 x Q165).

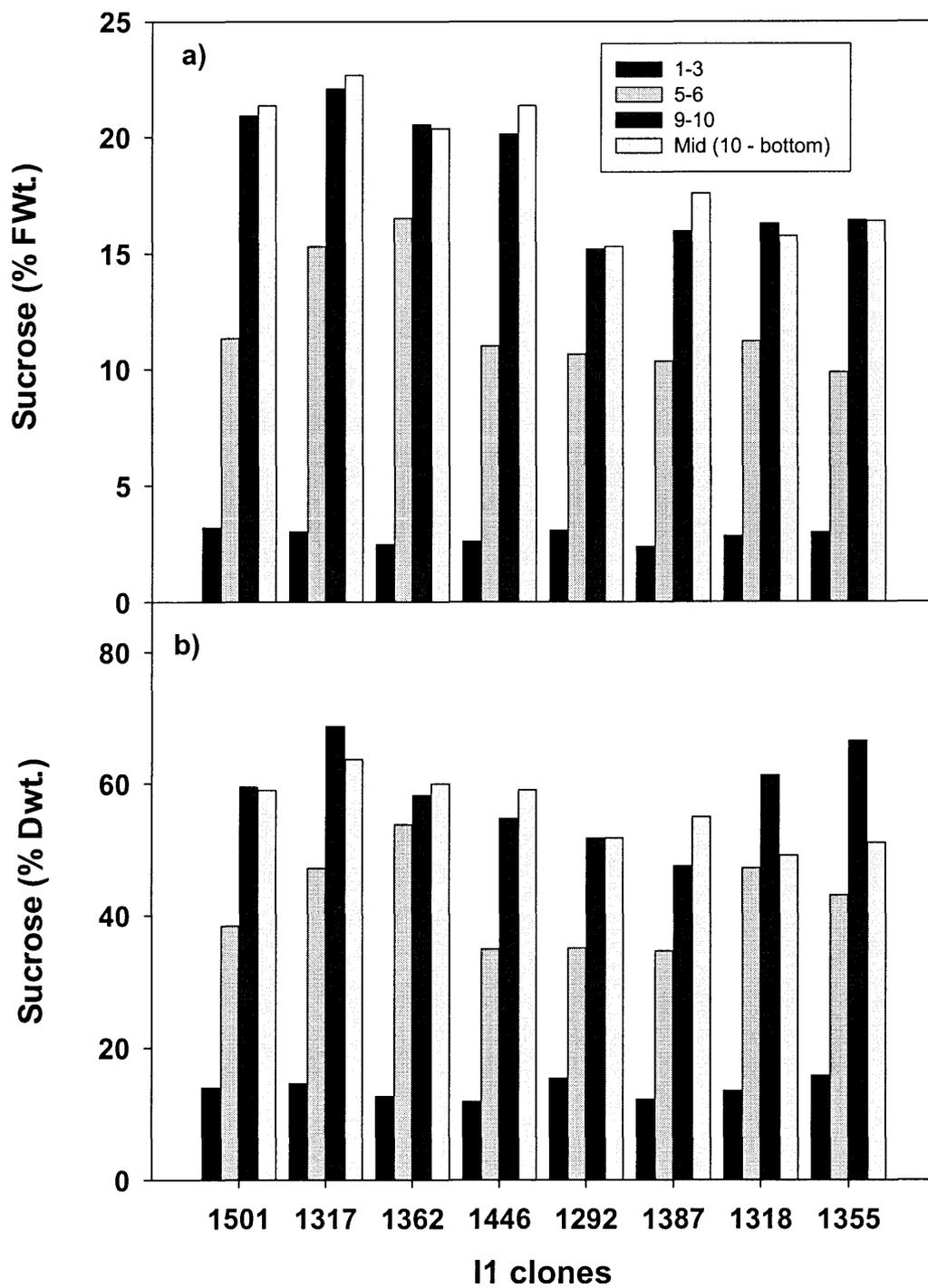


Fig. 2. Activity of key enzymes in high (○) and low (●) CCS clones from introgression population, IJ76-514 x Q165. Measurements performed in 2002 (a) – (c); measurements completed in 2004 (d) – (f). Panel (a) and (d) are sucrose phosphate synthase, (b) and (e) neutral invertase and (c) and (f) are vacuolar acid invertase. The units of activity are: a) and d) μg sucrose formed mg^{-1} protein min^{-1} b), d) and c), f) μg sucrose cleaved mg^{-1} protein min^{-1} . Each point is the mean \pm SE of six measurements.

