Transcriptome analysis reveals major transcriptional changes during regrowth after mowing of red clover *(Trifolium pratense)*

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Abstract

Background

Red clover (*Trifolium pratense*) is globally used as a fodder plant due its high nutritional value and soil improving qualities. In response to mowing, red clover exhibits specific morphological traits to compensate the loss of biomass. The morphological reaction is well described, but the underlying molecular mechanisms and its role for plants grown in the field are unclear.

Results

Here, we characterize the global transcriptional response to mowing of red clover by comparing plants grown under greenhouse conditions with plants growing on agriculturally used fields. Unexpectedly, we found that biotic and abiotic stress related changes of plants grown in the field overlay their regrowth related transcriptional changes and characterized transcription related protein families involved in these processes. Further, we can show that gibberellins, among other phytohormones, also contribute to the developmental processes related to regrowth after biomass-loss.

Conclusions

Our findings show that massive biomass loss triggers less transcriptional changes in field grown plants than their struggle with biotic and abiotic stresses and that gibberellins also play a role in the developmental program related to regrowth after mowing in red clover. Our results provide first insights into the physiological and developmental processes of mowing on red clover and may serve as a base for red clover yield improvement.

Background

Trifolium pratense (red clover) is an important worldwide forage crop and thus of great economic interest. This perennial plant offers several advantages like a high protein content and soil improving characteristics, which reduce the use of artificial nitrogen fertilizer and enhance livestock intake. Well-known disadvantages of red clover include poor persistence under several land use scenarios, like grazing or cutting [1–3]. *T. pratense* is a member of the Fabaceae (or legumes), which are, due to their economic value, among the most examined families in the plant kingdom with genome sequences available for species like *Medicago truncatula* (barrel clover) [4], *Lotus japonicus* (birdsfoot trefoil) [5], *Glycine max* (soy) [6], *Phaseolus vulgaris* (common bean) [7], *Cicer arietinum* (chickpea) *[8]*, *Vigna unguiculata* (cowpea) [9], *Trifolium subterraneum* (subterranean clover) [10] *T. medium* (zigzag clover) [11], and *T. pratense* (red clover) [12, 13].

Facing today's challenges such as an increased demand on food production in an era of global climate change, together with the aim to solve these problems in an environmental friendly and sustainable way, requires improvement of forage crops like *T. pratense* [14, 15]. *T. pratense* breeding aims to offer genotypes with improved key agronomic traits (dry matter yield, high quality, resistance to diseases and abiotic/biotic stress, persistency), while improving its regrowth ability [2, 16]. Unfortunately, the morphological investigations of several *T. pratense* populations showed a correlation of persistency with non-favorable traits, like small plant size and prostrate growth habit [17]. Moreover, most *T. pratense* cultivars or accessions are locally adapted and require their specific local conditions to show the favored traits [18, 19], which decreases the stability for individual traits in breeding efforts [20]. *T. pratense* exhibits significant intraspecific variation due to high intrapopulation genetic diversity, thus, persistence and performance in response to mowing or cutting depends on the variety as well as on the developmental stage at the moment of damage [21–24].

Persistency can be defined as a sustained forage yield over several growing periods [25] and is a complex trait influenced by a variety of abiotic and biotic factors, and the regrowth ability of a plant [26]. Plants with high regrowth ability can survive more frequent and intense biomass loss and are therefore more persistent. Decapitation or biomass loss due to herbivory or mowing triggers a complex reaction affected by environmental conditions, plant morphology, architecture, developmental stage and genotype [21]. After decapitation, the first known stress response in other legumes like *M. sativa* and *P. sativum* involves the production of phytohormones: cytokinins, auxins, and strigolactones [27–29]. In addition, the mobilization of energy reserves is activated [30]. Phenotypic plasticity of plant architecture in combination with alterations of hormone concentrations can be observed in *P. sativum* and *T. pratense* after decapitation [24, 29, 31]. However, the molecular processes allowing plants to thrive even after an enormous loss of biomass remain still unclear, even in *Arabidopsis thaliana* [32, 33].

Here, we compare the transcriptomes of mown (cut) vs. unmown (uncut) *T. pratense* plants from two different, well investigated field locations on the Biodiversity Exploratory "Heinich-Dün" [34] and greenhouse grown plants. Our field samples were subjected to standard agricultural treatment and we can thus discriminate transcriptional changes caused by abiotic factors and biotic interactions in the field from those that regulate regrowth. We present the identification and *in silico* characterization of putative developmental regulators differentially expressed in the regrowth phase after mowing in the field and in the greenhouse that may contribute to the regrowth response of *T. pratense* and demonstrate that gibberellin is a major regulator of specific aspects of the regrowth morphology in red clover.

Results

RNA-Seq results, *de novo* assembly, and functional description of contigs

The RNA-Seq produced a total number of short reads between 44.7 and 58.1 million for each library with two exceptions (Table S4) totaling 608,041,012 raw reads. The *de novo* assembly of the reference transcriptome of *T. pratense* produced 44,643 contigs, of which 41,505 contigs were annotated and 29,781 contigs were identified as plant specific. The minimal length of the contigs was 124 bp, the maximal length 15,551 bp (Table S5). After the *de novo* assembly

of the *T. pratense* transcriptome, each library was mapped back against the reference transcriptome to determine the overall alignment rate, which was between 77.85 % and 90.32 % (Table S6).

63 % of the 44,643 contigs could be mapped to a known locus of the *T. pratense* genome annotation [12, 35], 32 % could be mapped to an unknown locus of the *T. pratense* genome and 5 % could not be mapped to the *T. pratense* genome (Fig. S2). All plant-specific contigs were annotated with several databases (Table S3). To further verify the quality of our replicates, we identified the transcripts shared by the two replicates. We calculated TPM values for each transcript and discarded transcripts with TPM values < 1. The percentage of transcripts shared between the two replicates was between 90 % and 94 % for all treatments/localities, suggesting that the RNA-Seq data are highly reproducible (Table S8).

Differentially expressed gene analysis reveals diverse subsets of genes involved in regrowth influenced by location and environmental conditions

To identify gene expression responses underlying the regrowth response after mowing, a digital gene expression analysis was performed comparing field A mown vs. field A non-mown (FaM vs. FaNM); field B mown vs. field B non-mown (FbM vs. FbNM); greenhouse mown vs. greenhouse non-mown (GM vs. GNM) to identify DEGs (Table S9) from mown plants. Interestingly, using the |log2foldchange| ≥ 2, the number of differentially expressed genes (DEGs) is rather similar in all comparisons, ranging from 119 (GM vs. GNM) to 142 (FaM vs. FaNM) (Table 1).

Table 1: The numbers of differentially expressed transcripts (contigs) between libraries with changes equal or above |log2foldchange| 2. Upregulation for each comparison is shown.

