HCHL expression in hairy roots of *Beta vulgaris* yields a high accumulation of *p*-hydroxybenzoic acid (pHBA) glucose ester, and linkage of pHBA into cell walls

Laiq ur Rahman[a, 1], Hitomi Kouno[b], Yuya Hashiguchi[b], Hirobumi Yamamoto[a, 2], Arjan Narbad[c], Adrian Parr[c], Nicholas Walton[c], Toshihiko Ikenaga[b] and Yoshie Kitamura[b] *

[a] *School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*

[b] *Faculty of Environmental Studies, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*

[c] *Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK*

[1]Permanent address: Phytocellular Technology Division, Central Institute of Medicinal and Aromatic Plants, CSIR, P.O. CIMAP, Lucknow-226 015 India.

[2]Permanent address: Faculty of Life Science, Toyo University, 1-1-1 Itakura, Gunma 374-0193, Japan

* Corresponding author:

Y. Kitamura

Faculty of Environmental Studies, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan.

Tel/Fax: +81 95 819 2759; E-mail: k-yoshie@nagasaki-u.ac.jp

Abstract

As part of a study to explore the potential for new or modified bio-product formation, *Beta vulgaris* (sugar beet) has been genetically modified to express in root-organ culture a bacterial gene of phenylpropanoid catabolism. The *HCHL* gene, encoding *p*-hydroxycinnamoyl-CoA hydratase/lyase , was introduced into *Beta vulgaris* under the control of a *CaMV 35S* promoter, using *Agrobacterium rhizogenes* LBA 9402. Hairy root clones expressing the *HCHL* gene, together with non-expressing clones, were analyzed and revealed that one expression-positive clone accumulated the glucose ester of *p*-hydroxybenzoic acid (pHBA) at about 14 % on a dry weight basis. This is the best yield achieved in plant systems so far. Determination of cell-wall components liberated by alkaline hydrolysis confirmed that the ratio of pHBA to ferulic acid was considerably higher in the *HCHL*-expressing clones, whereas only ferulic acid was detected in a non-expressing clone. The change in cell-wall components also resulted in a decrease in tensile strength in the *HCHL*-expressing clones.

Keywords*: Beta vulgaris; metabolic engineering; p-hydroxycinnamoyl-CoA hydratase/lyase; p-hydroxybenzoic acid; cell wall.*

1. Introduction

Sugar beet (*Beta vulgaris*) is one of two important industrial crops for sucrose production and accounts for 25~27 % of the total world productivity of sucrose (Gurel et al., 2008; Zhang et al., 2008). Moreover, the tubers of sugar beet, with their large biomass allied to simple methods of harvesting and processing, provide in principle an attractive basis for engineering the biotechnological production of new or additional products besides sucrose. However, whilst important successful efforts have been made using genetic modification to improve the agronomic properties of sugar beet, notably by the introduction of a plant-derived nematode resistance gene, *Hs1pro*$^{-1}$ (Kifle et al., 1999), the pathogenesis-related S protein gene (Snyder at al., 1999) and the salt-tolerant vacuolar Na^+/H^+ antiporter gene (Liu et al., 2008), *B. vulgaris* is unfortunately still technically recalcitrant to regeneration following the insertion of exogenous genes (Gurel et al., 2008). This has so far substantially limited the development of transgenic sugar beet plants as vehicles for the production of new compounds.

Where whole-plant regeneration is currently problematic, as in *B. vulgaris, in vitro* culture systems, especially hairy root systems, are useful as demonstration models of the potential of field-grown plants for the development of new products; and they may also be attractive cell factories in their own right, on account of their potential for vigorous propagation without the need for added growth hormone, allied to a high productivity of secondary metabolites (Giri and Narasu, 2000). Thus, the production of the betalain pigments (betacyanin /betaxanthin),

which can be produced in restricted species belonging to the Caryophyllales, has been well characterised in hairy root lines derived from beetroot cultivars of *B. vulgaris* (Böhm and Mäck 2004; Suresh et al., 2004). Menzel at al. (2003) has reported the successful expression of bacterial polyester synthesis genes in *B. vulgaris* hairy roots, paving the way to a possible future biosynthesis of polyesters *in planta*.

One of the factors that influences the economics of sugar-beet cultivation is the production of lignified material, which must be removed as waste material during processing. A reduction in the amount of lignin biosynthesized could therefore offer an economic advantage, particularly if the carbon destined for lignin production could be re-routed into the production of a commercially-useful end-product. One such product is the industrial polymer feedstock, *p*-hydroxybenzoic acid (pHBA; Dong et al., 2001). This compound has been shown to be produced in transgenic plants expressing the gene encoding *p*-hydroxycinnamoyl-CoA hydratase/lyase (HCHL), which forms part of a bacterial catabolic pathway for *p*-hydroxycinnamic acids (Gasson et al., 1998; Narbad and Gasson, 1998; Mitra et al., 1999). Because it hydrates and then cleaves *p*-hydroxycinnamoyl-CoA substrates that are also intermediates of the plant phenylpropanoid pathway leading to lignin, it produces C_6-C_1 metabolites instead of lignin. To date, heterologous expression of *HCHL* has been reported in tobacco plants (Mayer et al., 2001; Merali et al., 2007), in *Datura stramonium* hairy roots (Mitra et al., 2002) and in sugar cane plants (McQualter et al., 2005). In these cases, the principal new products have been glucose conjugates of pHBA derived from endogenous

p-coumaroyl-CoA.

These various results have strongly encouraged us to examine the effects of expressing the *HCHL* gene in *B. vulgaris* (sugar beet). Furthermore, *B. vulgaris* (sugar beet) is known to be one of the most abundant sources of the *p*-hydroxycinnamic acid, ferulic acid, present in cell-wall material (ca. 0.5-1 % dry wt basis) (Clifford, 1999; Walton et al., 2002), as shown by an industrial trial (Couteau et al., 1997), suggesting that it might be a particularly promising target species for the re-routing of carbon from the phenylpropanoid pathway. We therefore examine here the metabolic perturbation exhibited by hairy root cultures of *B. vulgaris* (sugar beet) expressing the *HCHL* gene.