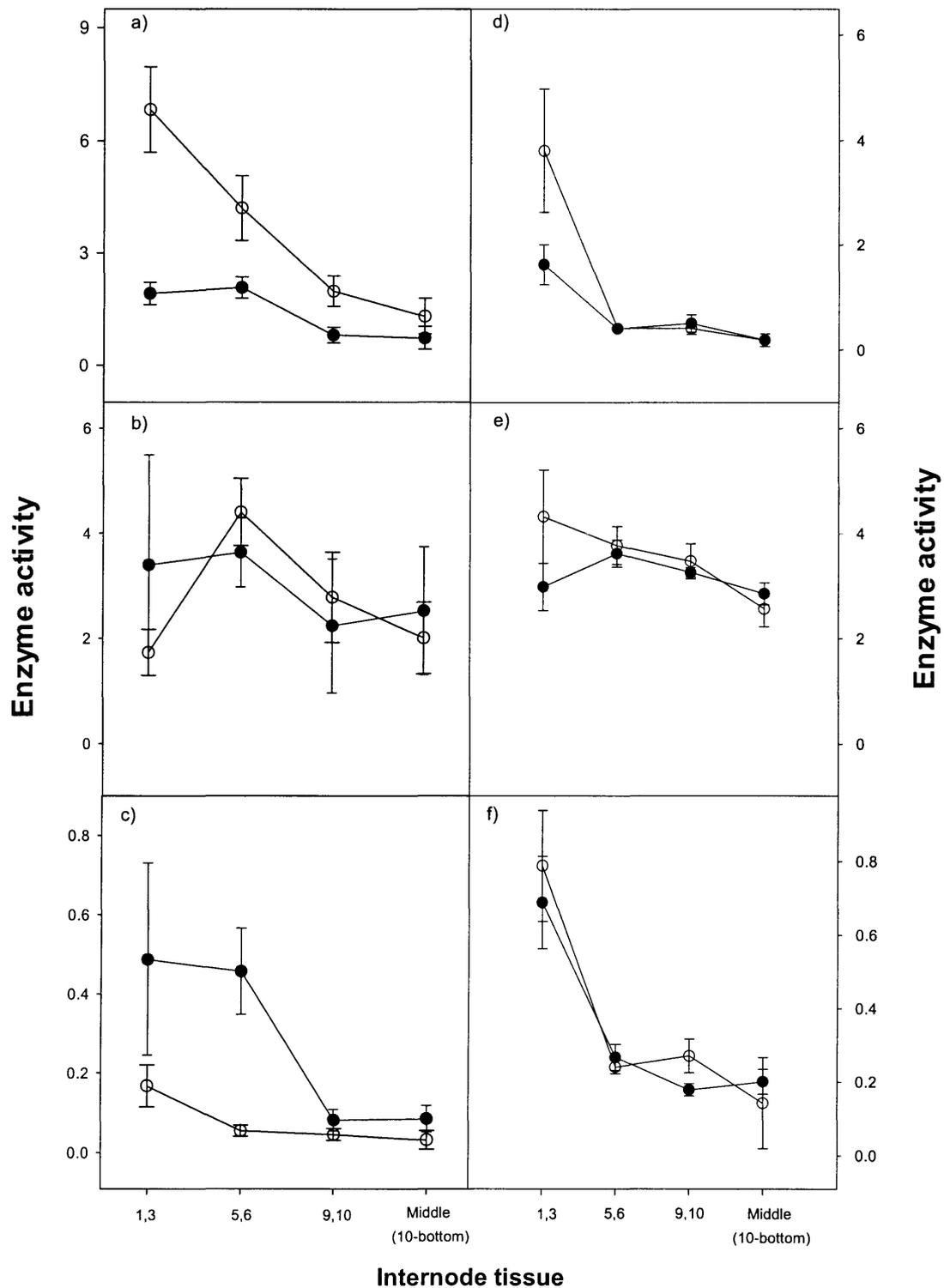


Fig. 3. Activity of key enzymes in four high (○) and four low (●) CCS clones from the backcross population KQ99-1410 x Mida. SPS (a), NI (b) and VAI (c). The units of activity are μg sucrose formed (a) or cleaved (b) and (c) mg^{-1} protein min^{-1} . Each value represents the mean \pm SE of twelve measurements.