Analysis	total DEGs	Number of upregulated transcripts in mown libraries	Number of upregulated transcripts in unmown libraries
GM vs. GNM	119	54	65
FaM vs. FaNM	142	49	93
FbM vs. FbNM	122	59	63

We were then interested to identify transcriptional changes in developmental processes required for the regrowth process. Thus, the results of the DEG analysis were restructured such that the DEGs were grouped in 16 descriptive classes defined by database and literature mining (Tables S3 and S10). Those classes (abiotic stress, abiotic/biotic stress, biotic stress, development, general cell function, growth, metabolism, photosynthesis, phytohormone, secondary metabolite biosynthesis, senescence, signaling, symbiosis, transcription, transposon, no available annotation) describe major functional groups and serve to broadly categorize the potential role of a gene (Table S10).

We compared the top 20 DEGs of mown vs. not mown plants and observed that the greenhouse plants displayed more DEGs (classes growth, transcription, and phytohormone) involved in regrowth processes (Fig. 1C, Table S3). Three DEGs involved in growth, two phytohormone genes, and two transcriptional regulators are among the top 20 DEGs, while ten DEGs are related to biotic and abiotic stress in the greenhouse (Table S3). The top 20 DEGs of field A grown plants include four growth related, three development related and five stress-related DEGs. The top 20 DEGs of field B grown plants included only two growth related and six stress-related transcripts. Taken together, the greenhouse grown plants showed most DEGs related to growth, transcription, and phytohormone actions indicative of a regrowth reaction, as they grew under less stressful conditions than the field grown plants, for which stress related DEGs were more dominant (Fig. 1 A-C /Table 2-4).

We then performed a GO enrichment analysis with the DEGs of each group to obtain a differential view on the transcriptional changes occurring in relation to regrowth (Table 2, 3, 4). The results revealed that GO terms involved mainly in general metabolic processes and pathways, as well as general reactions are enriched in non-mown plants including i.e. the GO terms “protein metabolic process”, “metabolic process”, “cellular process”, “catabolic process”, "biosynthetic process" (Fig. S3 and Table S11). Within mown samples we found the following GO terms enriched: “nucleic acid binding “(GM); GO terms related to photosynthesis ("photosynthesis", "thylakoid"), cell components and protein transport ("Golgi apparatus", "cytoplasm") and related to regrowth and stress response ("generation of precursor metabolites and energy", "cell growth", "cell communication") (Fig. S3 and Table S11). Within the GO term “cell growth” the contigs GIBBERELLIN-REGULATED PROTEIN 1 which is involved in cell elongation and ROOT HAIR DEFECTIVE 3, a protein involved in root hair growth are present. For FbM we found the GO term related to metabolic processes ("metabolic process", "lipid metabolic process"), cell related ("cytoplasm", "extracellular space"), enzymatic and catabolic processes ("enzyme regulator activity", "catalytic activity") and the GO term "binding", which included a contig encoding for “V”, a protein involved in the ethylene biosynthesis.

Interestingly, most functional groups differ between the field and greenhouse location (Fig. 1A-C), for example, more genes related to growth are upregulated in the non-mown Fa location but in the Fb and greenhouse location, they more genes are upregulated in the mown plants. Only genes related to biotic stress processes were upregulated in all unmown locations and more transposon-related genes are upregulated in mown plants (Fig. 1 A-C).

The photosynthesis- and phytohormone-related genes of field A show a similar pattern to the field B plants as do the phytohormone- and signaling related genes. Genes related to development, general cell functions and transcription are also similar between field A and the greenhouse grown plants, such that more transcription- and development-related genes are upregulated in mown plants. And unexpectedly, senescence-related genes are upregulated in mown plants of field B. However, as our analysis cannot discriminate between activating and repressing factors of senescence, we cannot conclude from our data weather the mown plants have activated or repressed their senescence program.

The largest group of differentially expressed genes is the one related to biotic stress with up to 38% differentially expressed genes in one location (field B, Fig. 1 B). This suggests that biotic stresses play a prominent role in non-mown plants. A similar phenomenon can be observed for growth related processes, where up to 24% genes were upregulated in the mown and unmown plants indicating that different growth programs are active in mown vs. unmown plants.

Taken together we can state that mown plants in all locations change their transcriptional programs upon mowing suggesting that they massively change their metabolism and signaling processes. However, the molecular answer to substantial biomass loss differed between all three locations.

To find similarly regulated genes between the treatments and/or locations, Venn diagrams were generated to compare the number of shared DEGs within the mown samples and the non- mown samples (Fig. 1 D-E, Table S12). Within the mown samples we detected no overlap between the groups with the exception of four upregulated DEGs in the two field transcriptomes (FbM and FaM (Fig. 1 D). Within the non-mown samples, also four genes were shared between the field transcriptomes (FbNM and FaNM)) and one was shared between the field B and the greenhouse (Fig. 1 E). No genes were shared between all three samples, neither in the mown treatment, nor in the non-mown treatment.

We were interested in the contribution of individual phytohormones to the regrowth reaction in *T. pratense*, as they are known to play a major role in the regulation of development and stress response. We identified DEGs related to phytohormone synthesis, homeostasis, transport, and signaling for all major classes of phytohormones in the datasets. The four phytohormones with the most associated DEGs were: abscisic acid (8 DEGs), gibberellin (8 DEGs), salicylic acid (6 DEGs), and auxin (5 DEGs) (Fig. 1 F). Abscisic acid and salicylic acid are well-known to be involved in response to drought and abiotic/biotic stress, respectively. Auxin is the major phytohormone required for growth and cell division regulation and thus, we expected DEGs related to these phytohormones to be abundant in our analysis. However, unexpectedly, eight DEGs with gibberellin association were found. As gibberellins regulate growth in response to stresses but have so far not been associated with regrowth after biomass loss, we suggest gibberellin as a novel candidate phytohormone to influence the regrowth response.

Specific transcriptional regulator families are differentially expressed during the regrowth process

As the regulation of stress response, growth and development depends on differential activity of transcription factors, we aimed to identify transcriptional relevant to the biological processes occurring two weeks after mowing by mapping the transcriptome to the PlnTFDB [36]. All members of a specific transcriptional regulator family (TRF) were identified *in silico* and their expression was compared between mown and unmown plants (Table S13). Fig. 2 shows TRFs with significantly differential expression between mown and unmown conditions in at least 10% of their members, Fig. S2 and table S13 includes also those TRFs with 5% of their members regulated differentially upon mowing.

17 TRFs were identified of which at least 10% of the members showed differential expression in mown versus unmown comparisons (Fig. 2): ABI3VP1, AP2-EREBP, C_2C_2-Dof, C_2C_2-GATA, GRAS, HSF, LOB, MADS, mTERF, MYB, NAC, PHD, SBP, SNF2, TCP, TRAF, WRKY.

On field A, the AP2-EREBP, LOB, MADS, MYB, NAC, PHD, SBP, TCP and WRKY TRFs were more prominent in unmown plants, and only the HSF TRFs were more prominent upon mowing. On field B, ABI3VP1, C2C2-Dof, GRAS, HSF, LOB, MADS, mTERF, SNF2, TRAF, and WRKY TRFs were reduced upon mowing. In the greenhouse-grown plants, members of ABI3VP1, C2C2-Dof, C2C2-GATA, and GRAS, show increased numbers in response to mowing. In addition, ARF, C2H2, homeobox, MYB, NAC, and TRAF TRFs show changes in expression in all locations, albeit with only between 5 – 10 % of the members being differentially expressed (Table S13).