2. Materials and methods

2.1. Chemicals

Standards of *p*-hydroxybenzoic acid-β-D-glucoside (pHBAG) and *p*-hydroxybenzoic acid glucose ester (pHBAGE) were kindly provided by Prof. Shigeo Tanaka (University of Tokyo Agriculture Science, Japan). *p*-Coumaric acid, caffeic acid, ferulic acid, *p*-hydroxybenzaldehyde, pHBA, sinapinic acid, vanillin, vanillic acid and 7-methoxycoumarin were purchased from Sigma-Aldrich (Japan).

Ampicillin sodium (Wako Chemicals) and kanamycin (Sigma) were obtained commercially.

2.2. Plant materials and bacterial lines

Seeds of *Beta vulgaris* (sugar beet) were obtained from CIMAP, Lucknow, India. Seeds were aseptically germinated *in vitro* after treatment with 0.5% sodium hypochlorite for 40 min and 0.1% $HgCl_2$ for 7 min, and then three times with sterile distilled water. One-to-two-week-old seedlings were used for transformation experiments.

Agrobacterium rhizogenes LBA 9402 harbouring either an empty pBin19 vector, or pBin19 containing *pmHCHL* under the control of the cauliflower mosaic virus (*CaMV*) *35S* promoter was used for transformation as described previously (Mitra et al., 2002). The bacteria were cultured in liquid YEB media (600A=0.8~1.0) before inoculation.

2.3. Transformation experiments and culture methods

Hypocotyls and cotyledons excised from *B. vulgaris* seedlings were inoculated directly using needles loaded with *A. rhizogenes*. These explants were cultured in darkness on solid Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962), containing kanamycin (50μg/ml) and ampicillin (250 μg/ml) as used previously (Rahman et al., 2004). Whenever roots formed and grew, root tips (*ca.* 1 to 2 cm in length) were separated from the original explants and transferred to the media described above. After the elimination of

bacteria, independent root lines were established on solid MS medium without antibiotics.

For solid culture, 3 root tips (*ca.* 1 cm in length) were introduced into plastic Petri dishes containing 30 ml of MS medium. For liquid culture, single root tips were introduced into 50ml flasks containing 15 ml of MS medium. Root tissues were harvested by vacuum filtration, immediately frozen in liquid nitrogen and stored at -70˚C until analysis.

2.4. Detection of gene insertion of HCHL/TL by PCR

Genomic DNA was isolated from fresh root material with CTAB according to the previous report (Rahman et al., 2004). To detect the insertions of *HCHL* gene and the *TL* genes of Ri plasmid (Bouches and Camilleri, 1990), putative transformants were examined using specific 25-to-26-mer oligonucleotide primers for amplification of 631 bp and 670 bp fragments, respectively. The forward and reverse primers used were as follows: antisense *HCHL1* (5’-ATCGCCATGGGCACATACGAAGGTC-3’); sense *HCHL* (5’-TTCGAGCAGGTTACG CGCCAGCTCG-3’); antisense *TL1* (5’-TAGCCGTGACTATAGCAAACCCTCC -3’); sense*TL3R* (5’-GGCTTCTTT CTTCAGGTT TACTGCAG-3’). PCR conditions were 95˚C for 5 min, 53˚C for 2 min and 72˚C for 3 min for the first cycle, followed by 29 cycles of 95˚C for 1 min, 53˚C for 2 min and 72˚C for 3 min and an extra cycle with a 10 min extension step at 72˚C. PCR products were separated on 1.5 % agarose gels and visualized with ethidium bromide staining. A 100 bp DNA ladder (Takara) was used as a marker.

2.5. Expression of HCHL gene

To confirm the expression of the *HCHL* gene, gene-positive roots were further checked by RT-PCR and partially by western blot. Total RNA was extracted from *ca.* 50mg of fresh root material with an RNeasy® Plant Mini kit (Qiagen, USA) according to the manufacturer's instructions. RNA samples were separated on 1 % formaldehyde/agarose gels and the yield was checked using bands visualized by ethidium bromide and measuring the absorbance at 260/280 nm. RT-PCR was performed using an mRNA-selective PCR kit (Takara, Japan) with the primers mentioned above. RT-PCR conditions were 45˚C for 25 min for the first cycle, followed by 35 cycles of 85˚C for 1 min, 45˚C for 1 min and 72˚C for 3 min. Products were separated and visualized as described for DNA.

For western blotting, protein was extracted as follows. Fresh root tissue (1g) was frozen in liquid nitrogen and ground into powder in the presence of 100 mg of insoluble PVP, using a chilled mortar and pestle. The powder was then mixed thoroughly with 3 ml of chilled extraction buffer (100 mM Tris/HCl, pH 8.5, containing 10 mM EDTA and 20 mM DTT). After centrifugation at 10,000 x *g* for 20min at 4˚C, supernatant (20 μg of protein) was examined by gel electrophoresis. SDS-PAGE of protein extracts was performed essentially as described by Laemmli (1970), with a resolving gel containing 12.5 % (w/v) acrylamide, and using an Atto AE 6530M apparatus (Atto Corporation, Japan). Proteins resolved by

SDS-PAGE were electroblotted onto PVDF transfer membrane (Atto Corporation) with a semi-dry Milliblot transfer system used according to the manufacturer's directions (Eido, Japan). As a molecular standard, visualized rainbow marker proteins (high pH range, Pharmacia) were included. Western analysis using the polyclonal antibodies raised to HCHL was performed essentially as described by Karakas Sen et al. (1999). Protein was assayed by the method of Bradford (1976), using commercially prepared reagent (Bio-Rad Laboratories) and bovine serum albumin as a standard.