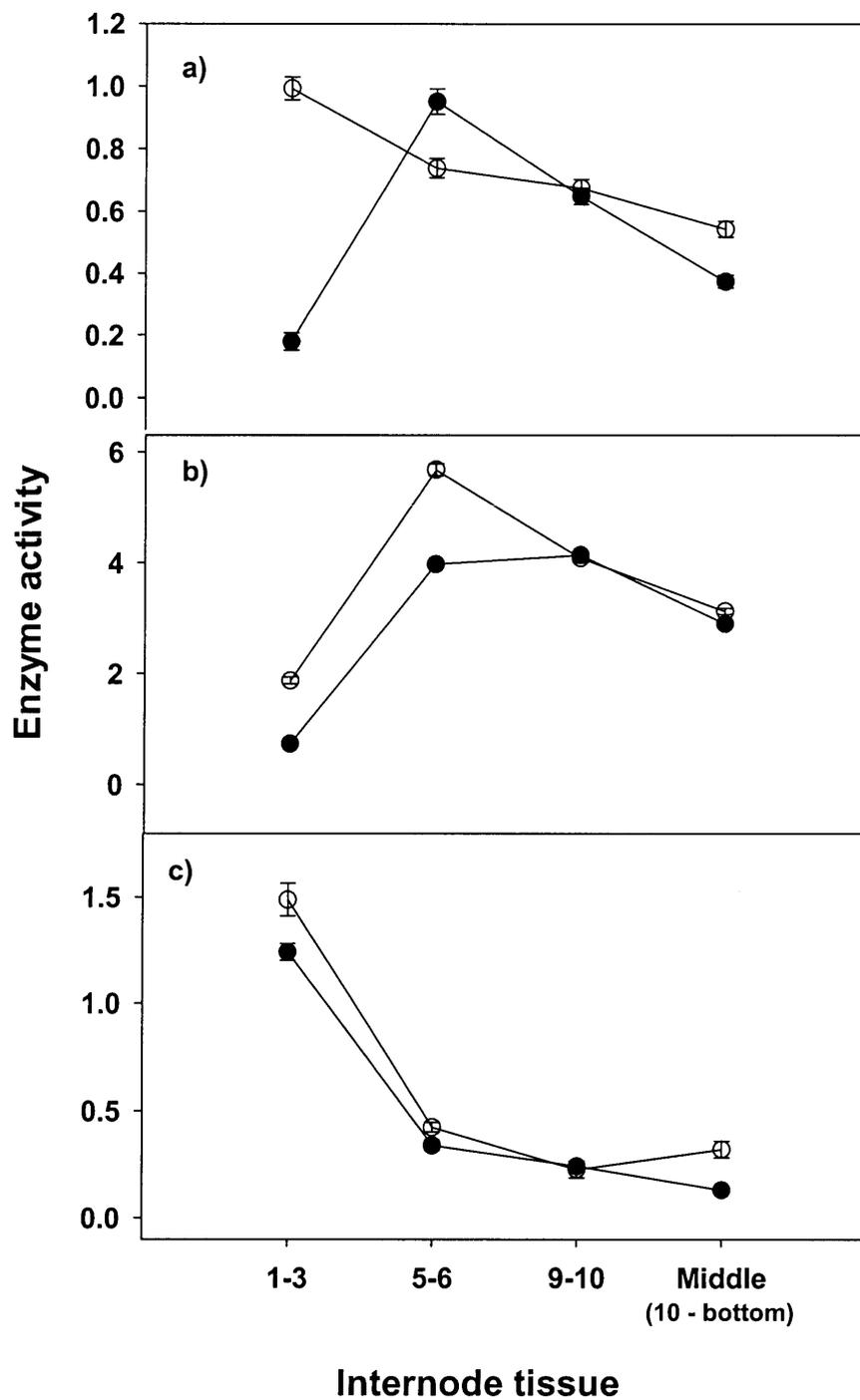