Two TRFs showed a repression of expression upon mowing: 10 % of the WRKY transcripts were less abundant in mown plants regardless of the provenance. In addition, MADS-box transcripts were found upregulated as well, but only in the field-derived transcriptomes. Generally, only four of the 17 TRFs analyzed here showed significant changes in expression towards mowing in the greenhouse-derived plants, suggesting that they react less strongly towards mowing than the field-derived plants. Six TRFs (AP2-EREBP, MYB, NAC, PHD, SBP, and TCP) showed transcriptional changes in reaction to mowing only in field A while only three TRFs (mTERF, SNF2, TRAF) showed this only in field B, suggesting that the combination of biotic and abiotic factors with mowing differed between the two field locations, and, in a similar way, between the field locations and the greenhouse.

Notably, only the C_2C_2-GATA TRF showed transcriptional changes in at least 10% of its members towards mowing under greenhouse but not under field-conditions, indicating that transcriptional changes in reaction to other biotic and abiotic factors may overlay the regrowth reaction. Taken together, the TRF analysis showed that the reaction towards mowing induces transcriptional changes in only a subset of TRFs, suggesting that those play a major role in relieving the stress of biomass loss and regrowth.

Table 2: Twenty most strongly differentially expressed genes of the GM vs. GNM analysis. Shown are the transcript name, |log2foldchange| ≥ 2 of the corresponding transcript, the library in which the transcript is upregulated (pattern), gene name based on *T. pratense* genome annotation, corresponding Phytozome description, gene name and species name of the next homologs and *A. thaliana* gene name, and locus name based on information available on Tair.

ID	Pattern	Contig ID	|log2foldchange| ≥ 2	Class (basis of classifiation)	Gene name *T. pratense*	Next homolog gene name	Next homolog species name	*A. thali* gene na
1	GHNM	tdn_99733	-9.5	Growth (*M. truncatula*)	Tp57577_TGAC_v2_mRNA4544.v2	Medtr4g029550.1	*M. truncatula*	-
2	GHNM	k41_54584	-6.3	Biotic stress (*T.pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA28349.v2	Medtr5g073620.1	*M. truncatula*	*ATEXO*
3	GHNM	tdn_92791	-5.5	Abiotic/biotic stress (*T.pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA20498.v2	Medtr1g041150.1	*M. truncatula*	*ATCPK*
4	GHNM	k41_130218	-5.5	-	-	-	-	-
5	GHNM	tdn_53091	-4.8	Phytohormone (*M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA39912.v2	Medtr4g010250.1	*M. truncatula*	-
6	GHNM	tgg_43136	-4.4	Transcription (*M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA29629.v2	Medtr4g098630.1	*M. truncatula*	*ANAC0*
7	GHNM	tdn_141837	-4.3	Abiotic stress (*T.pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA760.v2	Medtr2g022700.1	*M. truncatula*	*ATGPT*
8	GHNM	tdn_40997	-4.2	Abiotic stress (*T.pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA25718.v2	Medtr4g130540.1	*M. truncatula*	*HSP70*
9	GHNM	k71_5292	-4.1	Biotic stress (*T.pratense, M. truncatula*)	Tp57577_TGAC_v2_mRNA23166.v2	Medtr0163s0020.1	*M. truncatula*	*LECRK-*
10	GHNM	k59_6358	-3.9	Growth (*T.pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA12337.v2	Medtr3g435430.1	*M. truncatula*	*ATEXP*
11	GHM	tdn_86219	8.0	Biotic stress (*T.pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA29036.v2	Medtr4g066210.1	*M. truncatula*	*BGLU1*
12	GHM	k23_115785	8.0	Abiotic stress (*T.pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA22071.v2	Glyma.01G001000.1	*G. max*	-
13	GHM	tdn_91159	8.1	Biotic stress (*T.pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA7745.v2	Medtr4g035870.1	*M. truncatula*	-
14	GHM	k65_43517	8.3	Phytohormone (*T.pratense, A. thaliana*)	Tp57577_TGAC_v2_mRNA6281.v2	Medtr1g082750.1	*M. truncatula*	*ATAMI*
15	GHM	tgg_18067	8.4	-	Tp57577_TGAC_v2_mRNA32019.v2	-	-	-
16	GHM	k61_38813	9.0	-	-	-	-	-
17	GHM	k49_82496	9.0	Abiotic/biotic stress (*G. max, A. thaliana*)	Tp57577_TGAC_v2_mRNA37976.v2	Glyma.06G268800.1	*G. max*	-
18	GHM	k67_38815	9.1	Biotic stress (*T.pratense*)	Tp57577_TGAC_v2_mRNA41666.v2	Medtr0062s0020.1	*M. truncatula*	-
19	GHM	k45_11164	9.6	Transcription (*T.pratense*)	Tp57577_TGAC_v2_mRNA29953.v2	Medtr3g092510.1	*M. truncatula*	*ATRBP*
20	GHM	tdn_25484	9.6	Growth (*Phaseolus vulgaris*)	Tp57577_TGAC_v2_mRNA13093.v2	Phvul.006G033800.1	*Phaseolus vulgaris*	-

Table 3: Twenty most strongly differentially expressed genes of the FaM vs. FaNM analysis. Shown are the transcript name, |log2foldchange| ≥ 2 of the corresponding transcript, the library in which the transcript is upregulated (pattern), gene name based on *T. pratense* genome annotation, corresponding Phytozome description, gene name and species name of the next homologs and *A. thaliana* gene name, and locus name based on information available on Tair.