2.6. Analysis of soluble and cell wall-linkage phenolics

Frozen root material (0.1 g fresh wt) was powdered in a mortar and extracted with 1 ml of 80 % (v/v) MeOH and 100 μl of 5 mM umbelliferone as an internal standard. After centrifugation (8,800 x *g*, 15 min), the supernatant was applied to HPLC. Insoluble, cell-wall-bound phenolics were extracted from the tissue residue, which was then extracted with MeOH / 1 % (w/v) HCl and then washed with MeOH followed by acetone until it became colourless. The residue was treated with 2M KOH containing 1 % (w/v) ascorbic acid and 10 mM EDTA at 80˚C for 1h *in vacuo* and then allowed to stand at room temperature overnight as described previously (Nardini et. al., 2002). The reaction product was acidified with 4 M HCl, 5mM umbelliferone (100 μl) was then added as an internal standard and the solution was extracted with ethyl acetate (800μl). The ethyl acetate fraction was evaporated under reduced pressure and redissolved in MeOH (500 μl).

Samples were analysed by HPLC (Shimadzu LC-10) with a TSKgel ODS –120A column (Tosoh Corporation), using gradient elution at 40˚C and measurement at 254 nm and 322 nm. The gradient conditions for soluble phenolics were: solvent A, 1 mM acetic acid; solvent B, acetonitrile; flow rate, 1 ml min^{-1}; at 0 min, A = 93 %; at 7, 20, and 30 min, A = 15 %, 40 %, and 7 %, respectively. The conditions for phenolics liberated from cell walls were the same except for the time program: at 0 min, A = 12 %; at 20, and 30 min, A = 40%, and 12%, respectively. A Spectra FOCUS scanning diode-array detector (Shimazu SPD-M20A) was used to identify the phenolic compounds. Quantification was based on standard curves, with authentic *p*-hydroxybenzaldehyde, pHBA, ferulic acid, pHBAG and pHBAGE, and umbelliferone as an internal standard.

2.7. Mechanical analysis of roots

To analyse the mechanical strength of hairy roots, a tensile test was performed with an autograph (Shimazu AGS-J), using a 50 N load cell. Approximately 0.5 cm of root tip was fixed to the lower cell and *ca.* 1.0 cm of root was held between the upper and lower cells. Every root was measured accurately in respect of its diameter and length between the upper and lower cells. Each root was drawn at a tensile speed of 10.0 mm/min by the autograph linked to a computer. Trapezium 2 software (Shimazu, Japan) was used for analysis of root stress and strain.

3. Results

3.1. Establishment of transformants and gene expression

Roots were initiated from hypocotyls and cotyledons of seedlings within 3 weeks of infection with *A. rhizogenes* containing the *HCHL* gene. Nearly 33 % of hypocotyl segments showed root induction, whereas only 8 % of cotyledons did so. The roots were selected on 50 μg/ml of kanamycin, which was found to be lethal for the growth of normal roots as well as transformants without the vector gene. A total of 15 *HCHL*-gene-positive clones were established, plus 5 *HCHL*-negative clones. The presence of the *HCHL* gene as well as of the *TL* region of the Ri plasmid was confirmed by genomic PCR (data not shown). All of the *HCHL*-gene-positive as well as -negative clones showed the insertion of the *TL* region, together with typical hairy root characteristics (hormone-independence and profuse branching). No callus formation during growth was observed with any clone. Colour intensity, growth density and root appearance varied depending on root clones, but *HCHL*-positive clones could not be distinguished from *HCHL*-negative clones on the basis of appearance.

During the subculture of the transformants, the expression of the *HCHL* gene in 12 clones was monitored by RT-PCR at various stages of culture on solid media (data not shown). Of 12 *HCHL*-gene-positive clones, 6 clones expressed *HCHL* after 4 weeks, 7 clones after 6

weeks and 11 clones after 8 weeks; the expected band of 631 bp was observed in all *HCHL*-positive clones except for one clone which did not show expression even after 8 weeks and its growth pattern was typical of empty-vector transformants; this clone was therefore used as a further negative control. For further study, hairy roots were cultured in liquid medium and checked for *HCHL* expression again. According to the expression patterns in liquid culture, 3 representative *HCHL*-expressing clones and the negative control clone were selected and confirmed by western-blot analysis for the expression of 31 kDa HCHL protein (Fig.1). The result showed that Bv18 was the most promising clone.

3.2. Growth and production of soluble C_6-C_1 phenolics

When a root tip (*ca.* 1 cm) was cultured in a liquid medium for 6 weeks, the growth curves of three selected *HCHL*-expressing clones (Bv16, Bv18 and Bv42) and one non-expressing control clone (Bv48) were as shown in Fig. 2. Bv42 and Bv48 reached growth maxima after 6 weeks of culture and increased in fresh mass more than 740-fold and 810-fold, respectively, whereas the growth of clone Bv16 was about half that of Bv42; Bv18 was intermediate.

Analysis for the presence of C_6-C_1 phenolics in the roots and in the medium of these cultures by HPLC equipped with photodiode array revealed the production of *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid (pHBA), *p*-hydroxybenzoic acid-β-D-glucoside (pHBAG) and *p*-hydroxybenzoic acid glucose ester (pHBAGE) in the

HCHL-expressing clones, but not in the non-expressing clone (Fig. 3). *p*-Hydroxybenzaldehyde, the immediate reaction product of HCHL, was transiently detected at a relatively early stage of culture, but the amount was negligible. The major products of the *HCHL*-expressing clones in the roots were pHBAGE followed by pHBAG, together with much lower amounts of pHBA and *p*-hydroxybenzaldehyde. The main product in the medium was pHBA, together with some pHBAGE; however this was minor in total in relation to the pHBAGE and pHBAG in the roots. To reconfirm the production of pHBAGE, the root extract was analyzed after alkaline hydrolysis (2M NaOH); the peak corresponding to pHBAGE disappeared and, instead, a large peak corresponding to pHBA appeared.

Time-course production profiles of C_6-C_1 phenolics were compared for selected clones. Almost all of these products remained in the root tissues (Fig. 4), although approximately 1 %, 1.5 % and 2 % of total C_6-C_1 phenolics were found in the culture media of Bv16, Bv18 and Bv42, respectively (data not shown). pHBAGE was predominant in all clones; comparable amounts of pHBAG were detected in Bv16, but much smaller amounts of pHBAG were found in Bv18 and Bv42. Whereas the production of pHBAGE reached a maximum at 3 weeks in the case of Bv42, it continuously increased up to 6 weeks in the case of Bv16 and Bv18. Owing to the cessation of root propagation, followed by tissue necrosis, levels of C_6-C_1 phenolics decreased after 3 weeks in the roots of Bv42, but they continued to increase in the medium, even after 6 weeks in culture. In good agreement with the expression pattern (Fig. 1), Bv18 was the most productive of the clones analysed and was *ca.* 10 times and 20 times more productive than Bv16 and Bv42, respectively.