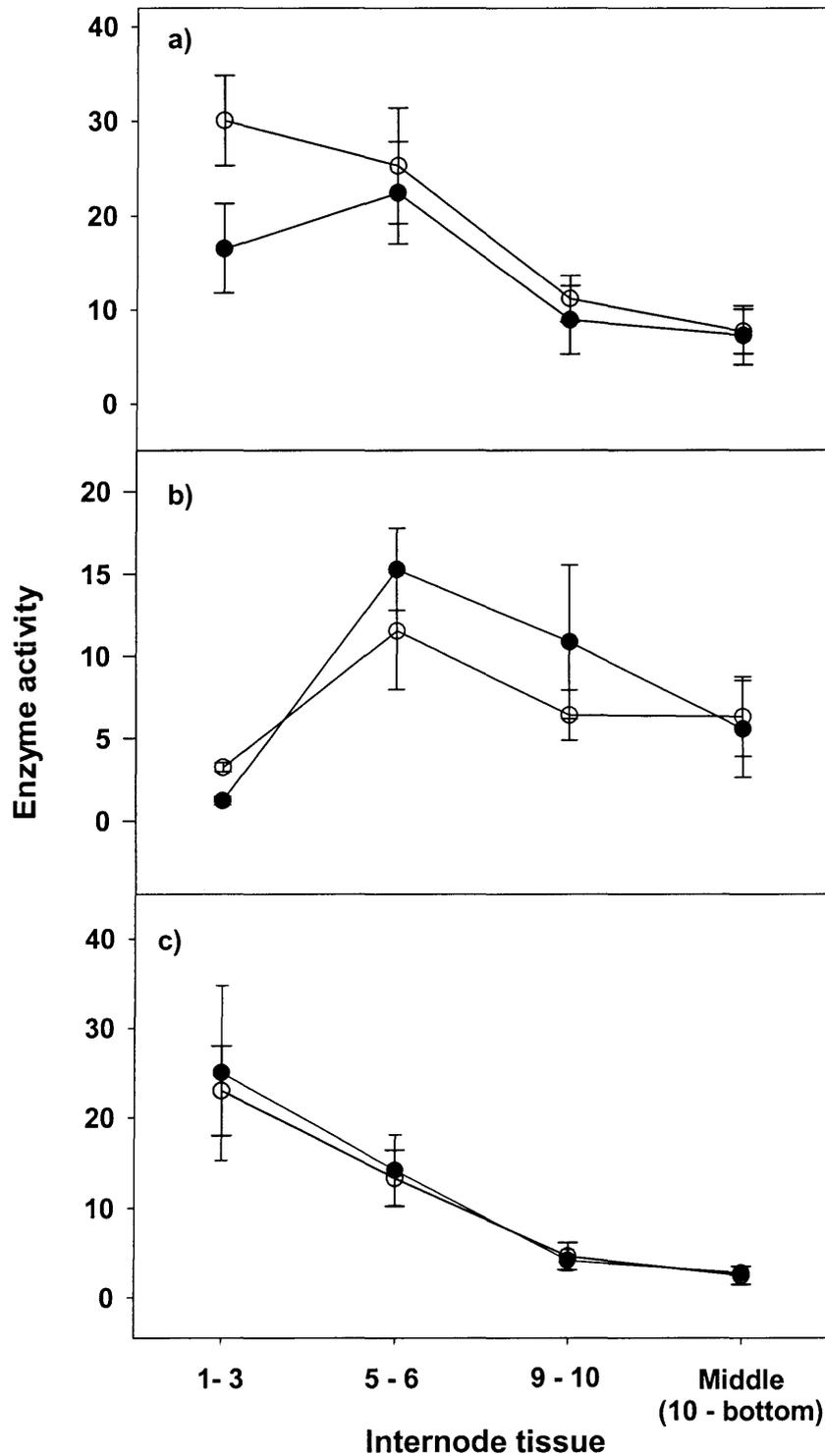