ID	Pattern	Contig ID	|log2foldchange| ≥ 2	**Class (basis of classifiation)**	Gene name *T. pratense*	Next homolog gen name	Next homolog species name	A g
1	TPNM2	k33_17052	-9,0	Biotic stress (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA21474.v2	Medtr4g079440.1	*M. truncatula*	r
2	TPNM2	k43_111792	-8,8	Biotic stress (*M. truncatula*)	Tp57577_TGAC_v2_mRNA26333.v2	Medtr8g101900.1	*M. truncatula*	C
3	TPNM2	tdn_34568	-8,6	-	Tp57577_TGAC_v2_mRNA9104.v2	Glyma.13G061800.1	*G. max*	-
4	TPNM2	tdn_49640	-8,6	-	-	-	-	-
5	TPNM2	tdn_58745	-8,5	Biotic stress (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA20190.v2	Medtr8g075200.1	*M. truncatula*	-
6	TPNM2	tdn_47209	-8,5	Growth (*M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA10703.v2	Medtr1g053315.1	*M. truncatula*	-
7	TPNM2	tdn_48478	-8,4	Biotic stress (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA19516.v2	Medtr2g099020.1	*M. truncatula*	-
8	TPNM2	k41_17597	-8,4	Growth stress (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA8526.v2	Medtr1g036490.1	*M. truncatula*	A A
9	TPNM2	k51_82581	-8,2	Growth (*T. pratense*)	Tp57577_TGAC_v2_mRNA23127.v2	Medtr2g436480.1	*M. truncatula*	K
10	TPNM2	tdn_82424	-8,1	Growth (*T. pratense*)	Tp57577_TGAC_v2_mRNA17103.v2	Medtr2g013740.1	*M. truncatula*	K
11	TPM2	k49_380	7,5	Development (*A. thaliana*)	Tp57577_TGAC_v2_mRNA37185.v2	SapurV1A.0885s0040.1	*Salix purpurea*	D
12	TPM2	tdn_49869	7,6	-	-	-	-	-
13	TPM2	tdn_54983	7,7	-	-	-	-	-
14	TPM2	k37_9029	7,8	-	-	-	-	-
15	TPM2	k45_6120	8,4	-	Tp57577_TGAC_v2_mRNA2166.v2	Medtr2g007510.1	*M. truncatula*	-
16	TPM2	k71_23808	8,4	Development (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA14131.v2	Medtr1g021320.1	*M. truncatula*	-
17	TPM2	k59_3541	8,4	Development (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA34193.v2	Medtr4g089030.1	*M. truncatula*	C
18	TPM2	k59_360	8,6	Metabolism (*Linum usitatissimum, T. pratense*)	Tp57577_TGAC_v2_mRNA21875.v2	Lus10012445	*Linum usitatissimum*	-
19	TPM2	k53_38903	9,0	Abiotic stress (*A. thaliana*)	Tp57577_TGAC_v2_mRNA37328.v2	Medtr8g063190.1	*M. truncatula*	P
20	TPM2	tdn_129978	9,6	-	Tp57577_TGAC_v2_mRNA9318.v2	Medtr7g062280.1	*M. truncatula*	-

Table 4: Twenty most strongly differentially expressed genes of the FbM vs. FbNM analysis. Shown are the transcript name, |log2foldchange| ≥ 2 of the corresponding transcript, the library in which the transcript is upregulated (pattern), gene name based on *T. pratense* genome annotation, corresponding Phytozome description, gene name and species name of the next homologs and *A. thaliana* gene name, and locus name based on information available on Tair.

ID	Pattern	Contig ID	|log2foldchange| ≥ 2	Class (basis of classifiation)	Gene name *T. pratense*	Next homolog gen name	next homolog species name	*A. thaliana* gene name
1	TPNM3	tdn_100726	-9,4	Biotic stress (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA24659.v2	Medtr4g094772.1	*M. truncatula*	*CYP81D*
2	TPNM3	tgg_49631	-8,0	Biotic stress (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA37846.v2	Medtr6g034470.1	*M. truncatula*	-
3	TPNM3	tdn_152262	-7,9	-	-	-	-	-
4	TPNM3	tdn_56712	-7,9	Biotic stress (*T. pratense, M. truncatula*)	Tp57577_TGAC_v2_mRNA30556.v2	Medtr8g027540.1	*M. truncatula*	-
5	TPNM3	tdn_87762	-7,9	Biotic stress (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA10533.v2	Medtr7g451400.1	*M. truncatula*	*ATMCP1E ATMCPB1*
6	TPNM3	tdn_86129	-7,1	General cell functions (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA10207.v2	Glyma.11G154500.1	*G. max*	*RPB5E*
7	TPNM3	k55_46241	-6,9	Growth (*T.pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA31452.v2	Medtr4g128150.1	*M. truncatula*	*histone 4*
8	TPNM3	tdn_55533	-6,2	Abiotic stress (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA39263.v2	Medtr5g007790.1	*M. truncatula*	*ATCRM1, ATXPO1*
9	TPNM3	tgg_51443	-4,7	Growth (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA37076.v2	Medtr5g019580.2	*M. truncatula*	*UGT72E2*
10	TPNM3	tdn_136706	-4,7	-	-	-	-	-
11	TPM1	tdn_140636	8,8	General cell functions (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA28209.v2	Medtr8g005980.1	*M. truncatula*	*C-NAD-MDH2*
12	TPM1	tdn_154158	8,9	General cell functions (*T. pratense, M. truncatula*)	Tp57577_TGAC_v2_mRNA39482.v2	Medtr3g114970.2	*M. truncatula*	-
13	TPM1	tdn_65187	9,1	Transposon (*T. pratense, Prunus persica A. thaliana*)	Tp57577_TGAC_v2_mRNA30115.v2	Prupe.4G011200.1	*Prunus persica*	-
14	TPM1	tdn_100956	9,2	Metabolism (*T. pratense, Capsella rubella, A. thaliana*)	Tp57577_TGAC_v2_mRNA9542.v2	Carubv10008027m	*Capsella rubella*	*AHA2*
15	TPM1	k63_21505	9,3	Biotic stress (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA19467.v2	Medtr3g022400.1	*M. truncatula*	-
16	TPM1	tdn_142681	9,3	Secondary metabolite biosynthesis (*T. pratense, M.*	Tp57577_TGAC_v2_mRNA15473.v2	Medtr8g074550.1	*M. truncatula*	-

				truncatula, A. thaliana)				
17	TPM1	k45_6120	9,6	-	Tp57577_TGAC_v2_mRNA2166.v2	Medtr2g007510.1	*M. truncatula*	-
18	TPM1	tdn_52922	10,1	-	Tp57577_TGAC_v2_mRNA41271.v2	mrna20290.1-v1.0-hybrid	*Fragaria vesca*	-
19	TPM1	tdn_65185	10,9	-	-	-	-	-
20	TPM1	tdn_109277	11,7	Transcription (*T. pratense, M. truncatula, A. thaliana*)	Tp57577_TGAC_v2_mRNA29560.v2	Medtr5g028610.1	*M. truncatula*	-

Gibberellins are also important regulators after mowing in red clover

We have shown previously (Fig. 1G) that genes related to gibberellins are also differentially expressed, even though GA is not well-known to regulate biological processes related to loss of biomass. We then wanted to know if GA is relevant for the regulation of regrowth and treated red clover plants exogenously with GA_3.

A weekly gibberellin application during the regrowth process led to significant and specific changes in morphology (Fig. 3). Previous work suggested that regrowing plants produce smaller and rounder leaflets with shorter petioles than non-mown plants [24]. Thus, number of leaves, shoots and inflorescences, leaf area, and the roundness of leaflets were measured in this experiment (Fig. 3, Fig. S4). The first visible effects of gibberellin treatment were recognized after 1.5 weeks, showing a significant higher leaflet area of gibberellin treated. Later it was observed that the petioles of treated plants were on average twice as long as petioles of untreated plants (16.7 ± 1.9 cm and 8 ± 1.2 cm, respectively). Leaflets of gibberellin treated plants had with 4.7 ± 0.9 cm² almost double the size when compared with those of untreated plants (2.4 ± 0.6 cm²). However, gibberellin treated plants produced only 30% more total leaf area than control plants. Other morphological traits such as number of inflorescences, leaves, and shoots remained unaffected by the gibberellin treatment (Fig. S4). In summary, mown plants normally produce leaves with shorter petioles, restrict their leaflet area and their leaves become rounder. Gibberellin treatment partially alleviated these developmental changes such that the mown, gibberellin treated plants produced larger leaves with longer petioles while the leaf shape was unaffected by gibberellin treatment.