3.3. Cell wall components and strength

The analysis of C_6-C_1 phenolics (Fig. 4) indicated that a considerable diversion of phenylpropanoid metabolism was occurring, away from cell-wall components including lignin. To investigate this further, hairy roots were first stained with phloroglucinol-HCl solution (Christiernin et al., 2005), but any staining was masked by betalain production. However, even after the removal of betalains from root tissues with 1 % HCl-MeOH prior to phloroglucinol-HCl staining, no detectable colour was formed in any selected clones. Since it was reported that cell-wall-bound ferulic acid (FA) was abundant (*ca.* 0.5-1 % dry wt) in beetroot (Clifford, 1999), we decided to measure cell-wall-bound phenolics in the selected clones. Cell-wall-bound phenolics were liberated by alkaline hydrolysis from cell-wall residues obtained after complete removal of soluble phenolics and were then analysed by HPLC. Interestingly, in the transgenic clones, pHBA was detected in addition to FA. Both compounds were readily detectable from *ca.* 100 mg fresh weight of hairy roots and hence their production was determined nearly every 10 days for 40 days using Bv16, Bv18 and the control clone, Bv48 (Fig. 5). In Bv48, only FA was detected and its content increased up to 30 days whereas in the *HCHL* expressing clones Bv16 and Bv18 pHBA was also detected. Especially in Bv18, the pHBA content increased linearly up to 40 days and was much greater than the content of FA. Bv16 contained lesser amounts of cell-wall-bound phenolics throughout the 40-day period.

We speculated that the chemical changes in cell-wall-bound phenolics would be likely to cause physical changes. Therefore, a tensile test was carried out on roots of each selected clone in order to obtain a stress-strain diagram. It was found that the roots of the transgenic clones Bv18 and Bv16 had less tensile strength than the roots of the control clone Bv48 (Fig. 6).

4. Discussion

Here we report for the first time the effects of heterologous expression of *HCHL* in hairy root cultures of *Beta vulgaris*. In *HCHL*-expressing clones, *p*-hydroxybenzaldehyde, pHBA, pHBAGE and pHBAG were all detected as soluble C_6-C_1 compounds (Figs. 3 and 4) and pHBA was also found in cell-wall-bound form (Fig. 5). *p*-Hydroxybenzaldehyde, the actual reaction product of HCHL, is probably initially converted to pHBA by an endogenous *B. vulgaris* dehydrogenase and then, except for the cell-wall-conjugated form, pHBA is further converted into its glucose conjugates, *i.e.* a glucose ester (pHBAGE) and a phenolic glucoside (pHBAG), probably by endogenous glucosyltransferases (GT) (Fig. 7).

Interestingly, the glucose ester of pHBA (pHBAGE) was far more abundant than the phenolic glucoside of pHBA (pHBAG) in *B. vulgaris* transformants (Fig. 4). This is in contrast to previous observations, which showed that the C_6-C_1 phenolic compounds produced in response to *HCHL* expression were accumulated mainly as pHBAG (Mayer et al., 2001;

Mitra et al., 2002; McQualter, 2005). Since pHBA must be liberated from glucose conjugates for industrial use, the glucose ester is preferable to the phenolic glucoside, due to its hydrolysis under much milder conditions. Why a glucosyltransferase producing the glucose ester should be dominant in *B. vulgaris* but not in other plants heterologously expressing HCHL is unclear at present. There may well be considerable variation between plant species; for example, carrot hairy roots even without metabolic engineering produced some free pHBA, but no pHBA glucose-conjugates (Sircar et al., 2007). Glucosyltransferases (GT) have been classified into at least 54 families according to sequence homology (Lim et al., 2002). Analysis of 14 GTs from *Arabidopsis* with activity towards various benzoates revealed a separation between those enzymes active on phenolic hydroxyl groups and those active on carboxyl groups (Lim et al., 2002). Easy access of pHBA to a specific GT in *B. vulgaris* with activity towards the carboxyl group of pHBA may lead to a preponderance of the glucose ester of pHBA, which is then probably transported to the vacuoles because the accumulation of large amounts of organic acids in plant vacuoles is commonplace (Rea, 1999; Lim et al., 2002). Further studies on *B. vulgaris* specific GTs and the transport system of pHBAGE would be of interest from biochemical, evolutionary and applicable perspectives.

In this paper, we have shown incorporation of pHBA into cell walls of *B. vulgaris* hairy roots expressing *HCHL* (Fig. 5). In an earlier study, changes in cell-wall-bound C_6-C_1 phenolics (*p*-hydroxybenzaldehyde, pHBA, vanillin and vanillic acid) and C_6-C_3 phenolics (including *p*-coumaric acid and ferulic acid) were investigated in stems of *HCHL* transgenic and syngenic tobacco plants and the findings indicated that the total of cell-wall-bound

phenolics in the transgenic plants tended to decrease in comparison to those in the syngenic plants (Merali et al., 2007). However, in that study, some C_6-C_1 phenolics were incorporated even into the cell walls of syngenic tobacco plants; and such incorporation was also observed in non-transgenic *Daucus carota* hairy roots (Sircar et al., 2007). In the present study, by contrast, C_6-C_1 phenolics were not detectable in non-*HCHL*-expressing roots of *B. vulgaris* and it is clear that an effect of HCHL was to cause the incorporation of pHBA into cell walls instead of ferulic acid. Presumably, both the depletion of FA and the abundant production of pHBA must lead to the conjugation of pHBA into cell walls. Since *p*-hydroxybenzaldehyde was not detected as a cell-wall component, the conversion from *p*-hydroxybenzaldehyde to pHBA must occur before binding to the cell walls (Fig. 7). The qualitative changes in cell-wall-bound phenolics affected root tensile properties (Fig. 6): this might reflect changes in the overall level of incorporation of phenolic compounds in the cell-wall, or be associated more specifically with the incorporation of pHBA in place of ferulic acid units.