Fig. 4. Changes in enzyme activities measured at four positions down the stem in two high (○) and two low (●) CCS clones selected from the IJ76-514 x Q165 introgression population. Each point is the mean \pm SE of six measurements. Activity of sucrose synthase forward (a) is expressed as μg sucrose produced $\text{mg protein}^{-1} \text{min}^{-1}$ whereas sucrose synthase reverse (b) is expressed as sucrose cleaved $\text{mg protein}^{-1} \text{min}^{-1}$. Cell wall invertase activity (c) is expressed as $\text{ng sucrose cleaved gFWt}^{-1} \text{min}^{-1}$.



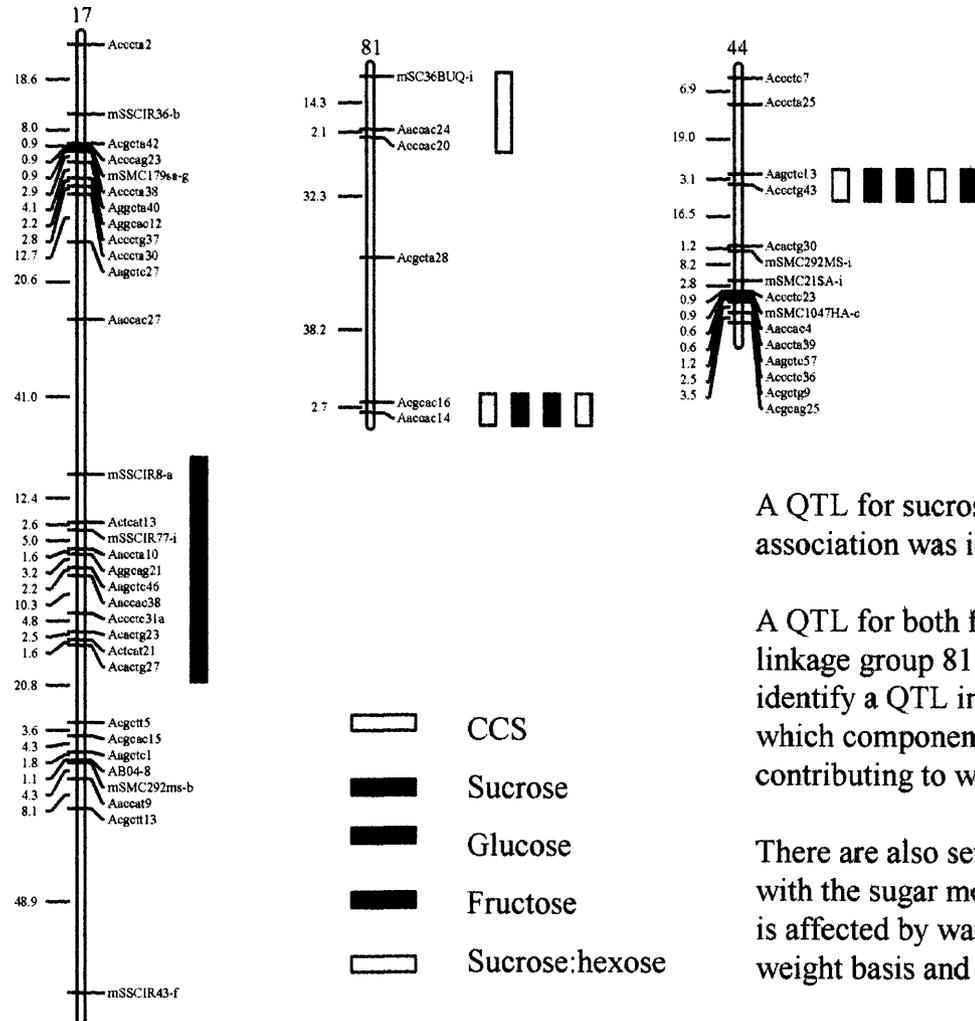
Appendix 4

Measurement of sucrose at internode level rather than whole stalk CCS alone can increase our understanding of sucrose accumulation in sugarcane.

Appendix 6

Identification of putative QTLs associated with sucrose, hexoses and CCS.

Some linkage groups from Q165 showing QTLs



A QTL for sucrose was identified on linkage group 17 (Q165^A) where no association was identified for CCS.

A QTL for both fructose and glucose was identified at the bottom of linkage group 81. There was no QTL identified for sucrose but CCS did identify a QTL in this region. This kind of analysis is starting to explain which components of the several variables that make up CCS is contributing to which QTL.

There are also several QTLs identified by CCS that are not associated with the sugar measurements (one is at the top of linkage group 81). CCS is affected by water and fibre independently of sugar content on a dry weight basis and composition.

Some linkage groups from IJ76-514 showing QTLs

First linkage group, QTL for CCS, glucose, fructose and sucrose:hexose ratio co-locate.

Second linkage group QTL for CCS only. Third linkage group QTL for glucose and fructose only.

Fourth linkage group QTL for CCS and sucrose co-locate.