Discussion

RNA-Seq and assembly

The *de novo* assembly in combination with a reference-based approach for the annotation led to the identification of 44,643 contigs of which 29,781 were annotated as plant-specific (Fig. S2). With the prior *de novo* assembly, 4,051 additional contigs were obtained that have not been found in the *T. pratense* 1.0 (GCA_000583005.2) genome [12, 35]. The estimated genome size of *T. pratense* is ~440 Mbp [27]. The *T. pratense* transcriptome data in this study was ~55 Mbp in size, corresponding to ~12.5% transcribed regions in the *T. pratense* genome, which is within the range of previously published transcriptomes (~10% (42 Mbp) [37]). Interestingly, we found plant-specific, previously unreported contigs suggesting that the *T. pratense* genome might need improvement in terms of sequencing coverage and protein coding sequence annotation.

Cell walls are remodeled after mowing

After massive biomass loss, like mowing inflicts on *T. pratense*, plants firstly need to seal wounded tissues. Several transcriptional regulators known to play a role in the tissue-reunion processes were identified in *Solanum lycopersicum*, *Cucumis sativus, andA. thaliana* (reviewed in [38]). Homologs of these genes were also identified to be differentially regulated in the *T. pratense* transcriptome after mowing (Table S14), such as several members of the Auxin Response Factor (ARF) family or the No Apical Meristem (NAM) family member *ANAC071,* the homolog of the most highly upregulated transcription factor in greenhouse grown plants after mowing (Table S14). *[39]* suggested that high levels of auxin induce the expression of *ANAC071* via ARF6 and ARF8 (in the upper part of incised stems), at the same time, reduced auxin levels directly after the cutting activate the expression of *RAP2.6L*. In addition auxin signaling via ARF6 and ARF8 influences jasmonic acid synthesis via the activation of *DAD1*, thus together with *LOX2* increases *RAP2.6L* expression during tissue reunion in *A. thaliana* (Table S14) [39]. Further it was demonstrated that *ANAC071* can initiate the expression of members of the xyloglucan endotransglucosylase/hydrolases family (XTH20 and XTH19) which recombine hemicellulose chains to drive the cell expansion during tissue reunion [40]. Interestingly, we found that all members of the cell wall remodeling pathway show distinct expression patterns- Some are upregulated in mown plants including for example *XTH32* (k69_7012, upregulated in FbM, tdn_94651, upregulated in GM, FaM and FbM), *XTH6* (tdn_91763, upregulated in GM), *XTH8* (k71_5058, upregulated in GM, FbM), *XTH9* (tdn_113578, upregulated in GM), *XTHA* (tdn_87930, upregulated in GM), *LOX2* (tdn_156279, upregulated FbM), and *ARF8* (tdn_156886 upregulated in GM, tdn_156890 upregulated in GM) (Table S9, S13 and S15). This implies that the early steps in the regrowth reaction are conserved in core eudicots and that the cell wall remodeling processes continue at least two weeks after mowing.

Loss of auxin control on axillary buds can be detected two weeks after decapitation

Axillary buds of decapitated *P. sativum* plants export auxin upon growth activation, a process mediated by the *P. sativum* PIN1 homolog PsPIN1. Upon the loss of apical auxin transport, PsPIN1 polarization changes and this new polarization is causing auxin export from dormant axillary buds and is required for their

activation [41]. Subcellular targeting and polarization of PsPIN1 starts about 6 hours after decapitation and then PsPIN1 expression increases. However, it remains unclear if the elevated PsPIN1 expression is maintained for a prolonged period [66]. Our data show a higher expression of the three PIN1 homologs in greenhouse-grown mown plants when compared to the non-mown control plants (Table S2), indicating a sustained expression of the homolog of PIN1 even after two weeks of biomass loss, which might help to activate the remaining dormant buds in *T. pratense*.

Biotic and abiotic stresses contribute to differential gene expression

Plant grown on fields face different stressors when compared to greenhouse grown plants and we were interested in how the field conditions contributed to differential gene expression. The transcriptome comparisons between locations revealed that the mown greenhouse plants showed the highest percentage of DEGs possibly involved in regrowth processes (Fig. 1 A-C). In contrast, the field transcriptomes displayed patterns of abiotic and biotic stress reactions. Comparisons of the top 20 DEGs of the unmown field transcriptomes showed that plants grown on field A and B faced more biotic stress than abiotic stress. One of the upregulated genes in field A is a chitinase homolog suggesting that those plants are under attack of fungi and/or insects. Follow-up analyses to correlate environmental conditions, as well as biotic and abiotic stresses monitored within the Biodiversity Exploratories with differential gene expression at the two field locations would be an interesting project but is beyond the scope of this work. In contrast, the top 20 DE transcripts of the greenhouse plants include phytohormone- and transcription-related genes, but also a high proportion of biotic and abiotic stress-related genes. This suggests that also these plants have to cope with stresses, but to a lesser extent emphasizing their regrowth reaction more strongly within the top 20 DEGs. Generally, the non-mown plants show a much higher number of upregulated biotic stress-related genes during a phase in their life when senescence commences and they become more susceptible to pathogen attacks. The mown plants during their regrowth phase are not senescing and their younger organs seem to be less affected by biotic stress.

Gibberellin-related genes influence regrowth of *T. pratense* in concert with other phytohormones

Wounding induces a first stress response, activating the interplay of the phytohormones jasmonic acid, salicylic acid, and ethylene. This allows an individual response to various abiotic and biotic stresses, and the differentiation between wounding inflicted by physical forces, pathogens, or herbivory [42–47]. Abscisic acid is required for the fine tuning of the jasmonic acid/salicylic acid/ethylene induced stress response by i.e. suppression of other phytohormones [48]. After the first stress response, additional phytohormones are involved in the regrowth of the plant. Auxin, cytokinin, strigolactone, and gibberellin become involved in a later stage. Following the initiation of shoot outgrowth induced by auxin and cytokinin, an increased gibberellin concentration allows for shoot elongation [31, 49–51]. In addition, auxin, cytokinin, and salicylic acid are involved in the shoot branching, where high levels of auxin and salicylic acid have a suppressing effect on lateral bud outgrowth. High levels of cytokinin promote shoot outgrowth which was shown in *A. thaliana*, *O. sativa*, and *P. sativum* [52–55]. As we were mainly interested in processes that happen approximately two weeks after cutting and as the role of those phytohormones was already studied, we concentrated on the role of gibberellin during regrowth, which was also found as one the phytohormones with the most associated genes in our transcriptomes. Gibberellins are involved in multiple aspects of plant development like cell elongation, flowering time regulation, and seed germination. Consequently, genes encoding for proteins involved in the synthesis, perception, and catabolism of the various gibberellins can be assumed to influence plant form. Our RNA-Seq data showed a high abundance of differentially expressed gibberellin associated genes (Fig. 1 F, table S13)) which may be connected to the morphological changes after mowing , such as rounder leaves, temporary dwarf-like appearance, and higher cumulative biomass production in mown plants [24].