The hairy root clone Bv18 established here is the most promising source of pHBA. After 4 weeks of culture starting from a root tip (*ca.* 1 cm, 50 mg FW), the amount of pHBAGE reached a steady level, around 40 μmol/g fresh weight, which represented around 1.3 % of tissue fresh weight and 14 % of tissue dry weight. This exceeds the best yield previously achieved elsewhere: 7.3 % of dry weight in leaves of transgenic sugarcane harboring the same *HCHL* gene, cultivated for 35 weeks (McQualter et al., 2005); and it compares very well with the yields of other useful plant secondary products achieved in other *in vitro* culture systems, notably anthocyanin, 15 % DW (Rajendran et al., 1992) ; berberine,

13.2 % DW (Sato and Yamada, 1984); and shikonin, 12.4 % DW (Fujita et al., 1981); ginsenoside, 1.7 % DW (Woo et al., 2004). Considering these results, 14 % DW in plant tissues seems to be near the maximum possible level for the accumulation of secondary metabolites, although the level of the primary metabolite, sucrose, can reach around 20 % in sugar beet (Gurel et al., 2008).

5. Conclusion

The bacterial enzyme *p*-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) converts C_6-C_3 4-hydroxycinnamates to C_6-C_1 4-hydroxybenzaldehydes. Here we report the successful expression of the *HCHL* gene in hairy roots of the important crop plant, *Beta vulgaris* (sugar beet). Plant hairy root systems are in principle very attractive cell factories in their own right, on account of their potential for massive growth allied to a high productivity of secondary metabolites. The C_6-C_1 product, pHBA, was mainly accumulated in soluble form as its glucose ester (pHBAGE), and to a lesser extent as its phenolic glucoside (pHBAG), although some pHBA was found in cell walls in conjugated form. One clone was able to produce about 14 % pHBAGE on a dry wt basis, substantially greater than previously achieved for pHBA conjugates in other plants heterologously expressing *HCHL*. Further extensive study using this clone and plants regenerated from it will be necessary to assess the potential for industrial production of pHBA .

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Figure Captions

Fig. 1. Profiles of the *HCHL* gene expression in hairy root clones of *B. vulgaris*

A) RT-PCR products of the *HCHL* gene (631bp): The left lane (M) is a 100bp molecular marker (bigger band is 500bp), while the following 6 lanes are hairy root clones. rRNA loadings are shown in the lower panel. Bv48 is used as a negative control hereafter.

B) Western blots of HCHL protein: The left lane is a rainbow molecular marker; upper, middle and lower bands are 60, 31 and 24 kDs, respectively. The following bands are from clones Bv18, Bv48, Bv16 and Bv42.

Fig. 2. Growth curves of 3 *HCHL*-expressing (positive) clones and 1 non-expressing (negative) clone of *B. vulgaris*

A root tip (*ca*. 1 cm, 3.6 ± 0.7 mg) was cultured for 6 weeks in 15 ml of MS basal liquid medium in the dark at 25℃ at 80 rpm.

Fig. 3. Profiles of HPLC chromatograms of the root (upper trace) and medium (middle trace) extracts from positive clone Bv18, and of root extract (lower trace) from the negative control clone Bv48

pHBA, *p*-hydroxybenzoic acid; pHBAld, *p*-hydroxybenzaldehyde; pHBAG, *p*-hydroxybenzoic acid-β-D-glucoside); pHBAGE, *p*-hydroxybenzoic acid glucose

ester; IS, internal standard.

Fig. 4. Culture-period-dependent production of soluble C_6-C_1 phenolics in the roots of 3 positive and 1 negative clones of *B. vulgaris*

Abbreviations are as for Fig. 3.

Fig. 5. Culture-period-dependent production of phenolics released by alkaline hydrolysis from cell walls of 2 positive and 1 negative clones of *B. vulgaris*

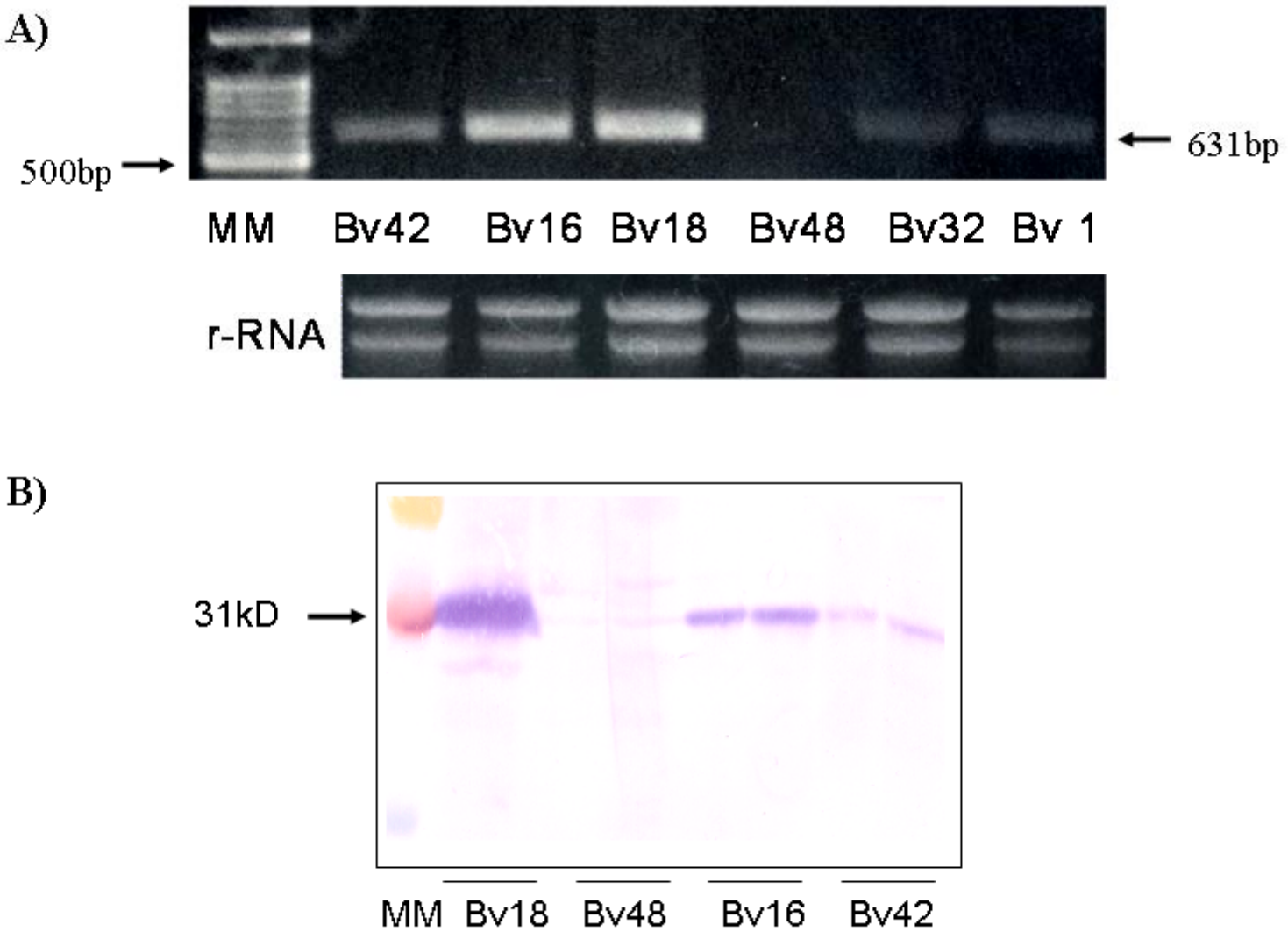
pHBA, *p*-hydroxybenzoic acid; FA, ferulic acid.

Fig. 6. Stress-strain diagram of 2 *HCHL*-expressing and 1 non-expressing hairy roots of *B. vulgaris* Mechanical strength of hairy roots was measured by an autograph (Shimazu AGS-J) using a 50 N load cell. Data was obtained using roots cultured for 2 weeks in liquid MS medium.

Fig. 7. Production of C_6-C_1 phenolics and involvement of probable enzymes *in vivo* in *HCHL*-expressing *B. vulgaris* hairy roots

DH, dehydrogenase; GT, glucosyltransferase. Other abbreviations are as for Fig. 3.

Fig. 1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Bv42
Bv16
Bv18
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Bv18
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Bv16
Bv42

Fig. 2

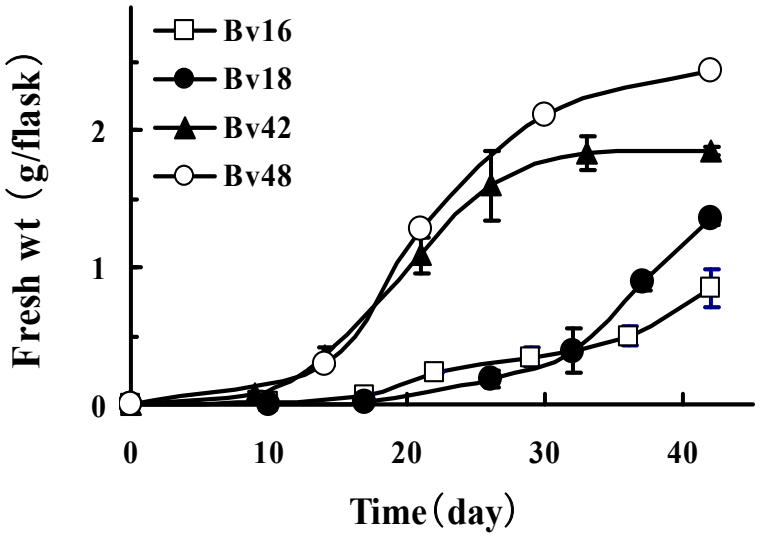
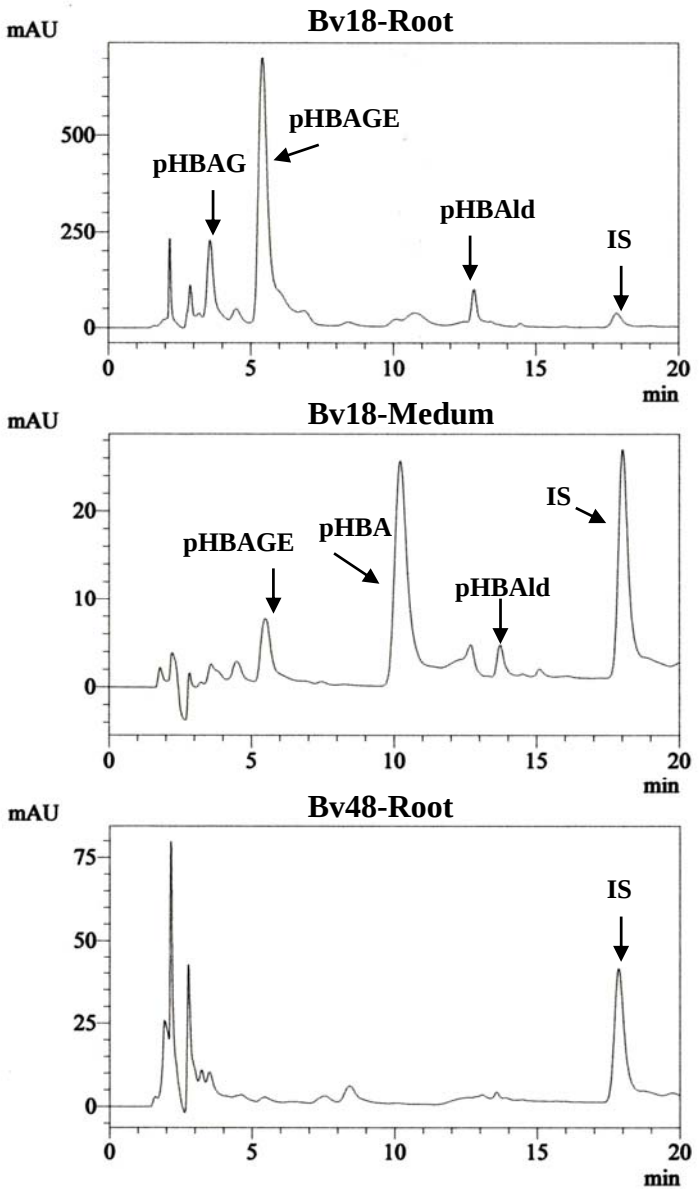