When analyzing the morphological effects of gibberellin application to mown plants (Fig. 3)), external gibberellin application led to the disappearance of specific traits typical for the mowing response. Mown plants developed shorter petioles and their leaf size area was smaller [24], but when treated with gibberellin, leaves and petioles grow up to the size seen in unmown plants.

The cell-expansion and proliferation promoting abilities of gibberellins via stimulation of the degradation of growth-repressing DELLA proteins are well established [56]. The length increase of petioles in gibberellin treated mown plants is in line with reported data from non-mown *Pisum sativum* plants, but in those, leaf sizes remained unchanged after gibberellin treatment [57], suggesting a more specific role for gibberellin in the regrowth reaction after biomass loss in red clover. Moreover, it was shown in *A. thaliana* that elevated gibberellin concentrations enhance cell-division rates in the distal end of leaves (reviewed in [58]). If these results are transferred to *T. pratense*, gibberellin treatment should result in longer leaflets after gibberellin treatment of mown plants. Interestingly, leaf shape did not change, but only the size increased suggesting a regrowth-specific shift of growth pattern which is unaffected by gibberellin but similar to leaf shape of juvenile plants [24]. However, one can also assume a cell-division pattern in red clover leaves that is distinct from the one reported for *A. thaliana* and may react more uniformly to enhance gibberellin concentrations.

Interestingly, gibberellin treatment of mown *T. pratense* plants does not generally lead to stronger longitudinal growth as leaves retained the round shape characteristic for untreated mown plants. These regrowth-specific characteristics can also be found in other species, for example in *A. thaliana*, *Fragaria ananassa*, *Duchesnea indica*, and *G. max*, gibberellin treatment causes elongated petioles and increased leaf sizes and a more erect growth habit [59–62]. This may suggest a new way to increase the accumulation of biomass, suitable for animal fodder. Previous experiments with the grasses *Leymus chinensis* and *Lolium perenne* showed gibberellin action to be limited by N fertilization [63, 64]. Red clover, living in symbiosis with nitrogen fixing bacteria, is not dependent on additional N fertilization and can produce high-protein content biomass without fertilizer on poor soils.

Materials And Methods

Plant growth conditions, gibberellin treatment, tissue sampling, RNA extraction, cDNA library construction, and RNA-Seq

Plant material for RNA-Seq was collected from three locations (fields and greenhouse, Fig. S1 and Table S1, thereby one field location includes two neighboring field sites). Field plant tissue for RNA-Seq was sampled on 06.11.2014 within the area of the long-term open research platform Biodiversity Exploratory "Hainich-Dün" [34], located in Thuringia, Germany. Collection permits from farmers and local authorities were obtained centrally by the Biodiversity Exploratory research platform. T. pratense is an agriculturally used species native to Germany and does not fall under the Nagoya protocol. ITS sequencing with subsequent database comparisons identified the species collected in the field. The Material was sampled on four neighboring sites; two mown pastures (FaM and FbM) and two meadows that were non-mown (FaNM and FbNM). In the year of the sampling, the non-mown pastures and meadows were not grazed upon or mown. The two treatments were comparable as these were the closest experimental plots neighboring one mown and one un-mown plot. The experimental plots were managed as normal agricultural fields and were populated with comparable red clover, as these are wild, established populations. For the greenhouse samples, seeds of regional *T. pratense* populations (from a region covering mainly Thuringia, Saxony, Saxony-Anhalt, Thuringian Forest and Uckermarck, Germany) were obtained from the Rieger Hofmann seed company (Blaufelden, Germany). Plants in the greenhouse were grown in 23 °C with 16 h of light in pots of 12 cm diameter, watered daily, and compound fertilizer (8'8'6'+) was given every ten days. After 122 days after sowing, half of the plants were cut to 5 cm (GM and GNM). Material from mown plants was sampled approximately 14 days after mowing/cutting, to avoid sequencing of the transcripts related to the first stress response [38]. After collection, the samples were snap frozen in liquid nitrogen. For each site and the greenhouse two biological replicates of four pooled plants (shoot and leaf material) each were collected. As reviewed in [38], the expected time for tissue reunion and wound closure accounts approximately seven days (cucumber and tomato) to 14 days (*A. thaliana*). Based on this information, we assumed that the first stress response and the initiation of regrowth in *T. pratense* will be approximately two weeks after cutting/mowing.

RNA was extracted using NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Preparation of the cDNA libraries and the strand-specific sequencing were conducted by Eurofins Genomics (Ebersberg, Germany). The RNAs of four individuals were pooled for each RNA-Seq library and sequenced on an Illumina Hiseq2000 platform with chemistry v3.0, creating 2x 100 bp paired end reads.

In order to assess the effect of gibberellin during the regrowth reaction of *T. pratense*, 14 red clover plants were mown as described in [24]. Of these plants, seven were used as control plants and seven plants were sprayed with 100 µM GA_3 (Duchefa Biochemie B.V, Haarlem, The Netherlands) once per week as described in [65]. Different morphological characters (leaf number, length/width of leaflets, petiole length, number of inflorescences, and number of main shoots) were measured every second day for four weeks.

Assembly of reference transcriptome and annotation

The raw-read-quality of the RNA-Seq data was analyzed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Illumina adapter and low quality regions were trimmed using Trimmomatic [66] with ILLUMINACLIP, SLIDINGWINDOW:5:20 and MINLEN:50 options. Quality trimmed reads were pooled and digitally normalized [67]. Multiple d*e novo* assemblies were computed using Trinity [68] and Oases [69] with all odd k-mer parameters between 19 and 85. In addition, a genome guided assembly was performed with Trinity using the draft genome of *T. pratense* 1.0 (GCA_000583005.2) [12, 35]. The resulting contigs were screened for potential coding sequences (CDS) using TransDecoder (https://transdecoder.github.io/). The EvidentialGene pipeline (http://arthropods.eugenes.org/EvidentialGene/about/EvidentialGene _trassembly_pipe.html) was used to merge and filter the contigs based on the TransDecoder CDS prediction. Completeness of the final contig was confirmed by computing the mapping-rate of the non-normalized reads to the contigs. The raw sequence reads can be found at NCBI: PRJNA561285.

The contigs were uploaded to the "Sequence Analysis and Management System" (SAMS) [70] for functional annotation with the SwissProt [71], TrEMBL [72] and Phytozome [73] (e-value cutoff of 1e-5) databases. Additionally, attributes like gene names or functional descriptions were extracted from the blast hits. Contigs were mapped to the *T. pratense* reference genome using gmap [74]. All non-Viridiplantae contigs were discarded. Transcription factors were identified using a blastp search of the protein sequences against the plant transcription factor database Potsdam (PlnTFDB) ([36], version 3.0 protein database with an e-value cutoff of 1e-20. All functional annotations oftranscripts can be found in the table S2).

Differential gene expression analysis, enrichment analysis, and classification of differentially expressed genes

Read counts for each contig of the final assembly in each sample were computed using RSEM [75] with bowtie mapping. To identify differentially expressed (DEGs) *T. pratense* genes, a pairwise comparison of all treatments was preformed using the DESeq2 [76] tool with FDR ≤ 0.01 and $|\log2foldchange| \geq 2$ between FaM and FaNM, FbM and FbNM; GM and GNM respectively. The top 20 DEGs were determined for each comparison based on the expression strength (log2foldchange). Homologs in the next closest species and *A. thaliana* for each *T. pratense* candidate gene were identified based on the *T. pratense* genome sequence deposited in Phytozome [73]. TPM (transcript per million) values were calculated to estimate contig expression level [77].

We used the description and gene names obtained from TrEMBLE and SwissProt to search the UniProt [78], NCBI [79] and TAIR [80] databases to obtain further information (Table S3). Raw reads that were assembled to contigs, exhibiting a gene structure (ORF) and attained a putative annotation referred to below as genes.

GO enrichment analysis and Blast2Go Analysis of *T. pratense* genomes

To further explore the digital gene expression results and to find more candidate genes/ to identify differentially expressed gene clusters, an enrichment analysis with Gene Ontology (GO) terms [81–83] was performed. For each pairwise comparison, the up-regulated genes were screened for enriched and

depleted GO terms using the GOSeq package [84] separately for each treatment. The results of this analysis were visualized with the program GOplot [85] implemented in RStudio [86] with the program R [87].

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the NCBI database repository, https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA561285

Competing interests

Not applicable

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Authors' contributions

DH acquired, analyzed, and interpreted the data and drafted the manuscript, TG analyzed and interpreted the data a drafted the manuscript, OR analyzed the data, AB conceived and designed the work, analyzed the data, and revised the manuscript All authors read and approved the submitted version.

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Third party submission

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Figures

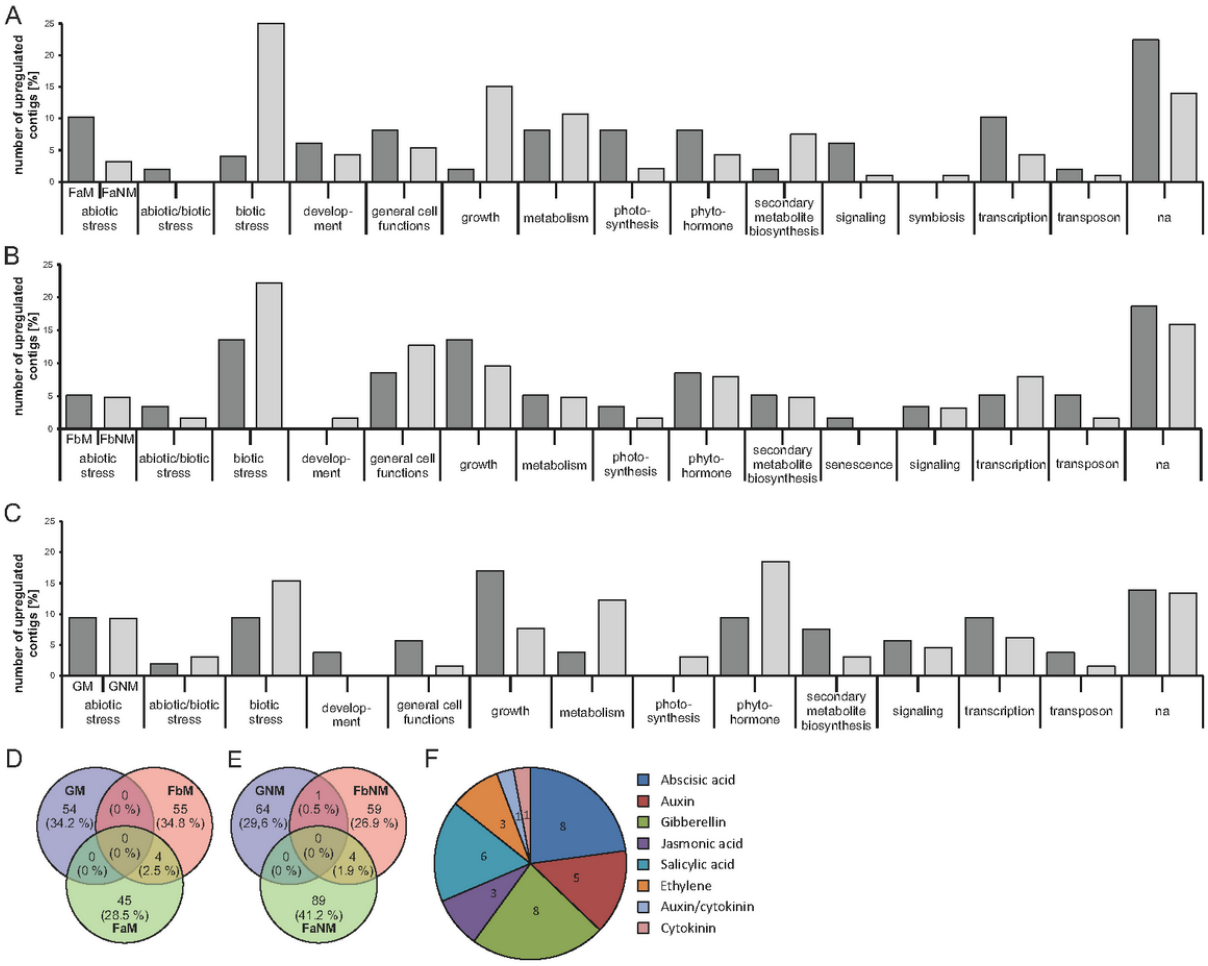

Figure 1

Transcriptome analysis showing biological processes relevant after mowing. A-C: Upregulation of DEGs with a |log2foldchange| ≥ 2, percentage share of each class to the corresponding gene list is shown in bar charts, dark grey bars show datasets from mown, light grey bars indicate datasets from non-mown plants A: Classes of DEGs from field A , B: Classes of DEGs from field B , C: Classes of DEGs contigs from greenhouse grown plants. D-E: Venn diagrams showing the number of shared upregulated genes within the mown samples (D) and the number of shared genes within the unmown samples (E) with a |log2foldchange| ≥ 2. Blue circles indicate greenhouse data, green field A and red field B. F: Number of genes belonging to the class “phytohormones” within the DEG list of the field and greenhouse transcriptomes. The pie chart shows the number of DEGs related to the different phytohormones (abscisic acid, auxin, genes common between the auxin and cytokinin pathway, cytokinin, ethylene, gibberellins, jasmonic acid, and salicylic acid).