Fig. 3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pHBAGE
pHBAld
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10
15
20
min
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mAU
20
10
0
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pHBA
pHBAld
IS
0
5
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IS
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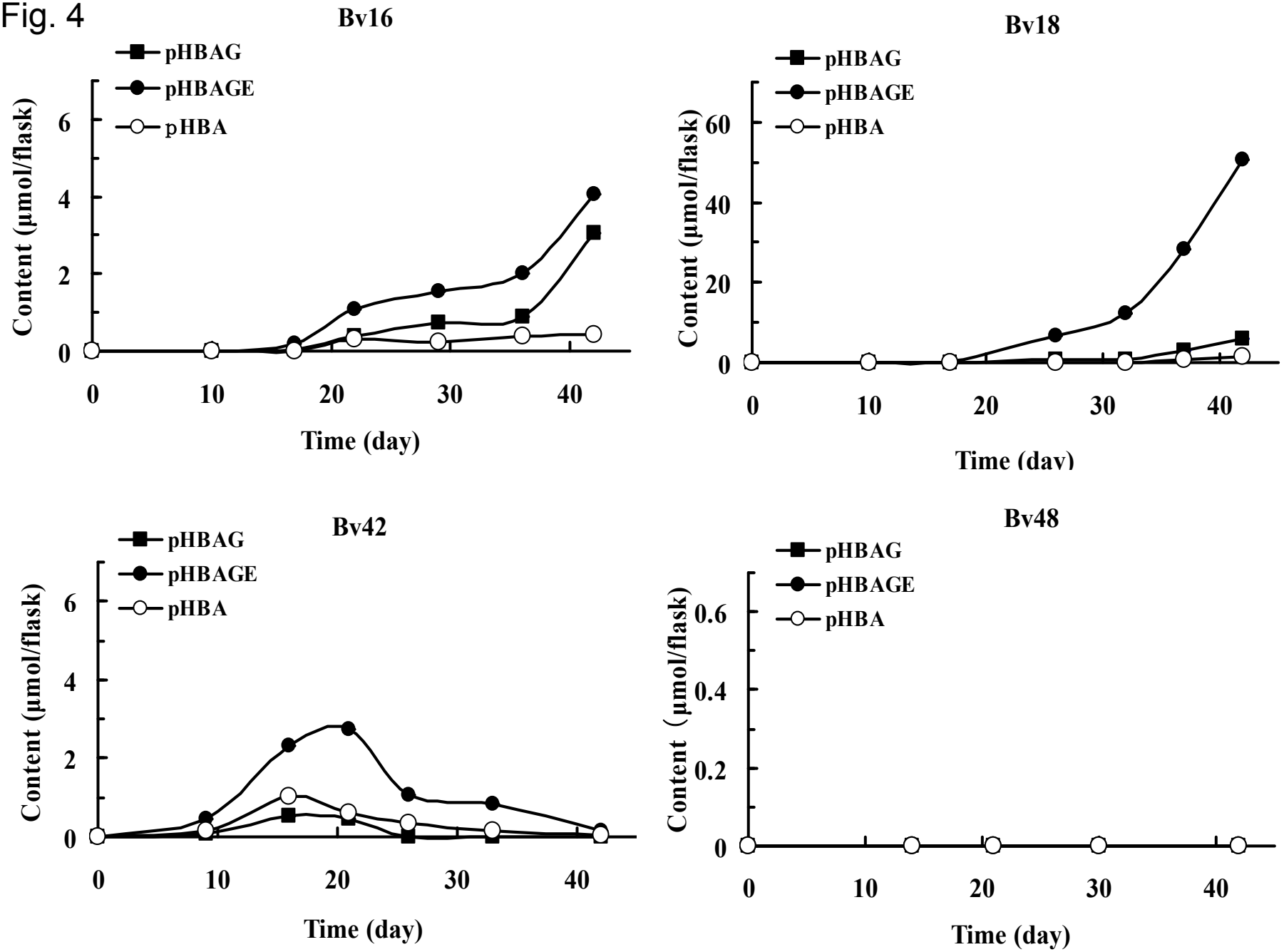
15
20
min

Fig. 4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10
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Time (day)
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pHBAGE
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Content (μmol/flask)
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20
40
60
0
10
20
30
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Time (day)
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pHBA
Content (μmol/flask)
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Time (day)
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10
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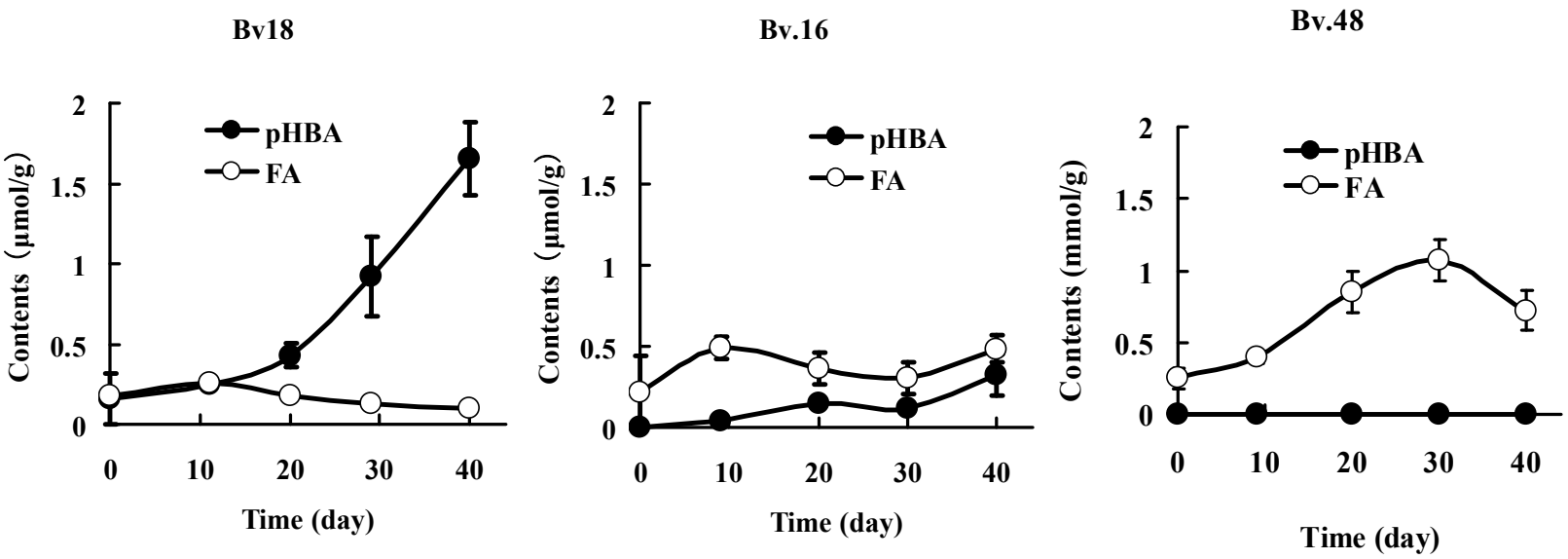
40
Time (day)

Fig. 5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1.5
2
0
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20
30
40

Fig. 6

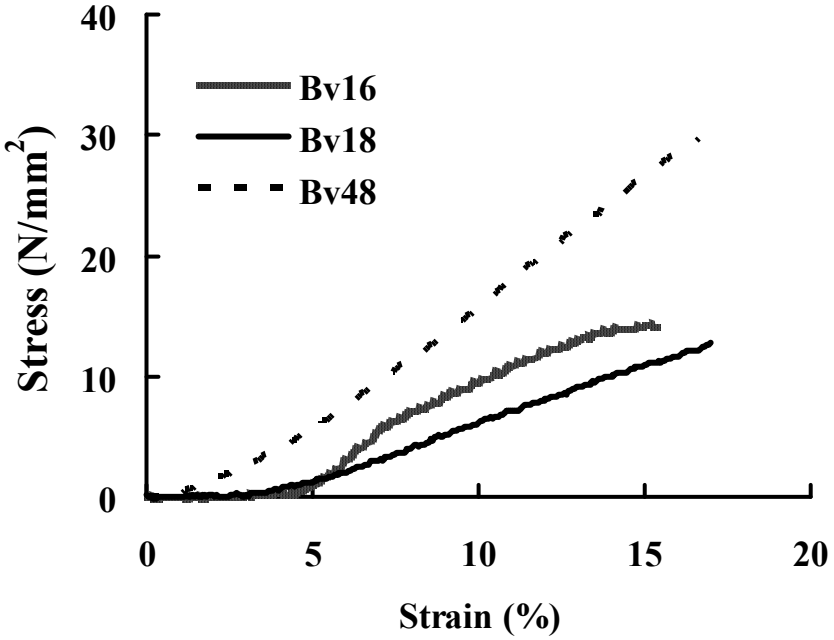
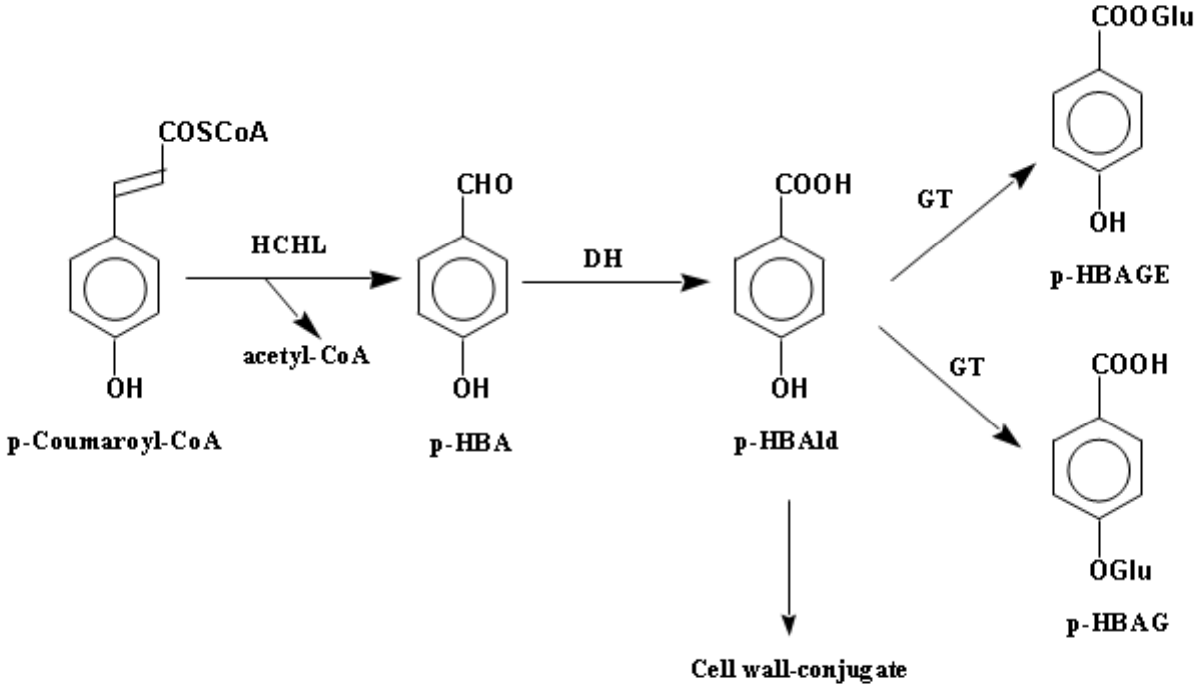


Fig. 7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GT
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OH
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GT
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OGlu
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