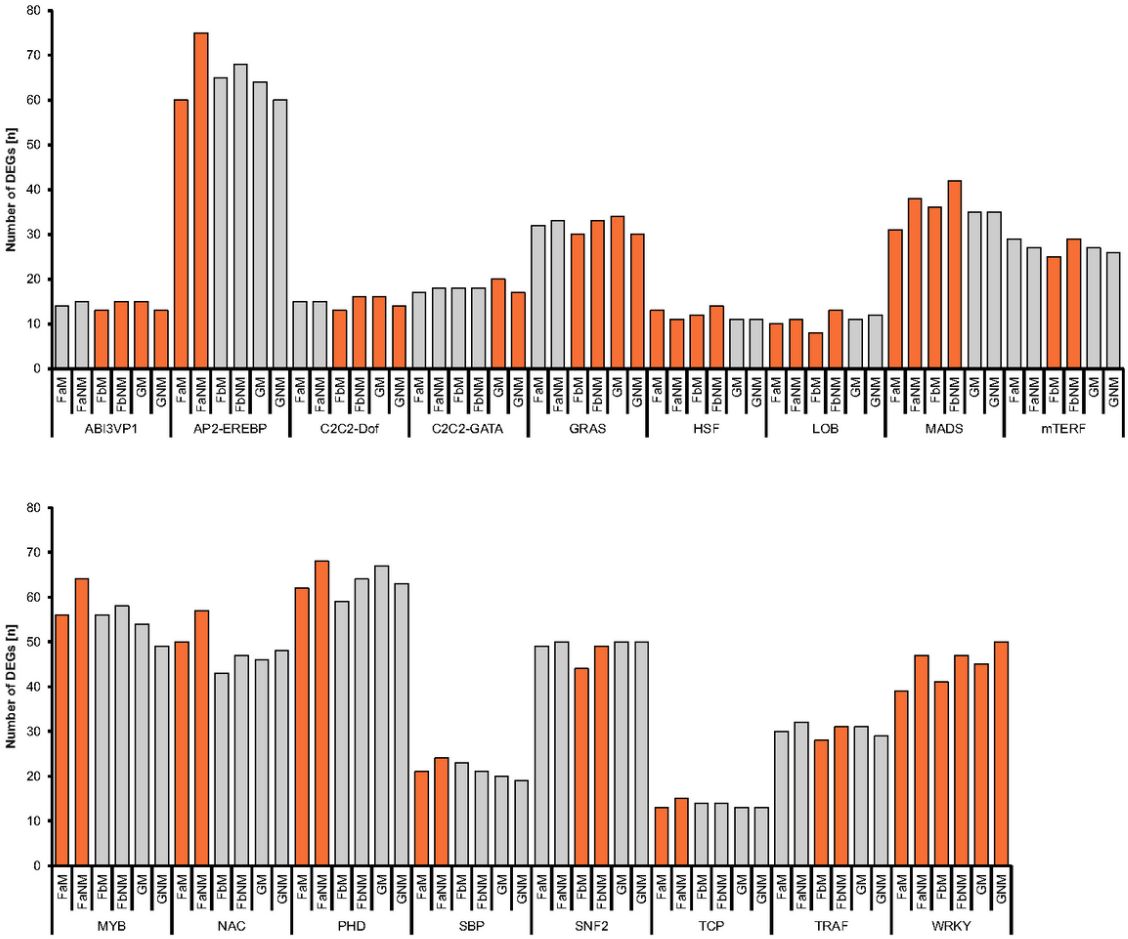

Figure 2

Differentially expressed TRF members in mown and non-mown T. pratense plants. The y-axis shows the number of expressed genes (TPM value over 5) that are members of the respective TRF. Names of the transcriptomes and TRFs are given on the x-axis. Expression of TRF members was compared in a pairwise manner (GM vs GNM, FaM vs FaNM, FbM vs FbNM). Shown are only those plant TRFs in which at least one of the comparisons resulted in a difference of more than 10% of the genes significantly upregulated in either the mown or the unmown condition (orange bars).

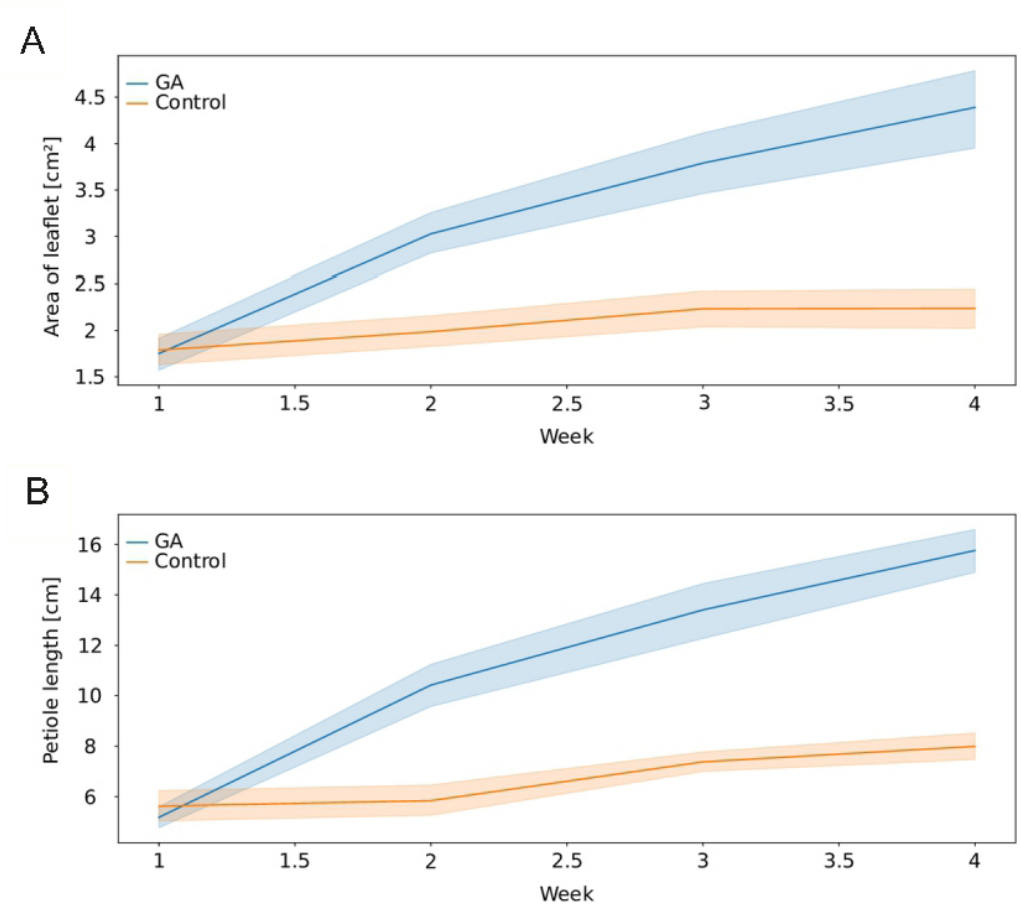

Figure 3

Gibberellin treatment affects the regrowth after biomass loss. A) leaflet area in cm², B) length of petioles in cm. The graphs show average values for each sampling date and the 95% confidence interval. Blue, GA3 treated; orange, mock-treated plants.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.docx
- supplement2.xlsx
- TableS11EnrichedGOTerms.xlsx
- TableS9AnnotationLog2DEGs.xlsx